

Biotechnological Support for the Development of New *Gladiolus* Hybrids

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ABSTRACT

The genus *Gladiolus* L. (*Iridaceae*) includes important ornamental species and hybrids which are successfully treated on the market since the last century. Recently breeders have selected new genotypes with the aim to increase the production in winter and late spring especially in countries bordering the Mediterranean Sea. In order to speed up the volume of the new hybrids and to produce virus-free stocks of mother plants, it is possible for *Gladiolus* to apply biotechnological tools of *in vitro* propagation through liquid culture or simple bioreactors such as temporary immersion system to reduce production costs and to enhance multiplication rate and cormel quality. *Gladiolus* micropropagation was reported first by Ziv *et al.* in 1970 and subsequently by several other authors exploring the performances of different species and varieties. Bulbs and corms of several species are commercially propagated in liquid culture in semi-automatic systems as temporary immersion; in *Gladiolus*, Ruffoni *et al.* presented in 2008 data about high efficiency micropropagation using temporary immersion compared with the culture on agar-solidified medium suggesting an efficient semi automatized protocol. The present paper takes into consideration the different ways for *in vitro* culture initiation and the efficiency of the meristem excision for the establishment of pathogen-free cultures including data coming from direct experiences and bibliography search. Moreover it will compare the different growth strategies (solid vs liquid micropropagation) evaluating finally the performances of the temporary immersion system.

Keywords: cormel production, liquid culture, meristem tip culture, somatic embryogenesis, temporary immersion, RITA®

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6- γ -(dimethylallylamino)-purine; BA, 6-benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, 1-naphthaleneacetic acid; LS, Linsmaier and Skoog, 1965; MS, Murashige and Skoog 1962; NASH, nucleic acid spot hybridization; PCB, paclobutrazol; RT-PCR, retro transcription polymerase chain reaction; SE, somatic embryos; TIS, temporary immersion system; ZEA, zeatin

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INTRODUCTION

Gladiolus L. belongs to *Iridaceae* family. It is a very popular plant for gardening and cut flower production. The species belonging to this genus are characterized by spikes carrying many flowers developing from a corm with annual growth. A new corm is produced each growing season and many cormels are formed at the tips of branched stolons (Cohat 1993). Summer flowering types originating from complex crosses are being produced since the middle of 19th

century particularly in France, in the United States and in the Netherlands. As the production season is spring and summer, new hybrids have been recently selected by a Spanish-Italian breeding group with the aim to increase the production in open air in winter and late spring (extra-season) in Mediterranean countries (Ruffoni *et al.* 2011).

Gladiolus is subject to severe damage by viral infection: *Bean yellow mosaic virus* (BYMV) and *Cucumber mosaic virus* (CMV) have the highest economic impact. It must be taken into consideration that virus diseases have a great

impact on the quality of the plant and the flower. Symptoms as “flower break” and “colour break” (Moran 1996) strongly modify plant behaviour and production. The production of healthy plants from cormels is impossible as vegetative propagation through corms allows virus transfer, effective physical and chemical treatments are unavailable for virus elimination and virus-resistant cultivars of *Gladiolus* are not available for breeding (Kamo and Joung 2007).

In the flower industry, the application of *in vitro* techniques is widely used but it is actually still underutilised because of high input of manual labour and little automation. The first note on *Gladiolus* micropropagation was by Ziv *et al.* in 1970. Sutter (1986) gave indications for the culture using LS medium supplemented with BA. Enhancement of corm production was then reported by Ziv (1989) with the application of growth retardant in liquid culture. Several papers have been published during 10 years focusing on aspects related to initiation in particular from corms. The micropropagation of selected cultivars of *Gladiolus* was reported by Mokshin *et al.* (2006) while Saha *et al.* (2011) focused their research on the biochemical and molecular diversity of five elite *Gladiolus* varieties. Nhut *et al.* (2004) described the importance of the type of the explant source and the presence of IBA in liquid shake culture. It is well known that liquid support can enhance the multiplication rate. Plant structures as buds, somatic embryos or micro bulbs can be easily propagated in liquid culture but, if the immersion is continuous, they can develop hyperhydricity. In order to overcome this physiological disorder they can be propagated in liquid culture in semi-automatic bioreactors that use a temporary immersion system (TIS). Some systems have been employed with tropical plants (Alvard *et al.* 1993; Lorenzo *et al.* 1998). TIS has been studied with laboratory size home-made vessels by several authors. The RITA[®] System (CIRAD, Vitropic, France; Teisson and Alvard 1995) is one of most widely used for the reproducibility of the results and for the availability of the jars. Other containers are used in commercial laboratories for their own production. Using this technique, an evident reduction of production costs has been reported by Kokko *et al.* (2002) for poplar. For ornamental species, increased quality and rooting ability were found for *Zantedeschia aethiopica* and *Anthurium andraeanum* in TIS by Ruffoni and Savona (2005).

