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ESTABLISHMENT OF *IN VITRO* PROPAGATION PROTOCOL OF *GERBERA JAMESONII* BOLUS EX HOOK F.: EXPLANT AND MEDIA SELECTION TO PLANTLET ACCLIMATIZATION

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ABSTRACT. Gerbera (*Gerbera jamesonii* Bolus ex Hook f.) is an important ornamental plant commodity with high economical value in Indonesia; however, development of the plant in larger scale commercially is constrained by the availability of qualified-planting materials. Conventional propagation is clearly not suitable to overcome the problem and therefore *in vitro* propagation protocol is importantly addressed. *In vitro* propagation protocol of *G. jamesonii* was successfully established in the study. Different explants (shoot tips, young leaves, petioles and petals) were selected and cultured on Murashige and Skoog (MS) basalt medium containing different plant growth regulators. Shoot tips and half-strength MS medium containing 1.5 mg l^{-1} thidiazuron (TDZ) and 0.25 mg l^{-1} N6-benzylaminopurine (BAP) were suitable explant and initiation medium for shoot formation with 75% explant regeneration and 5.5 shoots produced per explant. Shoots derived from selection stage were proliferated on half-strength MS medium supplemented with 0.25 mg l^{-1} BAP and resulting higher shoot regeneration up to 7.5 shoots produced per shoot with 22.8 leaves and 0.83 cm leaf length. Multiplication of shoots on the half-strength MS medium fortified by 0.25 mg l^{-1} BAP increased gradually from the first subculture till the fifth subculture with 9.1 shoots produced per shoot subcultured and reduced thereafter. Shoots were then rooted on half-strength MS medium augmented with 0.5 mg l^{-1} α-naphthalene acetic acid (NAA) and 1.5 g l^{-1} activated charcoal (AC) and produced 2.1 roots per shoot with 2.52 cm length of roots. Well-rooted shoots were acclimatized in a mixture of burned-rice husk and organic manure (1:1, v/v) with 95% survival rate and 114 plantlets grew well under this treatment.

Introduction

Gerbera is one of *Asteraceae* family members having high economic value as cut flower with high market demand in Indonesian and global floral industry (Naz *et al*., 2012; Statistic Indonesian, 2016a). The flower has a long vase life, resistance to transportation damage and no riskiness to obtain a good market price (Chung *et al*., 2016). In Indonesia, the flower is widely cultivated in several ornamental cultivation areas such as Bogor, Cianjur, Lembang-West Java; Semarang, Sukoharjo-Central Java; Malang-East Java; Tabanan-Bali, *etc*. with 198,846 m^2 total cultivation areas (Statistic Indonesia, 2016a); 7,5 million stem in 2015 (Statistic Indonesia, 2016b) and 38,5 stem m^{-2} of plant productivity (Statistic Indonesia, 2016c). In addition, the cut flowers are sold 9,000 to 25,000 rupiahs per bunch depending on variety and quality (Umkm-news, 2016; Ilham-florist, 2017). Though market demand to the flower increase year by year, however development of the plants commercially is constrained by availability and sustainability the qualified-planting materials.

Conventionally, gerbera is generally propagated both vegetatively and generatively (Kanwar, Kumar, 2008). The vegetative method is usually carried out by rhizome divisions and cuttings. Although this method maintains uniformity and genetic purity, the technique is laborious and time consuming with fewer results. While generatively, the plant is propagated by seeds. However, the method produces a higher number of regenerants, the technique results in varied-regenerants and their performances (Rukmana, 1995). Those methods cannot be used to develop the gerbera for commercial purposes and therefore clonal propagation via tissue culture works are importantly addressed in producing a large number of plants, uniform, vigorous and pathogen free in a short time (Mohammed, Azzambak, 2014).

Recently, several *in vitro* propagation protocols in different processes with varied results were established in producing high qualified-planting materials for gerbera. Akter *et al*. (2013) produced high shoots and plantlets by culturing flower buds and stalks in Murashige and Skoog (1962; MS) medium containing 5.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA for shoot initiation, 2 mg l^{-1} BAP for shoot multiplication, 0.2 mg l^{-1} indole-3-butiric acid (IBA) for plantlet preparation and soil for acclimatization of the plantlets with 100% survival rate. In different studies, shoot tips derived from *in vitro* seedlings were cultured on MS medium augmented with 2 mg l^{-1} N6-benzyladenine (BA) and 0.2 mg l^{-1} NAA for high shoot induction and proliferation; 0.5–1.0 mg l^{-1} NAA for rooting of shoots (Nazari *et al*., 2014). The highest number of micro-shoots derived from apical meristem were established in MS medium containing 10 mg l^{-1} BAP in induction and multiplication steps, while MS medium fortified by 10 mg l^{-1} NAA was suitable for rooting of shoots (Naz *et al*., 2012). Almost similar results were determined on MS medium containing 1 mg l^{-1} BAP, 0.030 mg l^{-1} IBA and 0.025 mg l^{-1} NAA for best initiation and proliferation of shoots. The half strength MS medium supplemented with 0.4 mg l^{-1} IBA was found best for a shoot *in vitro* rooting (Singh *et al*., 2016). These recent studies indicated that shoot tips, MS medium and BAP/BA were suitable explants, basal medium and cytokinin type for the establishment of *in vitro* propagation protocol of gerbera in different routes.

