**ACCLIMATION UNDER RUSTIC CONDITIONS OF *IN VITRO* PROPAGATED GLADIOLUS SEEDLINGS**

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**ABSTRACT**

In Mexico, the gladiolus (*Gladiolus grandiflorus* Hort.) asexual propagation is carried out with corms and cormels produced by the own farmers, but this type of reproduction is problematic when trying to obtain high quality corms because diseases spread more easily. Therefore, the aim of this study was to determine the most suitable environment for growth acclimation of *in vitro* and *Fusarium* free plants. As for *in vitro* propagation five phytohormone combinations were evaluated, and for acclimation studies, four rustic environments on a completely randomized experimental design were studied. The highest regeneration percentage, number of roots, plant height and 100 % free of fungus plants were obtained when using naphthaleneacetic acid (0.2 mg/l-1). The best acclimatization environment was when using 75 % shading mesh which promoted the highest survival (95 %), plant height (24 cm), total biomass (2.5 g/plant-1), corm biomass (1.20 g) and photosynthetic efficiency index (2.9); additionally, the variable and maximum fluorescence ratio was nearly the optimal (0.83). This procedure is an option for massive propagation of healthy gladiolus plants under rustic environments.

**Keywords:** Explants, ornamental plant, photosynthetic efficiency, production.

In Mexico, gladiolus is grown in open fields from corms and cormels (vegetative seed) which are mostly produced (80 %) by local farmers, such as in San Martín Texmelucan, Puebla (SMT, Pue.) (González-Pérez et al. 2011). The main problem of this traditional form of reproduction, when it is not totally controlled with fungicides, is the corm rot caused mainly by *Fusarium oxysporum* f. sp. *gladioli,* which demerits the physical, physiological and sanitary quality of the propagules (corms and cormels) (Chandel, Deepika 2010). In the field, the infected propagules can lead to new rotten propagules, wilting and death of the plants, and in extreme conditions to a total crop loss (González-Pérez et al. 2009).

Relying on integrated methods for mass propagation that guarantee the sanitary quality of the corm is of very high commercial interest; among the components of integrated management, an alternative is the *in vitro* vegetative propagation (Emek and Erdag 2007). In Holland, the gladiolus corms are reproduced by micropropagation in greenhouses under controlled conditions at a commercial level (Cantor, Tolety 2011); in India (Gupta, Prasad, 2010) the *in vitro* formation of seedlings and corms has been induced completing their vegetative cycle in the greenhouse. Furthermore of the vegetative reproduction by this technique, it is also necessary to establish the acclimatization process of the material (relative humidity, soil moisture, irradiance level, ground and air temperature, etc.) outside the greenhouse to promote its development (Roy et al. 2006). Since still such information is missing and that farmers generally do not have greenhouses, the aim of this study was to determine the most appropriate acclimatization environment under rustic conditions for growth of the plants generated *in vitro* and *Fusarium* free, with the hypothesis that the *in vitro* seedlings lack of the physiological mechanisms to survive in conditions without environmental control.

**MATERIALS AND METHODS**

**Explants samples.** In the winter of 2010, in a batch of 500 boxes of corms of the variety "Borrega Roja" produced in the study area (SMT, Pue, 19º12’18’’ NL, 98º26’54’’ WL, altitude 2425 meters over the sea level), 120 apparently healthy corms were randomly selected. Their robes were detached, washed in running water for 20 min (to remove impurities) and dried on sterile paper towels. Then with a stereoscopic microscope, a portion of the apical meristem (3-5 mm thick) was removed from each corm and used as the explant samples. **Explants disinfestation.** These samples were disinfected with 80 % ethanol (v/v) for 2 min, rinsed with sterile distilled water, dried on sterile paper towel and again disinfested with 1.5 % sodium hypochlorite for 3 min, and one time more rinsed and dried as indicated above (Remotti and Löffler, 1995).