In this paper the protocols for pathogen elimination coming from direct experiences of the authors and from a review of published works, are reported and compared. Moreover, the different growth strategies (solid vs liquid micropropagation) are evaluated comparing the *in vitro* performances focusing quality and quantity of cormels able to be directly sown in the field. The use of biotechnology to help in reducing the propagation costs is also discussed.

EXPLANT SOURCE AND REMOVAL OF PATHOGENS

Gladiolus, like other geophytes as Lily, has the possibility of an easy establishment of *in vitro* cultures, owing to the high reactivity and differentiation ability of the reserve organ tissues traditionally used for culture initiation. The problems of *in vitro* culture assessment are mainly related to the high degree of bacterial and fungal external contamination and to the necessity to propagate virus-free clones. The production of shoots able to multiply *in vitro* starts directly from the meristem which ensures optimal stability, or through direct regeneration of shoots or somatic embryos from leaves or corm fragments without an intermediate callus phase or, moreover, through indirect organogenesis from callus. It is important to note that the callus development pathway involves the risk of genetic or epigenetic modification (George 2003). The induction of embryogenic callus able to regenerate plants through somatic embryos has a high interest if related to the bioreactor culture assessment; in this case, the full automation could drastically reduce costs and bring the multiplication rates to levels

Table 1 Growth parameters and characters of the extra-season *Gladiolus* genotypes (BREA Breeding Farm, Migliarino (D)) (Ruffoni *et al.* 2011).

| | |
|--------------------|--|
| Growth environment | Open air in Mediterranean area (never below zero) |
| Temperature | 5-20 °C |
| Photoperiod | Short day |
| Programming | Planting at end of August - Blooming from 15th December up to the end of January |
| Flower quality | High |
| Features | <i>Fusarium</i> resistance |

comparable to *in vivo* propagation efficiency (Takayama 1998).

Meristem excision for virus eradication

The shoot apical meristem (SAM) is usually virus-free. Therefore, excision of SAM followed by outgrowth *in vitro* results in virus-free shoots. This system is very common to establish “clean” cultures avoiding an intermediate callus phase and that can guarantee the absence of virus contamination. It can be combined with a previous heat treatment to corms before sprouting and furthermore to a similar heat treatment to the *in vitro* growing shoots. An example of this two step strategy was reported for lily by Nesi *et al.* in 2009.

In our experiments, corms of 20 valuable extra-season *Gladiolus* genotypes were washed under tap water and allowed to sprout at 15°C in peat-perlite (1:1 V:V) sterile substrate. The physiological and cultural characteristics of the genotypes are summarized in **Table 1**. Meristems for each genotype were excised from the sprouted shoots (5 cm in length) and cultured in the dark at 15°C in MS (salts and vitamins) basal agar-solidified medium and subcultured 6 times every 15 days on the same medium. It was possible to obtain viable meristems in 18 out of 20 genotypes with a total mean of 30% asepsis from bacteria and fungi (Ruffoni *et al.* 2008). During 3 months in the hormone-free MS medium, the meristems were able to develop some axillary shoots confirming the sprouting potentiality of the SAM. Those genotypes, after transfer and growth in the field, did not show any virus contamination.

Direct shoot regeneration

Direct regeneration of shoots without an intermediate callus phase can be achieved from corm slices cultured on medium with BA (1 mg/l; Sutter 1986; Kamo 1995). The authors confirmed that plants regenerated vigorously and with a low risk of abnormalities in fact direct regeneration seems not to be genotype-dependent. Longitudinal corm sections resulted in more propagules than shoot tips, basal plate or daughter corm explants (Nhut *et al.* 2004).

Lateral corm buds (cormels) were used as explants for *Gladiolus anatolicus* micropropagation by Emek and Erdag (2007) that used 2 mg/l of BA and achieved a mean of 11 shoots/explant. Aftab *et al.* (2008) reported a direct regeneration protocol for several *Gladiolus* hybrids starting from corm slices that proved to be in this case the best explant producing a higher regeneration rate (16 shoots/vessel) than meristems (10 shoots/vessel).