A new and reliable route in *in vitro* propagation protocol of gerbera derived from shoot tip as explant source was established *via* initiation and proliferation of shoots, plantlet preparation and its acclimatization. The detail and different finding of each step discussed in this paper.

Materials and Methods

Planting material and its preparation

The material used in this study was *G. jamesonii* 'Black Jack' widely cultivated by a farmer at Ciwalen, Pacet-Cianjur, West Java-Indonesia. Shoots with 1–2 young leaves and young flower buds were harvested from the donor plants at farmer's location in 07.00–09.00 am. The shoots were then brought to tissue culture laboratory of Indonesian Ornamental Crops Research Institute for sterilization purpose.

Explant sterilization and preparation

Before explant sterilization, the shoots were prepared by separating shoot tip area and young leaves with their petioles. Furthermore, all explants were pre-treated under running tap water for 60 minutes (min). followed by immersing them in 1% soap solution with manual shaking for 30 min, pesticide solution (50% benomil and 20% kanamycin sulphate) for 30 min and rinsing 4–5 times (3 min each) using distilled water to remove all remain disinfection materials. The explants were then moved to laminar air flow cabinet for sterilization. They were soaked in 0.05% mercury chloride ($HgCl_2$) solution added by 5 drops of Tween 20 for 5 min, followed by rinsing with sterile distilled water (SDW) 4–6 times (3 min each), especially for shoot tip explant. For young leaves with their petioles and young flower buds, after the first treatment, the explants were further disinfected with 0.01% $HgCl_2$ for 3 min, followed by rinsing with sterile distilled water 4–5 times (3 min each). The young leaves with petioles were then prepared as cultured-explants by slicing of leaves and petioles transversally ± 5 mm in length. While young petals were prepared by pulling out directly using tweezers one by one gently.

The sterile explants were prepared by removing basal part of leaf petioles, one by one, using tweezers carefully until growing point easily observed. In each petiole basal part removing, omitting smooth hairs were carried out gently using small tweezers under the binocular microscope under 40 times magnification. The shoot tip was sliced vertically in four positions (± 1.5 mm in length and width) then sectioned transversally ± 3 mm in length from the tip-point. The isolated-shoot tips were further sterilized with 0.01% $HgCl_2$ for 3 min and then rinsing with SDW as previously described. The shoot tips were shortened by slicing transversally and longitudinally till the final size of the shoot tip was 1 × 1 × 1.5 mm in length, width and height. Pieces of the young leaves and young petals were cultured on initiation media in adaxial side down position, young petioles cultured horizontally and shoot tips vertically.

Selection of explant types and media for shoot initiation

Explant types selected in this experiment were (1) shoot tips, (2) young leaves, (3) young petioles and (4) young petals. While initiation media tested in this step were MS basalt medium supplemented with (1) 0.5 mg l^{-1} Kinetin, 0.5 mg l^{-1} GA3 and 0.5 mg l^{-1} NAA (Mohanty *et al*., 2005); (2) 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (Taha *et al*., 2010); (3) 0.5 mg l^{-1} BAP and 1 mg l^{-1} NAA (Ranjan, Gaurav, 2005); (4) 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} NAA (Hasbullah *et al*., 2011); (5) 2 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D; (6) 0.5 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D. All media were augmented with 20 g l^{-1} sucrose, 2 g l^{-1} gelrite and pH was adjusted in

5.8. Media were autoclaved in 121 ºC at 15 kPa for 20 min. The factorial experiment was arranged in a randomized complete block design (RCBD) with four replications. Each treatment consisted of 2 bottles and each bottle was cultured 4 explants. All cultures were incubated in dark condition for ± 1.5 months, then transferred to light incubation in 12 h photoperiod under a cool fluorescent lamp with 13 µmol m^{-2} s^{-1} till shoots regenerated and easily observed. Variables observed in this study were (1) percentage of explant regeneration (%) and (2) number of shoots per explant. The periodical observation was carried out to know the response of explants during incubation. All variables were recorded and measured ± 2.0 months after culture.

