INTRODUCTION

Gladiolus (Gladiolus spp., Iridaceae) is an important bulbous ornamental valued for its attractive spike with long-lasting and colorful flowers (Bhat, Nazki, Nelofar, & Burhan, 2017). In tropical countries like Indonesia, the production centres of gladiolus are located in highland areas of West, Central and East Java (Nuryani, Yusuf, Djatnika, Hanudin, & Marwoto, 2011). A breeding program in the country has produced and released more than 15 commercial varieties which are widely grown in production areas (Badriah, Wuryaningsih, & Rahayu, 2010). During in vivo maintenance, however, the parentals of the released varieties and other important genotypes have gradually been wiped out due to disease attacks and other disastrous abiotic stresses (Bakhtiar, Aswidinnoor, & Sutater, 2006; Nuryani, Yusuf, Djatnika, Hanudin, & Marwoto, 2011). These conditions threatened the gladiolus breeding program since the targeted characters to be incorporated should be available in the gene pool.

In vitro conservation has been extensively applied in preserving plant collections due to the limitation of in vivo maintenance. Compared to in vivo conservation, the technique needs less space and labor expenses. A huge amount of accessions can also be preserved using this method. In addition, tissue culture systems are considered safe for germplasm exchange in international schemes. A smaller sample size has made the cultures can be transferred in sterile conditions (Rodrigues, Arruda, & Forti, 2018; Saptari, Sinta, & Budiani, 2017).

Several authors have reported regarding the short and medium-term gladiolus in vitro. The method was dedicated to slower plantlet growth and postpone the subculture frequency (Ciuzan, Pamfil, & Rakosy-Tican, 2015; Rakosy-Tican, Bors, & Szatmari, 2012). The growth inhibitors fell into several categories i.e., growth regulator, osmotic pressure and nutrient modification. The growth regulator suppressed the growth of the cells through the inhibition of cell division and cell enlargement.

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ABSTRACT

In vitro conservation is recognized as one promising tool in conserving plant genetic resources like gladiolus when grown in the tropics. The study was conducted at the Indonesian Ornamental Crops Research Institute (IOCRI) from February 2016 to August 2018. A complete factorial experiment was set up to establish the combination of gladiolus cultivars and media compositions treatments for in vitro conservation. The results showed that the trends of plantlet survival rate and viability in every 4 months’ observation of both cultivars were similar. The highest plantlet death rate was detected during 12 – 16 months. After 24 months of storage, the differences in plantlet survival and viability were observed. Gladiolus ‘Nafa’ had higher plantlet survivals and viability than ‘Kaifa’. In respect to nutrient and sucrose concentration, full nutrient strength preserved 5% higher plantlet survival and viability than ½ MS. While media contained sucrose 90 g/l also supported plantlet survival and viability for more than 20% and 17%, respectively than lower sucrose media.
The substances include ABA, PAC, ancymidol and others that interfere with the biosynthesis and regulation of auxin, cytokinin and gibberellins (Bello-Bello, Poot-Poot, Iglesias-Andreu, Caamal-Velázquez, & de la Cruz Diaz-Sanchez, 2014). Some reports, however, indicated that the use of a growth regulator in the long period also could affect hormonal imbalance and decrease the viability of the cells (Hassan, Gomaa, Shahin, & El Homosany, 2013).

Considering the potential risk of growth regulator application over a long period, the use of osmotic pressure method and modification of nutrient and culture environment in single or in combinations were preferred in preserving the plant under in vitro conditions (D. P. C. da Silva, Ozudogru, dos Reis, & Lambardi, 2018). Higher sugar concentration in the media was usually used as an osmoregulator to increase osmotic pressure and limit the soluble ions and water. These conditions would reduce the nutrient uptake and finally slower the plantlet growth rate (Rahayu, Habibah, & Herlina, 2015). Nutrient modification has been reported to have a similar function as osmoticum to slower the plantlet growth on in vitro conservation. The reduction of one or several compounds or even the total nutrient strength was mostly applied to preserve suboptimal conditions for the conserved plantlet. These techniques have been successfully employed for cryopreservation in some plants, like Smallanthus sp., Musa sp., banana and pear genotypes (Edirisinghe, Denagamage, & Samarasinghe, 2017; Mqsood & Muhammad, 2010; (Skalova, Viehmannova, & Vitamvas, 2013; Tokoporo, Elhassan, & Ali, 2013). The use of sugar as osmoregulator and nutrient modification were predictably able to prolong plantlet survival during in vitro storage. This research was conducted to evaluate several culture media for medium-term in vitro conservation of gladiolus cultivars.