Callus mediated regeneration

Non-differentiated cells of most *Gladiolus* species show a high regeneration potential. Callus able to regenerate plants has been induced from various explants (inflorescence stalk, corm slices, basal meristem, shoot tips, ovaries and young leaves; **Table 2**). Plants have been developed from callus as buds and shoots regeneration (neo-organogenesis) but also from cells suspension via primary and secondary somatic embryogenesis. Regeneration from callus occurred on hormone-free medium and was enhanced by addition of 2 mg/l of kinetin (Kamo 1994). Kumar *et al.* (1999) found that prolific shoot regeneration can be obtained on MS medium following a heat shock at 50°C for 1 h and by culturing the

Table 2 List of several authors reporting callus mediated regeneration for culture initiation in *Gladiolus* spp.

| Explant type | Growth regulators for callus development | Growth regulators for regeneration | Type of regeneration | Author, year |
|----------------------------------|---|--|-----------------------------------|-----------------------------|
| Corm slices, young leaf base | 2,4-D (2 mg/l) NAA (10 mg/l) | None | Somatic embryos | Stefaniak 1994 |
| Middle section of cormels | 2,4-D (2 mg/l) Casein hydrolysate (1 g/l) | BA (0.05 mg/l) or ZEA (0.05 mg/l) | Somatic embryos | Remotti 1995 |
| Cormel slices, plantlets | Friable callus: NAA (10 mg/l) 2,4-D (0.5 mg/l) Dicamba (2 mg/l); Compact callus: BA (1 mg/l) | From friable callus: Kinetin (2 mg/l); none from compact callus | Somatic embryos | Kamo 1994 |
| Corm slices, inflorescence axes | BA (1.1 mg/l) + 2,4-D (1.1 mg/l) | BA (0.25 mg/l) and NAA (2 mg/l) | Shoots | Kumar 1999 |
| Primary leaves from corm | NAA (2 mg/l) | NAA (0.2 mg/l) and BA (2 mg/l) | Meristematic bud clusters | Prasad and Dutta Gupta 2006 |
| Leaves of <i>in vitro</i> shoots | NAA (5 mg/l) | BA (0.1 mg/l) and then BA (0.1 mg/l) + sucrose (20 g/l) | Somatic embryos | Emek and Erdag 2007 |
| Cormel slices | NAA (0.3 or 0.4 mg/l) or BA (1 mg/l) + 2,4-D (2 mg/l) | NAA (0.3 or 0.4 mg/l); 2,4-D (2 mg/l) and BA (1 mg/l) | Root induction; shoots (sporadic) | Aftab <i>et al.</i> 2008 |

callus with 0.25 mg/l of BA and 2 mg/l of NAA. The first histological documentation of somatic embryo formation followed by plant regeneration was reported by Stefaniak in 1994. Corm slices of *in vitro* grown plants were cultured with 2,4-D (2 mg/l) and NAA (10 mg/l) and using Phytigel as solidifying agent. Somatic embryos differentiated from the callus surface as further confirmed by other authors; the researcher did not observe vascular connections between SE and the mother tissue. Stefaniak described two callus types: embryogenic (friable, white, dry and crumbly) and non-embryogenic (white/yellowish and soft). The embryogenic callus was maintained for 2 years retaining the embryogenic capacity. The author obtained a 99% greenhouse survival of the regenerated plants via the SE pathway and for the genotypes considered, no off-types were found. In contrast, Remotti (1995), for the genotype *Gladiolus x grandiflorus* 'Peter Pears', found regenerated plants coming from secondary embryogenesis with a small number of albino plants indicating a certain degree of somaclonal variation.

For the use of the embryogenic cell cultures in bioreactors, the first step is the development of an embryogenic suspension culture able to maintain a high level of differentiation ability. A clear definition of suspension culture referred to *Gladiolus* has been given in 1995 by Remotti: "well dispersed clusters of 25-50 cells that double in packed cell volume (PCV) in two weeks". Cell suspensions were first reported by Simonsen and Hildebrandt in 1971 using MS liquid medium supplemented with 0.1 mg/l NAA. Callus induced in the presence of 2,4-D is mostly friable and has been used to establish suspension cultures particularly suitable for transformation by particle gun method (Kamo 1995). No reports have been published about the automation of the production of *Gladiolus* somatic embryos.

Virus eradication by chemical treatment to the callus

An alternative system for virus eradication was reported in *Gladiolus psittacinus* var *Hookeri* cv 'Red' by Singh *et al.* (2007). They started the *in vitro* culture from the cormel basal plate of infected plants. Callus developed and it was treated for 6-8 weeks with virazole (ribavirin, 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide). Calli were analysed with several techniques for the virus indexing (electroblot immunoassay, NASH and RT-PCR or duplex RT-PCR) and the calli showing absence of virus particles were transferred for regeneration to half-strength MS salts supplemented with 2 mg/l of Kinetin and then to the MS base medium supplemented with 0.25 mg/l of NAA for cormel differentiation. The plants obtained were tested again for the virus presence; virus-free *Gladiolus* plantlets were produced after treatment with 40 mg/l of virazole. The com-

Table 3 Composition of the media used for the growth in temporary immersion (liquid medium) or supplemented with 0.8% of technical agar (solid medium).

| | Shoot-producing medium | Corm-producing medium |
|--|--------------------------------|--------------------------------|
| Microelements, macroelements, vitamins | Murashige and Skoog, 1962 (MS) | Murashige and Skoog, 1962 (MS) |
| 2 iP | 0.6 mg/l | - |
| IBA | - | 1 mg/l |
| sucrose | 30 g/l | 30 g/l |
| pH | 5.7 | 5.6 |

parison among the different systems to detect the virus presence and their reliability is discussed in the same paper (Singh *et al.* 2007).