Optimization of media for initial shoot proliferation

In the stage, shoots derived from the first experiment were subcultured on optimization media (OM). The media were half-strength MS medium containing (1) 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP (OM-1), (2) 0.75 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP (OM-2); (3) 0.25 mg l^{-1} BAP (OM-3); (4) 0.5 mg l^{-1} BAP (OM-4); (5) 1.0 mg l^{-1} BAP (OM-5); (6) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} Picloram (OM-6); MS medium supplemented with (7) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} IAA (OM-7), (8) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} 2,4-D (OM-8). All media were prepared as the previous experiment. The experiment was arranged in RCBD with three replications. Each treatment consisted of 3 bottles and each bottle contained 4 explants. Similar variables as the previous experiment were recorded and measured ± 2.0 months after culture.

Study on multiplication of shoots

To multiply of shoots, single shoots derived from the initiation stage were subcultured periodically on two selected media (SM) *i.e.* (1) half strength MS medium containing 0.25 mg l^{-1} BAP (SM-1) and (2) MS medium fortified by 0.2 mg l^{-1} BAP and 0.02 mg l^{-1} NAA (SM-2) till peak shoot production established. SM-2 was a high potential medium for shoot proliferation established from the preliminary study (Data not shown). Each treatment consisted of 10 bottles. Each bottle contained 5 single shoots. All cultures were incubated under light incubation as described previously for ± 1.5 months. Variables observed in this study were (1) number of shoots per single shoot culture, (2) total number of leaves and (3) length of leaves (cm). All variables were recorded and measured ± 1.5 months after culture.

Root formation of shoots

In root formation of shoots, different rooting media (RM) *viz*. half-strength MS medium containing 0.1 mg l^{-1} BAP (RM-1), 0.5 mg l^{-1} IAA (RM-2); 0.5 mg l^{-1} NAA (RM-3); and 0.5 mg l^{-1} IBA (RM-4). All media were added with and without 1.5 g l^{-1} activated charcoal (AC). The factorial experiment was arranged in completely randomized design with four replications. Each treatment consisted of five bottles. Each bottle contained 5 shoots. Variables observed in this study were (1) number of roots pe shoot and (2) root length (cm). The periodical observation was carried out to know root formation response on shoots culture during incubation. All variables were recorded and measured ± 1 months after culture.

Plantlet acclimatization

Plantlet acclimatization was prepared by pulling out well-rooted shoots from culture bottles gently using tweezers. Plantlet roots were put under running tap water to remove remains of agar attaching them. The plantlet roots were then immersed in 1% pesticide solution (50% benomil and 20% kanamycin sulphate) for 3 min, air-dried plantlets on paper for a while, then cultured on plastic trays containing a mixture of burned-rice husk and organic manure (1:1, v/v) watered sufficiently. The plastic trays were then placed in a plastic box and covered by plastic transparent for 15 days. Each tray was planted 24 plantlets. Total plantlets acclimatized the step were 240 plantlets. Variables observed in the stage were (1) percentage of plantlet survivability (%) and (2) number of survive plantlets. The variables were recorded ± 2 months after acclimatization.

Data analysis

Quantitative data in all experiments were analysed by analysis of variance (ANOVA). Significant differences between means were assessed by Tukey test, $P < 0.05$.

Results

Selection of explant types and media for shoot initiation

Under periodical observation, it was known that there were different responses of explants on shoot initiation. Shoot initiation was easily observed and regenerated from shoot tip explants (Figure 3A and 3B). Regeneration of the initial shoot was clearly observed 7–10 days after culture, while on other explants, initial shoots were obviously observed 25–35 days after culture *via* callus formation on 15–20 days after culture. A number of shoots derived from different explants was varied from 1–8 shoots with a high number of shoots per explant exhibited by shoot tip explants (Figure 3C).

Different explant types and initiation media gave significant effect on shoot formation capacity. Shoot tip explant was the suitable explant stimulating high explant regeneration as high as 51.1% with 1.1 shoots produced per explant (Table 1). The result was also higher compared to young petioles and leaves in all variables observed. While from 7 initiation media tested in the study, IM-6 was the most appropriate medium for shoot formation. Though there was no significant different compared to IM-3, the medium induced high percentage of explant regeneration up to 66.3% with 1.4 shoots per explant (Table 2). The second best medium was indicated by IM-3, whereas other media were not able to stimulate shoot regeneration.

Table 1. Effect of explant types on shoot formation of Gerbera

Explant type	Percentage of explant regeneration (%)	Number of shoots per explant
Shoot tips	51.1[a]	1.1[a]
Young leaves	50.7[ab]	0.2[bc]
Young petioles	42.9[b]	0.3[b]
Petals	32.9[c]	0.0[c]
CV (%)	19.85	12.30

CV – coefficient of variation. Means followed by the same letter in the same column are not significantly different based on Tukey test, *P > 0.05*.