***In vitro* explants**

**Phytohormones treatments.** The explants were cultured on MS medium (Murashige, Skoog, 1962) supplemented with 30 g L-1 sucrose, 4 g/l-1 agar and growth regulators (auxins and cytokinins). Five treatments were prepared: three concentrations (mg/l-1) of dichloro phenoxyacetic acid (2, 4 D; 0.2, 0.6, and 1.0), one of naphthalenacetic acid (NAA; 0.2), one of indoleacetic acid plus kinetin (IAA/ KN, 0.2 / 0.05), and a control with no growth regulators. The pH of the medium was adjusted to 5.8 before autoclaving (Isolable, Abrogate GMBH) at 120 °C during 15 min. From each treatment, 20 ml of solution were poured in flasks of 120 ml capacity and four explants per flask were seeded; five replicates per treatment were done in aseptic conditions inside a laminar flow chamber (ESCO). **Incubation phases.** Explants went through two stages of incubation in a bioclimatic chamber at different time intervals. The first one was a photoperiod of 16/8 h light/dark at 26 ± 2 °C during 15 days; at the end of this phase, the explants with roots and shoots were transferred to plastic cups containing peat (Premier PRO-MIX® PGX) previously disinfested and hydrated, where the second phase was carried out under the same conditions of incubation for another 18 days. **Variables evaluated.** At the end of the second phase the following morphogenetic responses in 10 seedlings were evaluated: regeneration (%; frequency of explants with root, stem and leaf), number of roots per corm and seedling height (cm) taken from the base of the corm to the leaf apex.

**Propagation and transplantation.** With the best treatment and same incubation conditions, 400 plants were generated. Under aseptic conditions the seedlings were washed with distilled water, dried on sterile paper towels and transferred to 0.5 l plastic pots (two seedlings per pot), 12 cm in diameter and containing sterilized peat at 120 °C for 20 min. In order to keep the RH around the plant, the pots were covered with 1.0 l clear plastic cups and incubated in bioclimatic chamber at 16/8 h light/dark and 26 ± 2 °C for 10 days.

**Acclimation of propagules**

**Pre-climatic adaptation.** At the end of the bioclimatic phase, the plants were removed from the chamber and placed in a tunnel with a 50 % shading mesh of 25x15 threads per inch-2 (BIOMALLA; HDPE monofilament yarn) in rustic environmental conditions without temperature and humidity control for seven days (Schiappacasse et al. 2006). Three side holes were done to the 1.0 l clear plastic cups and removed three days later. During the acclimatization two light waterings were applied.

**Environments tested.** Seven days after transplanting the seedlings, they were transferred to new 1.0 l pots of 16 cm in diameter and containing sterilized and humid peat. An experimental design of completely random four experiments was established, the four acclimation experiments were the following: 50 % reduction shading mesh (25x15 threads per inch-2), 75 % reduction shading mesh (44x14 threads per inch-2), milky white polyethylene plastic cover with 70 % light transmission, and an open air control experiment. In each environment 50 pots (two seedlings per pot) were placed. During the acclimation process, light watering was applied three times per week and twice per week a fertilization with the formula 20N - 30P - 10K. The response variables underwent a variance analysis by using the Statistical System (SAS Institute 2009) software and a Tukey means comparison (p ≤ 0.05). The variables measured in percentage were transformed with the arcsine formula √ (100/X).

**Evaluated parameters**

**a) Environmental variables.** In order to record the air temperature (°C) and RH every 30 min, a Data Logger (HOBO H8-032-08, Onset Computer Corporation) was placed on each environment. The average, maximum and minimum temperatures were recorded and calculated, as well as the days with temperatures below 0°C (frosty days). In addition, daily irradiance values were obtained from the meteorological station at Campo Experimental del Colegio de Postgraduados Campus Puebla (19° 04’ 27.7’’ NL and 98° 15’ 38.14’’ WL and 2135 masl) located 5.0 km from the experimental area.

**b)** **Plant variables.** The percentage of plant mortality on each environment was recorded every 4 d, and the final percentage of survival (SV) was calculated at the 42nd d. Also, after 42d of being transferred to different environments, the registered variables from six randomly selected plants per treatment were: plant height (measured from the ground level to the apex of the longest leaf, PH, cm), total biomass (included root, corm and leaves, TB; g) and corm biomass (CB; g); additionally, the temperature of the widest and erected leaf (of six random and well hydrated plants) was measured with an infrared thermometer (TN408LC ZyTemp) at noontime with clear skies and no wind.

**c)** **Photosynthetic efficiency.** Chlorophyll fluorescence was measured 42 days after the plants were transferred to their environments with a Handy PEA (Hansatech Instruments, United Kingdom) in six plants per environment; measurements were done in the widest leaf of the plants with more than two leafs. The fluorescence was induced by a pulse of a second red light (640 nm) emitted by six diodes (600 W/m-2) (Strasser et al. 2000) and read with a PIN photodiode with a 50 % transmission filter at 750 nm. The values registered were: Variable fluorescence (Fv), maximum (Fm) and minimum (Fo), Fv / Fm ratio, and the photosynthetic efficiency index (P. index).