In this case, no information was released about the genetic stability of the regenerated plants; in general the risk of modifications is higher in the case of callus differentiation than in the meristem culture.

MICROPROPAGATION IN SEMI-SOLID MEDIUM

Shoots or buds obtained by axillary branching or regeneration were first placed on agar-solidified media (semisolid media) containing cytokinins for proliferation induction. Most authors used BA at a wide range of concentration, depending mainly on the genotype. In the case of our Mediterranean genotypes, two alternative multiplication strategies have been evaluated: the use of 2iP 0.6 mg/l compared with IBA 1 mg/l (Table 3). It was assessed that corms can develop either using IBA or 2iP with different efficiency level, depending on the genotype; it is clear that in the presence of the cytokinin 2iP either corms and shoots can develop from the mother explant (Fig. 1) but in the presence of IBA the growth of shoots was strongly inhibited (Fig. 2) (Ruffoni *et al.* 2008).

MICROPROPAGATION IN LIQUID CULTURE

The culture in semisolid media usually reduces the size of the plant probably because of the poor level of water flow; the agar mechanically prevents the gaseous exchange and the nutrient uptake of embedded portions of the tissue (Prasad and Dutta Gupta 2006). The liquid culture permits the plant tissues enlargement together with internode increase and stimulates root emission; this is important for the further acclimatization phase in the greenhouse. On the other hand, explants fully immersed in liquid medium may accumulate too much water in the apoplast resulting in physiological, anatomical and morphological abnormalities

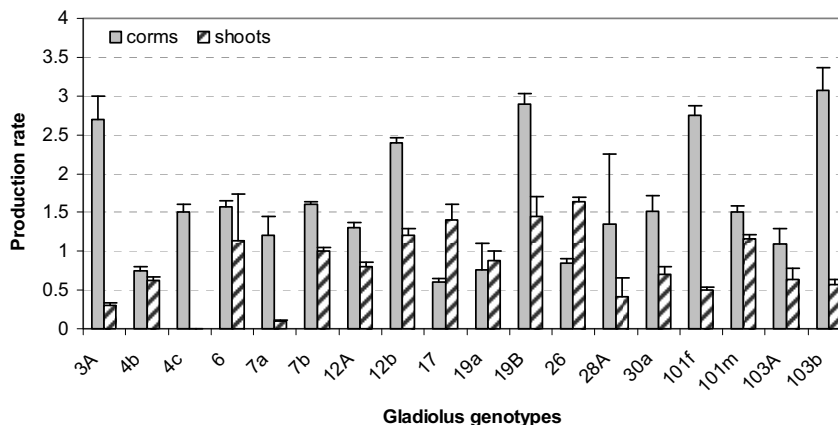


Fig. 1 Multiplication of *Gladiolus* on semi-solid medium. Evaluation of the propagation rate (shoots or corms \pm S.E.) per explant after 40 days in the medium containing 0.6 mg/l 2iP with 18 genotypes (improved from Ruffoni *et al.* 2008).

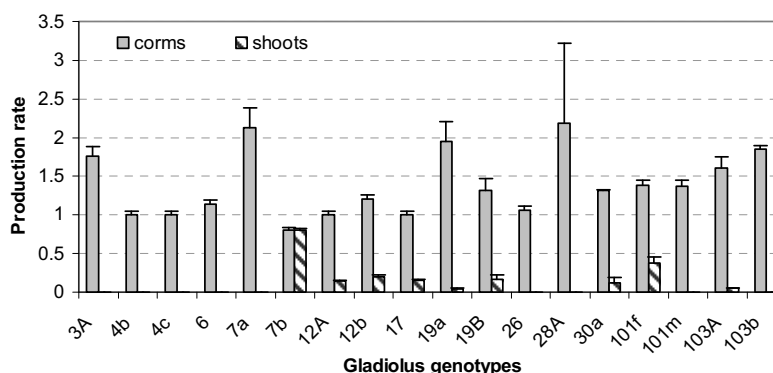


Fig. 2 Multiplication of *Gladiolus* on semi-solid medium. Evaluation of the propagation rate (corms or shoots \pm S.E.) per explant after 40 days in the presence of 1 mg/l IBA with 18 genotypes (improved from Ruffoni *et al.* 2008).

known as hyperhydricity. This makes the tissues recalcitrant and the plantlets unfit for field acclimatization.