Table 2. Effect of media on shoot formation on Gerbera

Media	Percentage of explant regeneration (%)	Number of shoots per explant
IM-1	25.0[d]	0.0[b]
IM-2	38.1[c]	0.0[b]
IM-3	52.5[a]	1.4[a]
IM-4	46.3[b]	0.0[b]
IM-5	43.8[bc]	0.0[b]
IM-6	66.3[a]	1.4[a]
IM-7	38.8[c]	0.0[b]
CV (%)	19.85	12.30

Note: IM-1, MS medium supplemented with 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} NAA; IM-2, MS medium containing 0.5 mg l^{-1} Kin, 0.5 mg l^{-1} GA3, and 0.5 mg l^{-1} NAA; IM-3, MS medium augmented with 4.0 mg l^{-1} Kin and 0.5 mg l^{-1} IAA, IM-4, MS medium fortified by 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA; IM-5, MS medium containing 0.5 mg l^{-1} BAP and 1.0 mg l^{-1} NAA; IM-6, half-strength MS medium supplemented with 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP; and IM-7, half-strength MS medium fortified by 0.02 mg l^{-1} BAP and 0.01 mg l^{-1} NAA. Means followed by the same letter in the same column are not significantly different based on Tukey test, *P > 0.05*.

Different explant types and initiation media also indicated significant interaction effect on shoot formation, where the explant types gave higher effect compared to initiation media. Shoot tips cultured on IM-6 were the most suitable treatment combination to produce high shoot formation. The combination induced the highest percentage of explant regeneration up to 75% with 5.5 shoots produced per explant (Table 3 and 4).

Table 3. Interaction effect of explant types and media on percentage of explant regeneration (%)

Media	Explant types			
	Shoot tips	Young leaves	Young petioles	Petals
IM-1	25.0[c]	35.0[b]	25.0[c]	6.0[c]
IM-2	37.5[bc]	45.0[b]	35.0[bc]	35.0[abc]
IM-3	50.0[abc]	60.0[ab]	55.0[a]	45.0[ab]
IM-4	65.0[ab]	50.0[ab]	45.0[ab]	25.0[bc]
IM-5	45.0[abc]	50.0[ab]	45.0[ab]	35.0[abc]
IM-6	75.00[a]	75.0[a]	60.0[a]	55.0[a]
IM-7	60.0[abc]	1.0[b]	35.0[bc]	20.0[c]
CV (%)	19.42	15.47	16.98	20.96

Means followed by the same letter in the same column are not significantly different based on Tukey test, *P > 0.05*.

The second best combination was determined on shoot tips cultured on IM-3 medium. The medium also successfully induced regeneration shoots up to 1.8 shoots produced per explant for young petioles and 1.3 shoots per explant for young leaves. Whereas other combinations, though they successfully stimulated callus formation, failed to regenerate shoots.

Table 4. Interaction effect of explant types and media on number of shoots per explant

Media	Explant types			
	Shoot tips	Young leaves	Young petioles	Petals
IM-1	0.0[c]	0.0[b]	0.0[b]	0.0[tn]
IM-2	0.0[c]	0.0[b]	0.0[b]	0.0[tn]
IM-3	2.5[b]	1.3[a]	1.8[a]	0.0[tn]
IM-4	0.0[c]	0.0[b]	0.0[b]	0.0[tn]
IM-5	0.0[c]	0.0[b]	0.0[b]	0.0[tn]
IM-6	5.5[a]	0.0[b]	0.0[b]	0.0[tn]
IM-7	0.0[c]	0.0[b]	0.0[b]	0.0[tn]
CV (%)	10.78	19.82	8.22	–

Means followed by the same letter in the same column are not significantly different based on Tukey test, *P > 0.05*.

Optimization of media for initial shoot proliferation

Subculturing shoots derived from the first experiment on different optimization media gave a different effect on initial shoot proliferation. New shoots generally initiated in the basal part of subcultured-shoots 10–15 days after culture. A number of the new shoots regenerated in the stage were 1–12 shoots per single shoot subcultured with a 3–26 total number of leaves and 0.5–2.0 cm leaf length (Figure 3D). The results indicated an improvement on shoot regeneration. Interesting results were determined in the study. Subculturing shoots on selected medium established in the first experiment could not produce a high number of shoots per shoot. For initial shoot proliferation, omitting TDZ in half-strength MS medium and maintaining 0.25 mg l^{-1} BAP (OM-3 medium) led to improving high new shoot regeneration up to 7.5 shoots produced per shoot subcultured with 22.8 leaves and 0.83 leaf length (Table 5). The second best medium was proved on the OM-4 medium by increasing BAP concentration from 0.25 to 0.5 mg l^{-1}. While other media tended to reduce initial shoot proliferation.