**MATERIALS AND METHODS**

The study was conducted at the Indonesian Ornamental Crops Research Institute (IOCRI) from February 2016 to August 2018. A completely randomized design (CRD) experiment was set up to establish the combination of two factors: first was the gladiolus cultivars, namely 'Nafa' and 'Kaifa', the second factor was media compositions for in vitro conservation, i.e. MS + 30 g/l sucrose (control 1), ½ MS + 30 g/l sucrose (control 2), MS + 60 g/l sucrose, MS + 90 g/l sucrose, ½ MS + 6 g/l sucrose and ½ MS + 9 g/l sucrose. The chronological steps of the experiment were described in the following details.

**Plantlet Establishment**

The plantlets with the diameter of ± 0.5 cm from both tested cultivars were selected for uniformity and planted at sterile carbonized rice husk under a protected environment. To maintain the cormels, irrigation and pesticide were applied twice a week for about two weeks. The cormels were brought into the laboratory for further disinfection processes; they served as a source of explants.

The plantlets were cleaned up from the dirt under continuous water flow. They were then soaked in aquadest containing detergent for 10 minutes, twice rinsed with aquadest and disinfected using 1% NaClO for 2.0 minutes and then 1.5% of the same solution for 1.0 minute. The plantlets were then immersed into 70% ethanol and gently shaken for 1.0 minute, rinsed with aquadest and air-dried inside di LAFC.

The cormels were inoculated into solidified MS + 1 ppm BAP + 3% sucrose for direct shoot induction. After 2 months, the newly emerging shoots were excised into the same media for further multiplication. Deflasking and inoculation in the same media were made every 2 months after which the shoots were excised and planted in MS + 0.1 ppm BA to induce the formation of cormels.

**Cormel Subcultures into Conservation Media**

Micro cormels formed after 2 months-induction in MS + 0.1 ppm BA, were selected for uniformity (diameter of 0.3 - 0.5 cm). They were excised and planted in a culture flask containing 15 ml solidified media. Every treatment was replicated 5 times and every replication had 10 plantlet samples. The cultures were then stored in a growth chamber at 18-21°C and 16 hours illumination for about 2 weeks. The temperature inside the growth chamber were gradually lowered (± 2°C every two days) until a constant 4°C in dark condition was achieved for preconditioning the cultures.

The viability of plantlets was evaluated and checked every 4 months during the 24-months storage; they were subcultured into the induction medium. Prior to subculture, the cultures were placed into another growth chamber with a gradual temperature increase up to 16-18°C (for one
week) under 16 hours-illumination. The survival rate, the viability of plantlets after storage and other pertinent phenomena were recorded. Statistical analysis was carried out using LSD (α = 5%) and pertinent photographs were presented for visual descriptive data.

RESULTS AND DISCUSSION

Analysis of variance showed that no significant interaction existed between gladiolus cultivars and conservation media in all parameters observed. There were variations on survival rate and percentage of plantlet viability; these were referred to as single effects of the respective gladiolus cultivar and conservation media treatments.

Performance of the Conserved-Plantlets Between Gladiolus Cultivars

The percentage of plantlets survival and viability of gladiolus cultivars during low temperature storage are presented in Table 1. Plantlet survival of both cultivars was considered high after 4-months storage; the death of plantlets was initially observed after 8 months. The differences on the percentage plantlet survival on both cultivars were detected after 12 months of storage; ‘Nafa’ had higher plantlet survival than ‘Kaifa’ until 24 months storage. These can be attributed to the respective genotypes and their adaptation capability. A decrease in plantlet viability was also detected after 8 months of storage on both cultivars. The capability to withstand extreme environmental conditions during low-temperature storage is related to the genetic construction of the tested cultivars (Cruz-Cruz, González-Arnau, & Engelmann, 2013). Genotypes respond differently to stressed conditions during in vitro conservation in coffee (Bertrand-Desbrunais, Noirot, & Charrier, 1992), potato (Nasiruddin & Islam, 2018) and sweet potato (Arrigoni-Blank et al., 2014).