To avoid or minimize hyperhydricity in the liquid culture the use of inert support materials such as glass beads, polyurethane foam, rock wool and coconut coir has been suggested and tried in several plant species (Conner and Meredith 1985; Afreen-Zobayed *et al.* 2000). A further development of those techniques is the temporary immersion (TIS).

Another strategy to avoid hyperhydration, can be applied only to bulbous plants and consider the possibility to work on the reserve organ inducing multi-propagule structures with less developed leaves able to be multiplied in automatized systems and to survive outside. For these reasons the authors dealing with this strategy explored the chance to use growth retardants together with the modulation of the carbohydrates levels (Ziv 1989).

Liquid shake flask culture

The first experiments in liquid culture were performed in agitated Erlenmeyer flasks. Steinitz *et al.* in 1991 tried combinations of PCB, an inhibitor of GA-biosynthesis, and sucrose. They found that total plant dry weight increased when the sucrose concentration was raised from 20 to 60 g/l. At each sugar concentration, the addition of PCB, reduced the plant dry weight. However, PCB increased the percentage of *Gladiolus* plants with corm formation and corm fresh weight reached the highest value with addition of PCB and 60 g/l of sucrose. This result demonstrates that all the *Gladiolus* plant benefited from high sugar presence but a differential promotion of corm development by sucrose alone was never noticed. In the absence of growth retardants, the leaves showed succulent tissue and continued to elongate (Ziv 1989). On the contrary, with PCB, leaf expansion and root elongation were limited with sucrose in the medium and sucrose was utilized almost exclusively for

corm filling. So, in *Gladiolus*, as in other plant systems (Davis *et al.* 1988), PCB shifted assimilate allocation towards the storage organs. Comparing the effect of other growth retardants, Ziv (1989) found that PCB induced in *Gladiolus* cv 'Eurovision' the highest number of buds per explants (38) while ancymidol and Majic 27 and 24, respectively; the buds turned into protocorm aggregates and the largest size protocorms was observed in explants treated with ancymidol and Majic. Steinitz and co-workers (1991) proposed a procedure to obtain large corms in *Gladiolus* hybrids (*grandiflorum* \times *tristis*) that includes treatment with BA in semi-solid medium followed by PCB in shake culture. Good induction of bud proliferation is related to the correct ratio between number of initial explants and volume of liquid medium (Nhut *et al.* 2004).

Liquid culture in coir

Liquid medium with an alternative matrix as coir from coconut husk, which is an ecofriendly biodegradable matrix, was tested by Subhash *et al.* in 2006 for *Gladiolus* 'Pacifica'. The development of micro corms started within 45 days and the maturation within 90 days. They found that corms coming from the liquid culture reached higher size than those developed in the control semi-solid cultures (up to 25 mm rather than up to 10 mm). Then the corms were dried in desiccator for 60 days prior the use as seeds.

Liquid culture with support systems

Prasad and Dutta Gupta (2006) reported results about a system that consider a divisory structure at the interface between plant tissue and liquid medium; they compared the effects of the polypropylene Membrane Raft (MR, Osmotek™, Rehovot, Israel) with those of 0.3 μ m pore size or the Duroplast Foam of 0.3 cm thickness (DF, polyurethane foam, Sheela Foam Pvt. Ltd, Ghaziabad, India). In the



Fig. 3 (A) Temporary immersion system (TIS) with RITA[®] containers, for *Gladiolus* the flooding time was set up for 3 minutes every 3 hours. (B) *Gladiolus* TIS culture, development of new corms, 30 days after culture initiation in IBA. (C) *Gladiolus* TIS culture, corm development together with leaf sprouting, 45 days after culture initiation in medium with IBA. (D) *Gladiolus* TIS culture, corm development together with leaf sprouting, 90 days after culture initiation in medium with IBA. (E) *Gladiolus* corms with diameter < 5 mm after drying and ready to be stored at 6°C. (F) *Gladiolus* corms with diameter > 5 mm after drying and ready to be stored at 6°C. (G) *Gladiolus* micropropagated material after storage under slow-growth conditions for 6 months. The explants are ready to be transferred to liquid multiplication medium. (H) *Gladiolus* storage under slow-growth conditions. (I) *Gladiolus* plants after transfer of tissue-culture corms to soil.

Gladiolus hybrid ‘wedding bouquet’, they found that a combination of NAA and BA (1 mg/l and 2 mg/l respectively) gave the best results for the MR-system (33 shoots per cluster), while the combination of NAA and BA (0.5 and 4 mg/l, respectively) was the best for the DF-cultured plants (24 shoots/cluster). They evaluated also the trend of shoot multiplication and elongation in the two systems through an original calculation of regression coefficients. Shoot regeneration was linearly correlated to the incubation period (in MR system the relative rate was 5.7% every 7 days of culture) in both systems while shoot elongation varied significantly with culture type. The use of MR support has several advantages: better aeration and dispersion of phenolic exudates in the medium. In this case, the immobility of the support nullified the shear stress and the mechanical injuries.