Table 5. Effect of optimization media on initial shoot proliferation of Gerbera

Optimization media (OM)	Number of shoots per explant	Number of leaves	Leaf length (cm)
OM-1	1.5[tcd]	6.5[tcd]	1.20[tabc]
OM-2	1.3[tcd]	4.5[td]	1.53[tab]
OM-3	7.5[ta]	22.8[ta]	0.83[tabc]
OM-4	5.8[tbc]	17.0[tab]	0.65[tc]
OM-5	3.8[tbc]	12.8[tbc]	0.73[tbc]
OM-6	1.8[tcd]	7.5[tcd]	1.55[ta]
OM-7	0.7[td]	4.5[td]	0.88[tabc]
OM-8	0.7[td]	4.5[td]	0.80[tabc]
CV (%)	14.73	19.92	17.52

Note: Half-strength MS medium containing (1) 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP (OM-1), (2) 0.75 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP (OM-2); (3) 0.25 mg l^{-1} BAP (OM-3); (4) 0.5 mg l^{-1} BAP (OM-4); (5) 1.0 mg l^{-1} BAP (OM-5); (6) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} Picloram (OM-6); MS medium supplemented with (7) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} IAA (OM-7), (8) 0.25 mg l^{-1} BAP and 0.02 mg l^{-1} 2,4-D (OM-8). Means followed by the same letter in the same column are not significantly different based on Tukey test, *P > 0.05*.

Study on multiplication of shoots

Subculturing shoots on two selected media for multiplication of shoots also gave varied results in the shoot multiplication with a similar trend. SM-1 (half-strength MS medium containing 0.25 mg l^{-1} BAP) was a more suitable medium for multiplication of shoots. An average number of shoots produced per subculture period increased gradually from first subculture period to second, third, fourth till the fifth subculture with the highest average of the new shoots produced per shoot in the fifth subculture of 9.1 shoots and reduced thereafter (Figure 1). The qualified shoots under subculture period could be maintained till ninth subculture (Figure 3F). From one active grown shoot could stimulate 6–12 new shoots ± 1.5 months after of incubation. The regenerated shoots in the stage began to produce root in the sixth subculture with 0–2 roots per cluster of shoots.

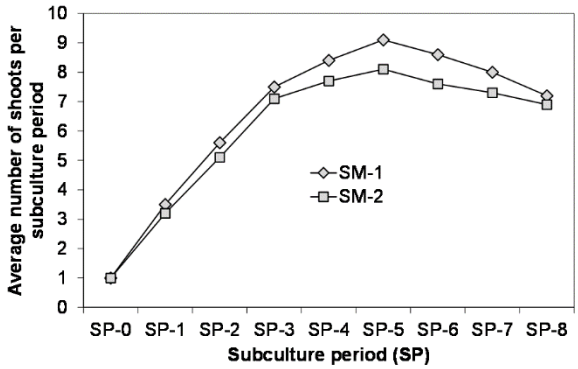

Figure 1. Multiplication of shoots under periodical subcultures on two selected media. SM-1, half-strength MS medium containing 0.25 mg l^{-1} BAP. SM-2, MS medium supplemented with 0.2 mg l^{-1} BAP and 0.02 mg l^{-1} NAA. Each periodical subculture was ± 1.5 months.

Root formation on shoots

Culturing single shoots derived from ninth subculture period on different rooting media stimulated different results on root formation. Initiation of root was obviously observed 10–13 days after culture. A number of root per shoot was varied from 0–3 roots with 0–1.2 cm length of roots. High root formation was generally recorded on rooting media containing AC.

Application of different AC concentrations and rooting media gave significant effect on root formation. Adding AC on rooting media stimulated the high formation of roots compared to non-AC media. The media induced 1.2 roots per shoot with 1.4 cm root length. While the non-AC media failed to induce roots (Figure 2A). Whereas RM-3 was a suitable medium for rooting of the shoot than others. The medium successfully regenerated 1 root per shoot with 0.76 cm root length (Figure 2B). Other media gave low results on root formation.

Acclimatization of plantlets

Acclimatization of plantlets, carried out by removing remains of agar under tap water (Figure 3H), immersing plantlet roots in 1% pesticide solution for 3 min (Figure 3I), culturing them in two different acclimatization media, covering with plastic transparent for ± 15 days after acclimatization (Figure 3J and 3K), gave different results. A mixture of burned-rice husk and organic manure (1:1, v/v) were a suitable medium for acclimatization of plantlets compared to burned-rice husk without organic manure. The medium successfully induced high survivability of plantlets 91.7–100% with 95% in average and 22.8 survival plantlets (Figure 3L). A number of plantlets were acclimatized under the suitable medium were 114 plantlets. While total plantlets successfully acclimatized in the study were 204 plantlets.