The survival rate of the plantlets of both cultivars decreased as the storage period increased. However, no significant differences were observed in the percentage survival rate of the plantlets between the cultivars under each storage period. The same was true for the plantlet viability. On both cultivars, the highest plantlet death rate was detected during 12–16 months of storage. The diminishing survival rate in these periods reached 29.4 – 30%, followed by those during 8 – 12 months that grasped 21.3 – 25.5%. Similar phenomena were also observed on plantlet viability. The sharp decrease of plantlet viability was detected during 8–12 and 12-16 months storage that reached 22.91-24.5% and 31.7% respectively. The high diminishing survival rates during in vitro conservation might be due to several factors, i.e. plantlet propagule (Hanan, Youssef, & Saleh, 2013), oxidative stress (Purohit & Agarwal, 2017), toxic environment (Niino & Arizaga, 2015), permanent abnormal plantlet growth (Lopez-Puc, 2013), nutrient imbalance (Tokoporo, Ellhassen, & Ali, 2013) and others. Since the symptoms of hyperhydricity in all conserved plantlets were absent and the plantlets were vigorously recovered after transplanting in induction medium, the high death rates of plantlets during 8 to 16 months storage than were suspected due to initial plantlet propagule. The size of cormels (0.3 – 0.5 cm) was considered too small and had a lower capability to withstand under extreme condition during in vitro storage (Noor-Ul-Amin et al., 2013). In longer periods, the inability to adapt the dark and low temperature environment during storage induced irreversible turgor loss and caused the death of cells, then resulted in permanent failure to retain active growth in the induction medium (Shehata & Al-Khayri, 2013).

Table 1. Percentage of plantlet survival and viability of gladiolus ‘Nafa’ and ‘Kaifa’ during low temperature storage (4°C)

| Gladiolus cultivars | 4   | 8   | 12  | 16  | 20  | 24  |
|---------------------|-----|-----|-----|-----|-----|-----|
| Plantlet survival (%) |     |     |     |     |     |     |
| Nafa                | 100 a | 87.51 a | 66.24 b | 36.86 b | 21.07 b | 14.75 b |
| Kaifa               | 100 a | 87.82 a | 62.32 a | 32.32 a | 16.31 a | 9.32 a  |
| Plantlet viability (%) |     |     |     |     |     |     |
| Nafa                | 100 a | 91.21 a | 70.31 a | 48.02 a | 21.02 a | 9.72 a  |
| Kaifa               | 100 a | 89.67 a | 67.70 a | 46.22 a | 19.33 a | 8.02 a  |

Remarks: * Values followed by different letters under the same duration storage at each parameter differ significantly under LSD (α ≤ 5%)
Effect of Conservation Media on Plantlet Survival and Viability

The percentage of survival and viability among gladiolus plantlets stored under different conservation media are presented in Fig. 1. Among the evaluated media, plantlets conserved under MS + 90 g/l sucrose had higher survival and viability in every observation period. After 24 months of storage, plantlets stored under ½ MS + 90 g/l sucrose and MS + 60 g/l sucrose positioned secondly after MS + 90 g/l sucrose in terms of plantlet survival and viability. Sucrose concentration within the media seemed to have effects on plantlet survival and viability. Higher plantlet survival and viability in higher sucrose concentration-media inferred that sucrose induced plantlet resistance during storage through the increase of plantlet adaptability under an extreme environment like low temperature (T. L. da Silva & Scherwinski-Pereira, 2011).

Sucrose or other sugar alcohols are usually employed in in vitro conservation media subjected to osmotic agents. Osmotic agents acted as a growth retardant by causing osmotic stress to the plantlet under in vitro conservation. When supplemented to the medium, sucrose reduced the hydric potential and restricted water availability to the plantlet (Muñoz, Díaz, Reinún, Winkler, & Quevedo, 2019). The osmotic agent inhibited mineral uptake through differences in osmotic retention, thereby retarding plantlet growth (Nufus, Saptadi, & Yulianti, 2018). The added sucrose also served as the carbon source of the conserved plantlets (Maqsood & Muhammad, 2010) and when applied in higher concentration, sucrose was considered less detrimental to the conserved plantlet compared to sugar alcohols (Ozudogru et al., 2013). Higher plantlet survival and viability under higher sucrose concentration within the medium after a certain period at low-temperature storage were also reported in jojoba (Bekheet, Matter, Taha, & El-Ashry, 2016), wild lily (Du, Li, Zhang, He, & Jia, 2012), Epidendrum chlorocorymbos orchid (Lopez-Puc, 2013) and others.