Liquid culture in TIS

In our experience, *Gladiolus* corms were cultured in TIS using RITA[®] vessels (CIRAD, France) (Fig. 3A). The

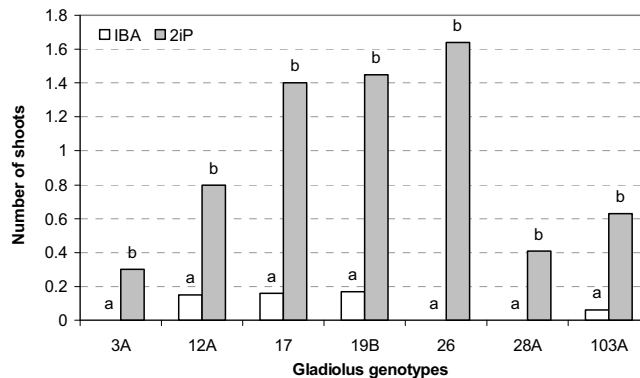


Fig. 4 *Gladiolus* TIS culture. Evaluation of the number of shoots per explant obtained in the presence of 2iP or IBA (within genotype, different letters indicate values that differ at $p=0.05$ SNK test) (improved from Ruffoni *et al.* 2008).

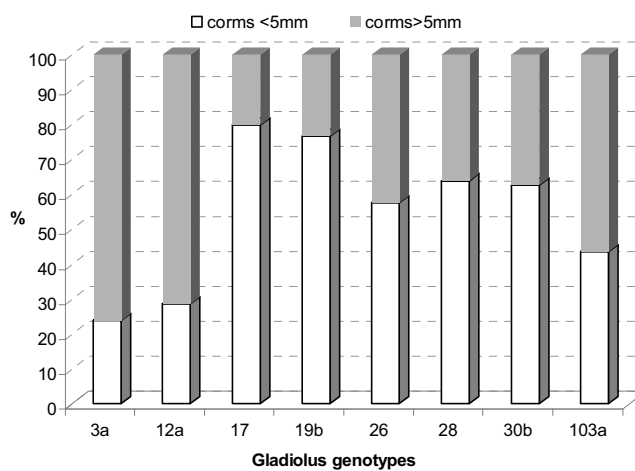


Fig. 5 *Gladiolus* TIS culture with IBA 1 mg/l. Evaluation of the percentages of large and small corms (improved from Ruffoni *et al.* 2011).

Table 4 Quantitative and qualitative evaluation of the *Gladiolus* corms produced in TIS.

| Clone | Diameter (cm ± S.E.) | Weight (g ± S.E.) | Corm number (± S.E.) |
|-------|----------------------|-------------------|----------------------|
| 3 a | 0.660 ± 0.027 | 0.221 ± 0.023 | 46 ± 2 |
| 12 a | 0.725 ± 0.028 | 0.358 ± 0.045 | 28 ± 5 |
| 17 | 0.616 ± 0.023 | 0.147 ± 0.014 | 213 ± 7 |
| 19 b | 0.556 ± 0.013 | 0.128 ± 0.011 | 167 ± 5 |
| 26 | 0.566 ± 0.017 | 0.123 ± 0.017 | 92 ± 19 |
| 28 a | 0.620 ± 0.027 | 0.204 ± 0.026 | 94 ± 14 |
| 30 b | 0.610 ± 0.017 | 0.192 ± 0.014 | 150 ± 24 |
| 103 a | 0.777 ± 0.043 | 0.380 ± 0.057 | 62 ± 12 |

RITA[®] system consists of an autoclavable polypropylene 500 ml vessel in which the liquid medium is pushed up by an air pump at regular intervals providing the immersion of the plantlets in liquid medium for a short period of time. After preliminary trials, 3 min of immersion every 3 hours was used. We used 150 ml liquid medium per vessel containing 0.6 mg/l 2iP or 1 mg/l IBA. TIS culture was carried out at 24°C and 16 h light at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. Replications with 12 explants each were carried out for each condition for 7 genotypes of Mediterranean *Gladiolus* hybrids. Proliferation and corm diameter were evaluated after 90 days of culture. In the RITA[®] vessels the plants grew faster than in solid medium and it was not necessary to subculture the material up to the 90th day of culture. After 90 days, it was possible to recover corms very easily. A time-course summary of the RITA[®] cultures condition for *Gladiolus* corms is shown in Fig. 3B-C-D. The presence of 2iP increased the vegetative development and more