Significant interaction effect from both treatments was also noted from the experiment. Half-strength MS medium supplemented with 0.5 mg l^{-1} NAA and 1.5 g l^{-1} AC were the most appropriate combination treatment to obtain the highest formation of roots. The combination treatment induced 2.1 roots per shoot with 2.52 cm length of roots (Table 6 and 7). While other combinations stimulated low root formation. The rooted shoots were then prepared for acclimatization (Figure 3G).

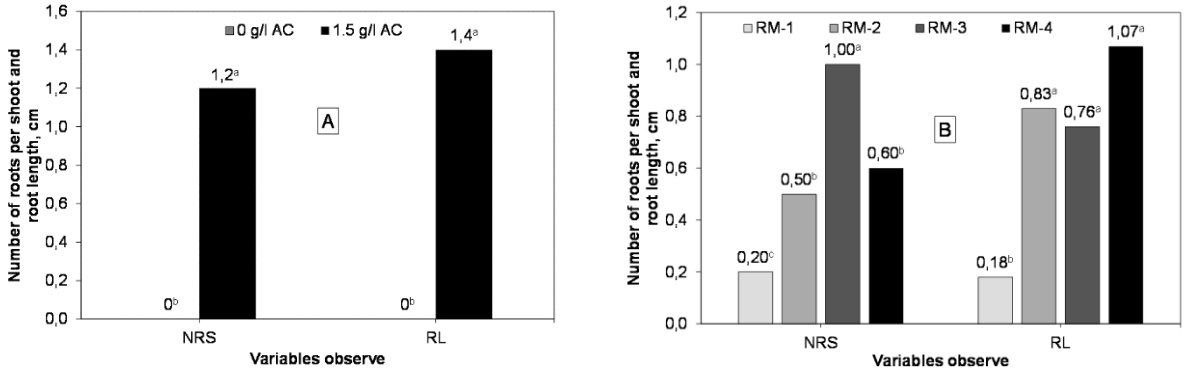

Figure 2. Effect of AC concentration and rooting media (RM) on root formation. A. Effect of AC concentrations on root formation. B. Effect of rooting media (RM) on root formation. Rooting media (RM) was half-strength MS medium containing 0.1 mg l^{-1} BAP (RM-1), 0.5 mg l^{-1} IAA (RM-2); 0.5 mg l^{-1} NAA (RM-3); and 0.5 mg l^{-1} IBA (RM-4). NRS-number of roots per shoot, RL-root length. Histogram with a similar letter in the same cluster is not significantly different based on Tukey test, *P > 0.05*.

Table 6. Interaction effect of activated-charcoal concentrations and rooting media on number of roots per shoot

Rooting media (RM)	Activated-charcoal concentration (g l^{-1})	
	0	1.5
RM-1	0.0^{tn}	0.3^{c}
RM-2	0.0^{tn}	1.1^{b}
RM-3	0.0^{tn}	2.1^{a}
RM-4	0.0^{tn}	1.2^{b}
CV (%)	–	16.78

Means followed by the same letter in the same column are not significantly different based on Tukey test, $P > 0.05$.

Table 7. Interaction effect of activated-charcoal concentration and rooting media on root length (cm)

Rooting media (RM)	Activated-charcoal concentration (g l^{-1})	
	0	1.5
RM-1	0.0^{tn}	1.35^{b}
RM-2	0.0^{tn}	2.93^{a}
RM-3	0.0^{tn}	2.52^{ab}
RM-4	0.0^{tn}	2.92^{a}
CV (%)	–	20.44

Means followed by the same letter in the same column are not significantly different based on Tukey test, $P > 0.05$.