***Fusarium* evaluation.** In order to assess *Fusarium* presence in seedling corms produced *in vitro* with the best phytohormones treatment, twenty corms which were transferred to pots, were washed with tap water for 20 min and dried on sterile paper towel inside a laminar flow chamber, then they were cut longitudinally and disinfested as mentioned above for explants. Corms halves were sown in Petri dishes with PDA medium (potato dextrose agar) and incubated for 10 d at 21 °C in 40 W (36 μE/cm-2/s-1) lamps of continuous white light. After seven days, the number of colonies of *Fusarium* was counted and identified (Booth 1971; Leslie, Summerell 2006) as well as other fungi.

**RESULTS AND DISCUSSION**

***In vitro* explants**

There were significant differences (p ≤ 0.05). The fourth treatment with NAA resulted in the highest regeneration percentage, highest number of roots and the highest seedlings height (Table 1). The other treatments produced fewer seedlings (15-50 %), whereas in the control experiment without hormones there was no regeneration at all.

In the five treatments, except for control, meristem elongation started 4 d after the *in vitro* establishment and rooting began the 6th d and ended at 12th d. The root length was not statistically different (p ≤ 0.05) and all explants had 1.3 ± 0.2 cm average length.

In general, results of various research investigations of *G. grandiflorus* with NAA have been successful. Torabi-Giglou and Hajieghrari (2008) obtained similar results to those reported in this work at 0.5, 1.0 and 2.0 mg/l-1 concentrations in the “Pink” cultivar; while Faheen et al. (2008) found better responses on Murashige and Skoog medium supplemented with 0.5 mg L-1 NAA in “Pink and White” hybrid cultivars. In another gladiolus species, *G. anatolicus*, Emek and Erdag (2007) obtained better rooting with 0.2 mg/l-1 NAA. Other authors report that 0.1 mg/l-1 NAA promotes the root differentiation without generating callus and at 2.0 mg/l-1 leaves production is induced (Aftab et al., 2008). In horticulture, auxins are used to accelerate rooting because they stimulate cell division in the cambium and the xylem and phloem differentiation, and according to Beyl and Trigiano (2008) they are involved in the roots induction and they can cause positive effects on the seedlings regeneration because of the carbohydrates redistribution that can be modified during the rooting process (this depends on the physiological condition of the explant). The NAA presence in the culture medium confirms that root induction is a function of the incorporation of growth regulators. The absence of hormones in the control, as expected, did not allow the development of structures because of the absence or the insufficient concentration of endogenous hormones in the explant, which prevented the differentiation process, similarly to Remotti and Löffler (1995) reports who mentioned that the explants do not generate structures in the absence of hormones.

**Propagation and transplantation**. The survival of seedlings generated under the NAA best treatment (4) was 100 %. With this treatment, the leaf size and the root length increased, some plants showed a second leaf, and the differentiation of the new corm started.

**Acclimation of propagules**

**Pre-climatic adaptation.** All seedlings survived during the 7 d that were under the 50 % reduction shading mesh, although there was no apparent growth or development during this phase.

**Environment.** The average and maximum air temperature were similar in the open air treatments to those carried out under 50 and 75 % irradiance mesh, and they were higher under the plastic cover. The lowest average mean temperature was recorded in the open air treatments and there were eight days with temperatures ≤ 0 °C, while in the other three environments just one frosty day was recorded (Table 2). In this regard, Cantor and Tolety (2011) reported that the maximum temperature for gladiolus growth is 32°C and the lowest is 5 °C, therefore, open air acclimation is not recommended. Memon (2012) reports that gladiolus seedling generated *in vitro* and transferred to greenhouse conditions show better survival at cool temperatures (18 ± 2 °C) in mesh treatments.

**Plant variables.** There were significant differences (p ≤ 0.05). The lowest percentage of plant mortality and therefore, the highest survival was obtained with the 75 % reducing mesh, followed by the 50 % mesh (Table 3); however, with the 75 % mesh the best quality characteristics of the plants developed were obtained, and they were completely different from the other treatments (Table 3). In contrast, open air was the less favorable treatment.