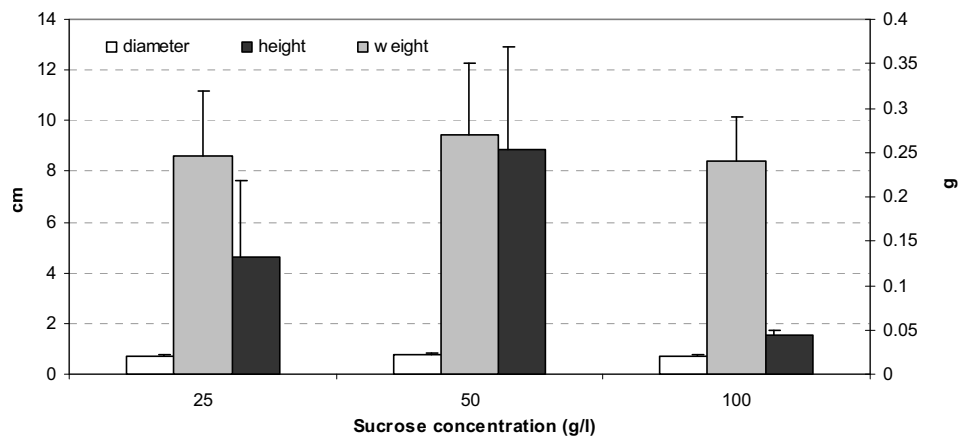


Fig. 6 *Gladiolus* TIS culture with IBA 1 mg/l. Evaluation of length of the shoots and the diameter and weight of the corms in the presence of different concentration of sucrose (mean of three genotypes \pm S.E.) (Ruffoni *et al.* 2011 – assembled and improved).

Table 5 *In vitro* storage of 8 *Gladiolus* hybrids.

| Genotype | Growth regulator in the medium (mg/l) | Corms with cormels (%) | Multiplication rate | Corms showing new shoots (%) | Corm weight (g) |
|----------|---------------------------------------|------------------------|---------------------|------------------------------|-----------------|
| 3a | 2iP 0.6 | 41 | 1.6 | 32 | 0.462 |
| | IBA 1 | 55 | 1.5 | 14 | 0.430 |
| 30a | 2iP 0.6 | 51 | 1.7 | 30 | 0.424 |
| | IBA 1 | 52 | 1.5 | 35 | 0.422 |
| 103a | 2iP 0.6 | 23 | 1.3 | 43 | 0.244 |
| | IBA 1 | 52 | 1.8 | 7 | 0.277 |
| 7a | 2iP 0.6 | 50 | 1.6 | 5 | 0.436 |
| | IBA 1 | 44 | 1.4 | 25 | 0.830 |
| 103b | 2iP 0.6 | 19 | 1.2 | 0 | 0.272 |
| | IBA 1 | 43 | 1.6 | 3 | 0.393 |
| 12b | 2iP 0.6 | 48 | 1.7 | 19 | 0.325 |
| | IBA 1 | 30 | 1.3 | 26 | 0.670 |
| 19a | 2iP 0.6 | 62 | 1.9 | 26 | 0.348 |
| | IBA 1 | 61 | 1.7 | 56 | 0.504 |
| 4c | 2iP 0.6 | 54 | 1.8 | 0 | 0.244 |
| | IBA 1 | 36 | 1.6 | 0 | 0.746 |

shoots appeared with 2iP than with IBA (Fig. 4). Corms developed in both media with significant differences among the genotypes. Very interesting productions were achieved with genotype 17, 19b and 30b with 213, 167 and 150 cormels per vessel, respectively. In these genotypes a great percentage of cormels had a diameter $<$ 5 mm (Fig. 5). Diameter and weight of the cormels $>$ 5 mm are shown in Table 4.

In a trial focused on three promising genotypes, testing different sucrose concentration (25-50-100 g/l), the addition of 50 g/l of sucrose in the IBA containing multiplication medium increased significantly the height of the shoots but did not affect the weight or the diameter of the corms (Fig. 6). The same result was observed by Steinitz *et al.* (1991).

Cormels were then recovered, dried in laminar air flow for 24 hours (Fig. 3E and Fig. 3F) and maintained at 5°C for 6 months. After cold storage, the cormels were treated as seeds (Fig. 3G).

After this screening of a significant number of genotypes, good performances in terms of production rate, quality and field emergence were obtained and these results are particularly interesting for some of them. This suggests that the production of cormels seed-size could be done in automatized vessels with reasonable costs that could be supported for a commercial exploitation.