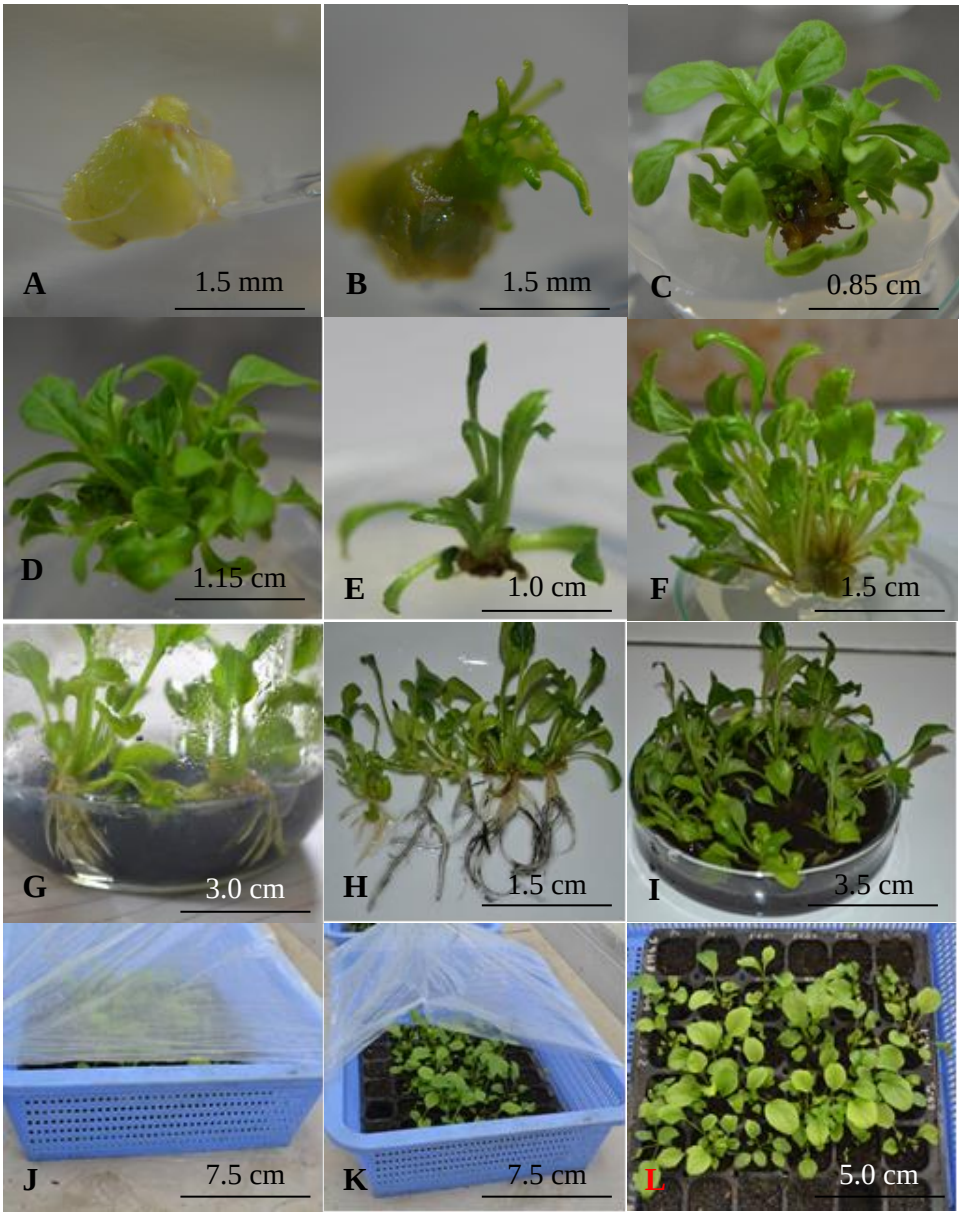

Figure 3. *In vitro* propagation protocol of Gerbera using shoot tip as explant source. A. Shoot tip explant 3 days after culture initiation. B. Initial shoots regenerated from shoot tip 15 days after culture on half-strength MS medium containing 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP. C. Shoots regenerated from shoot tip 1.5 months after culture on half-strength MS medium containing 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP. D. Regenerated shoots derived from initial shoot proliferation on half-strength MS medium supplemented with 0.25 mg l^{-1} BAP. E. Single shoot isolated for multiplication of shoot. F. Regenerated shoots derived from multiplication stage on half-strength MS medium supplemented with 0.25 mg l^{-1} BAP after ninth subculture. G. Rooted shoots on half-strength MS medium fortified by 0.5 mg l^{-1} NAA and 1.5 g l^{-1} AC 1.5 months after culture. H. Plantlets prepared for acclimatisation after cleaning roots from remain of agar under tap water. I. Plantlets immersed in 1% solution pesticide for 3 min before planting in acclimatisation media. J. Plantlets acclimatised under the transparent plastic covering 7 days after acclimatisation in a mixture of burned-rice husk and organic manure (1:1, v/v). K. Acclimatized-plantlets 15 days after acclimatisation. L. Acclimatized-plantlets 40 days after acclimatisation.

Discussion

Development of *in vitro* mass propagation technology was affected by several factors such as plant and explant types; medium, initiation, proliferation and multiplication of shoot; shoot rooting and acclimatization of plantlets (George *et al*., 1993; Hartmann *et al*., 1997). Each plant and explant type have a specific response and different behaviour in *in vitro* tissue culture (Ranjan, Gaurav, 2005; Sharma *et al*., 2014). Each stage in *in vitro* culture of plants also needs suitability responses of explant, culture medium and culture condition (Winarto *et al*., 2013). Therefore, the establishment of each *in vitro* mass propagation protocol of plants, including Gerbera, resulting in different and specific routes. A new route in *in vitro* mass propagation protocol of Gerbera was successfully established in this study started from a selection of explant types and media for initiation till acclimatization of plantlets.