Plantlets conserved under higher sucrose concentrations also produced roots. After 18 months storage visible roots were detected on the plantlets conserved under MS + 90 g/l sucrose and ½ MS + 90 g/l sucrose. The plantlet roots, however, were less developed when conserved under lower sucrose concentrations in MS + 60 g/l sucrose, ½ MS + 60 g/l sucrose, MS + 30 g/l and ½ MS + 30 g/l sucrose (Fig. 2). The visible root development on the plantlet conserved under higher sucrose concentrations inferred that higher osmotic pressure had induced the root growth. The mechanism of plantlets’ response to such conditions still needs further investigation. The existence of root was putatively related to higher plantlet adaptability during storage, as shown in the higher percentage of plantlet survival and viability. Similar phenomena were also reported on habanero pepper (Montalvo-Peniche et al., 2007), heliconia (Rodrigues, Arruda, & Forti, 2018), and chrysanthemum (Budiarto & Rosario, 2017) during in vitro storage.

![Plantlet survival (%)](image)

Fig. 1. (a) Percentage of survival and (b) viability of the gladiolus plantlets during in vitro storage under various conservation media

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Plantlet survival and viability also decreased along with the duration of storage in all evaluated media. Until 24 months of storage, the pattern of plantlet death rates was similar among the conservation media (Fig. 1). The increase of number of the plantlet death along the storage duration indicated the maximum resistance of plantlet to adapt under low temperature conditions. These conditions referred to the capability of the respected conservation media to support the plantlet life. The media volume of 15 ml per flask was considered not sufficient to support the plantlet life for a long time. Nutrient and carbon source in the media were predictably exhausted, the plantlets stored in certain media were all desiccated and the observation could not be recorded beyond 24 months. Perhaps culture could be stored for a longer period if the volume and culture tubes were bigger or more suitable vessels were used (Nasiruddin & Islam, 2018).

**Effects of Combined Treatments on the Conserved Plantlets**

The percentage plantlet survival and viability of gladiolus cultivars stored under various conservation media after 24 months are presented in Table 2. The highest survival and plantlet viability were detected on the plantlets of ‘Nafa’ stored under MS + 90 g/l sucrose, followed by ‘Kaifa’ in the same conservation media. The least plantlet survival and viability of both cultivars were detected on their plantlet conserved under ½ MS + 30 g/l sucrose. In every conservation medium, ‘Nafa’ showed higher plantlet survival and viability than ‘Kaifa’ indicating the respected genotype had more capability to adapt a suboptimal environment during low-temperature storage.
Combination of different nutrient and sucrose concentrations within the media affected the plantlet survival and viability on a longer storage period. On both tested cultivars, the full strength of MS medium supported the plantlet life longer, especially at higher sucrose concentrations. The variation in plantlet survivals and viability was also observed in different sucrose concentrations. Higher sucrose concentrations induced osmotic potential within the media and inhibited mineral absorption by the plantlets (Lopez-Puc, 2013). When the mineral uptake was restricted, plantlet growth was slowed. The mineral within the medium was depleted more slowly and enabled to support the plantlet survival longer (Hassan, Stino, Gomaa, & Al-mousa, 2014). In lower sucrose concentrations, the growth inhibition was perhaps less restricted, thus the support of media for longer plantlet survival was limited.

Plantlet viability represented the capability of the survived plantlet to retain the normal growth after low-temperature storage. The viability of the conserved plantlet in the induction medium was in line with the percentage of survival after 24 months of storage (Table 2). These conditions indicated that the survived plantlet still had their physiological integrity to exhibit normal growth in induction medium after stored under low temperature storage (Weilan, Xu, & Zhu, 2018). Further evaluations were needed regarding the proper volume of medium, type of osmoticum, and temperature during storage to find out the more efficient and maximum duration of in vitro storage of gladiolus accessions.

### CONCLUSION

Studies on in vitro conservation of two gladiolus cultivars were conducted for 24 months under low-temperature conditions. Plant genotypes affected the percentage of plantlet survivals and viability, though the trends of both cultivars were similar in every observation period. The highest plantlet death rate was detected during 12 – 16 months (29.4 – 30%). Survival percentage decreased as the length of storage increased especially in low sucrose content. Full nutrient strength preserved 5% higher plantlet survivals than ½ MS. Greater sucrose within the media also supported higher living plantlet and viability at more than 20% and 17%, respectively. Both gladiolus cultivars showed higher plantlet survival percentage and viability when inoculating in MS + 90 g/l sucrose and stored for 24 months.

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