IN VITRO STORAGE

An additional application of the *in vitro* techniques is slow-growth storage. We evaluated *in vitro* conservation of tissue-cultured material in 8 clones (Table 5). The corms were stored at $2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 18 h per day, at 15°C in semi-solid medium containing MS base salts and vitamins

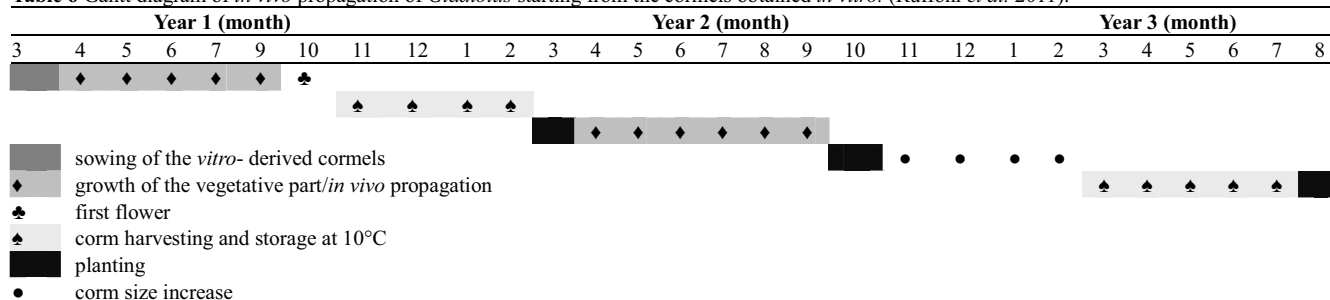
and 2iP (0.6 mg/l) or IBA (1 mg/l). Evaluation of corm proliferation, multiplication rate, the occurrence of vegetative growth, weight of the corms and viability of the material were performed every 40 days for a period of 8 months of storage (Fig. 3H).

Storage of the *in vitro* material as corms allowed to maintain viability in all genotypes without losing growth potential (shoot emergence). The maximum survival rate was achieved in genotype 19a (54%). A low, but constant rate of proliferation guaranteed a rapid growth recovery after transfer to the standard growth conditions. No weight differences related to the tested growth regulators in the medium were observed in 3 genotypes (3a, 30a, 103a) while in the other 5 genotypes the presence of IBA in the medium permitted a higher accumulation of reserve substances than the medium supplemented with 2iP (Table 5). The possibility to store the material in this way and recover viable material is, by a commercial point of view, a new opportunity to decrease the general propagation costs.

TRANSFER TO SOIL

The acclimatization phase has always been a difficult step in micropropagation, in particular for woody and semi-woody plants. In the case of *Gladiolus* some difficulties have been reported. Ziv (1979) observed hindrances in transplanting shoots of cv. 'Eurovision' to soil. The protocol included culture of shoots on half strength MS medium with activated charcoal (0.3%), a low sucrose level (1.5%) and high light intensity. The acclimatization percentage depended on the extent of roots (Lilien-Kipnis and Kochba 1987).

In order to obtain high acclimatization percentages is better to transplant cormels rather than shoots. The success

Table 6 Gantt diagram of *in vivo* propagation of *Gladiolus* starting from the cormels obtained *in vitro*. (Ruffoni et al. 2011).

is also due to the cormel size and the corm diameter is directly proportional to the sucrose concentration (6-10%) (Danjtu and Bhojwani 1995). After TIS culture in RITA[®] vessels, single cormels were recovered and dried. They were then maintained for 6 months at 5°C. The cormels were then sown directly in soil resulting in 100% sprouting (Fig. 3I). All plants developed the first flower after 5 months. In our experience, the cold storage was essential for a fast bud emergence.

CONCLUSIONS

It was shown that several tissue culture strategies could be combined fruitfully to support breeding programs for *Gladiolus*. From the selected new genotypes for growth in Mediterranean area, virus-free plants were obtained and a fast and reliable micropropagation system was established. This system was first set up essentially to prepare mother stock plants in perfect sanitary conditions, to be used to multiply the corms in field but *Gladiolus* corms, grown in the semi-automatic bioreactor RITA[®] produced high numbers of propagules (cormels) that could be considered as “seeds”, easy to plant *in vivo*. Furthermore, a scheme of the *in vivo* propagation starting from the cormels obtained *in vitro* was set up by the growers of the same breeding group that supplied the material (Table 6).

Like other tropical species that are already commercially propagated by liquid culture in temporary immersion, *Gladiolus* demonstrated to be particularly suitable for the growth in TIS.

The *in vitro* efficiency is proved to be related to the genotype, also for *Gladiolus*. Some genotypes are more productive and for these it could be possible to compare the costs of *in vitro* propagation with those of traditional *in vivo* propagation considering also the possibility to save at least one year to introduce a new genotype on the market. In addition, the propagation *in vitro* through cormels rather than shoots, seems to be very attractive mainly for the possibility to produce single propagation units that can be stored *in vitro* or *in vivo* and that don't require acclimatization. This opportunity is normally considered to be made through somatic embryogenesis and consequently artificial seeds, but, in the case of *Gladiolus*, this differentiation pathway needs more research efforts to guarantee genetic stability.

We propose a strategy with the combination of the meristematic explants excision, virus control and then the Temporary Immersion System that, after a deep evaluation, could be economically competitive to the traditional propagation in the field.

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