In the control experiment, the open air low acclimation was due to the average minimum temperature recorded (1.5 °C) and the occurrence of 8 frosty days (Table 2, Figure 1). This is considered on the basis that temperatures below 5 °C are the cause of poor survival of gladiolus plants (Cantor, Tolety 2011); besides, it is well known that most mortality occurs when plants in a controlled environment are moved to an uncontrolled one (Memon 2012), and this particularly occurs when plants are transferred to natural conditions (23 ± 2 °C) (Torabi-Giglou, Hajieghrari 2008). In this research, transplantation was not restricted to seedlings, and during acclimation it varied from 93.8 % mortality in the control treatment (Figure 1) down to 3.2 % in 75 % reducing mesh (Table 3) due to environmental conditions.

It is possible that the highest PH and TB values were due to the low light intensity under the 75 % shading mesh (Memon 2012), which in this study was 2.6 MJ/m-2/d-1 in the photosynthetic band with an average maximum of 116 W/m-2/s-1, and based on the fact that gladiolus is very sensitive to light during the appearance of the first leaf (Hazarika 2006). This is the reason why in environments with higher light intensity, or open air (average daily photosynthetic irradiance of 10.7 MJ/m-2/d-1 with average maximum of 466.6 W/m-2/s-1), the PH is reduced. Moreover, the light intensity has a synergistic effect with temperature in the plants, because a higher accumulation of plant biomass occurs in long days with cool temperatures (15 °C) (Memon 2012). In open air treatments, the lowest PH and TB were due to the low development, premature senescence of the plants and to the high number of days with temperatures below or equal to 0 °C (when 5 °C is the minimum growth temperature of gladiolus) (González-Pérez et al. 2011).

Lastly, the high gladiolus CB values obtained under 75 % reducing mesh are attributed to the corms weight (Table 3); and under the plastic cover and open air environments, the plants wilted before the differentiation of stem stage and the normal corm production. Under the 50 % reducing mesh environment, the period of differentiation could occur later.

**Leaf temperature.** Plants under 75 % reduction mesh showed an average of 9 °C lower, which gave as a result better development because they did not suffer heat stress (Memon 2012). Treatment under plastic cover showed the highest leaf temperature (Table 3) which was close to the maximum temperature for gladiolus growth (González-Pérez et al. 2011) and this led to the death of plants under this environment (Figure 1), since the seedlings under this state of growth cannot regulate the transpiration via stomata and very easily they dehydrate and die (Aftab et al. 2008).

**Photosynthetic efficiency.** There were no significant differences for minimum fluorescence. The two treatments under reduction mesh showed same significance level and they were the best in all the analyzed variables (Table 4). The variable fluorescence/maximum fluorescence (Fv/Fm) ratio was 0.8 in both treatments under reducing mesh, whereas it was 50 % lower in plants grown in the open air and under plastic cover (Table 4). Such Fv/Fm value near to 0.83 is considered optimal in a number of species, C3 and C4 (Mugnai et al. 2009).

Similarly, significant differences the in photosynthetic efficiency index (P. index) between environments (Table 4) with the highest P. index were in 75 % reduction mesh, and the lowest in the open air and plastic cover environments; meaning that the plants in these last two treatments had a low photosynthetic performance, and according to Zlatev and Cebola (2012), the decrease in efficiency of the photosystem II (PSII) is due to water stress, extreme temperatures or low solar radiation, which corroborates the results of this research as the plants in open air and under plastic cover showed stress because of the low and high temperatures. Furthermore, it is also possible that in these treatments the wide temperature range would have caused photoinhibition, considering that plants under these environments were exposed to high levels of solar radiation. According to Zlatev and Cebola (2012), it is considered that photosynthetic processes can be limited by non- stomatic causes due to extreme temperatures. The largest measured area was in the reducing mesh treatments and this was due to the font size of photosystem II.

***Fusarium* evaluation**. In the culture medium no *Fusarium oxysporum* f sp. *gladioli* colonies were developed nor any other fungus. This proved that *in vitro* generated seedlings from the apical meristem in NAA, were free of corm fungus.

**CONCLUSIONS**

The pre-climatic phase helped in the environmental adaptation although it did not enhance the vegetative growth. The best acclimation environment was under the 75 % reducing mesh, which resulted in 95 % seedling survival, good index of photosynthetic potential (2.920) and 0.80 optimal variable fluorescence. This protocol represents an option for mass propagation of gladiolus plants under rustic conditions and *Fusarium* free.

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