In the initiation stage, high shoot formation with 75% of explant regeneration and 5.5 shoots per explants was derived from shoot tip explants cultured on half-strength MS medium containing 1.5 mg l^{-1} TDZ and 0.25 mg l^{-1} BAP. In other studies, culturing shoot tip explants on MS medium supplemented with 0.4 mg l^{-1} NAA was applied to produce axillary shoots (Data not shown) (Nazari *et al*., 2014). The shoot tips cultured on MS medium fortified by 5 mg l^{-1} BA only gave 30% of explant regeneration and a low number of shoots per explant (Mohammed, Özzambak, 2014). Apical meristems were cultured on MS medium supplemented with 2 mg l^{-1} BAP and 0.5 mg l^{-1} IAA to produce optimal shoot formation (NS) (Naz *et al*., 2012). While shoot buds were cultured on MS medium augmented with 2 mg l^{-1} BAP and 1 mg l^{-1} IAA successfully producing higher shoot number up to 8.7 shoots per explant (Mishra *et al*., 2014). Though our results were not as high as Mishra *et al*. (2014) results, results of the study had an important role in initial establishing *in vitro* propagation protocol to prepare qualified seedlings as good planting materials to develop Indonesian gerbera's agribusiness.

Shoot proliferation in keeping with the multiplication of explants was successfully carried out in different culture media with varied-results. In the study, initial proliferation was not successfully induced in MS medium containing TDZ. The initial proliferation and multiplication of shoots were clearly determined on half-strength MS fortified by 0.25 mg l^{-1} BAP that resulted in 7.5 shoots produced per shoot with 22.8 leaves and 0.83 leaf length. A number of shoots reached the highest number up to 9.1 shoots in the fifth subculture period. Naz *et al*. (2012) maintained high multiplication of shoots up to 9 shoots per explant and 7 cm shoot length on MS medium supplemented with 10 mg l^{-1} BA. A number of multiple shoots up to 28.6 shoots per clump with 4.6 shoot length was recorded on MS medium augmented with 2 mg l^{-1} BAP (Akter *et al*., 2013). MS medium containing 2 mg l^{-1} N6-benzyladenine (BA) and 0.2 mg l^{-1} NAA successfully produced 10 shoots per explant (Nazari *et al*., 2014). Multiple shoots of 8.7–10.5 shoots derived from shoot buds with 4.8–6.0 leaves per shoot were proved on MS medium supplemented with 2 mg l^{-1} BAP and 1.0 mg l^{-1} IAA (Mishra *et al*., 2014). A maximum number of shoots (12.7 shoots) with 44.7 leaves and 3.48 cm leaf length were established on MS medium with 1 mg l^{-1} BAP, 0.03 mg l^{-1} indole-3-butyric acid (IBA) and 0.025 mg l^{-1} NAA (Singh *et al*., 2016). Though our results were not as high as compared to some previous reports, but our results were higher than 6.4 shoots per division and 3.9 cm shoot length on MS medium with 2 mg l^{-1} BAP (Bhargava *et al*., 2013), 7.3 shoot per explant on MS medium containing 2 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 100 mg l^{-1} adenine sulphate (Kadu, 2013), and 7.2 shoots per explant with 5.6 leaves per shoot on MS medium with 5.6 mg l^{-1} BAP (Kozak, 2011).

Till now there was few study on multiplying shoots under subculture periodically. In the study, an average number of shoots produced per subculture was increased gradually from 3.5 shoots in the first subculture to 9.1 shoots in the fifth subculture, then declined afterwards. The qualified-shoots produced could be maintained till the ninth subculture. Other subculture studies were reported by Tyagi and Kothari (2004), Akter *et al*. (2013) and Rahman *et al*. (2014); however subculture periodically to follow multiplication of shoots was not published.

Preparing well-rooted shoots in *in vitro* culture of gerbera had a significant effect on acclimatization as one of the critical stages. Bhargava *et al*. (2013) found that 6.2 roots per clump with 3.3 cm root length was established on half-strength MS medium supplemented with 2 mg l^{-1} IBA; 4–6 roots per shoot with 3.5 cm root length on MS medium containing 1 mg l^{-1} NAA (Nazari *et al*., 2014); 80% of rooting of shoots with 4 roots per shoot recorded on MS medium containing 0.3 mg l^{-1} IBA (Rahman *et al*., 2014); 4 roots per shoot with 1.96 cm root length on MS medium fortified by 1 mg l^{-1} IBA (Shylaja *et al*., 2014). While in the study best root formation with 2.1 roots per shoot and 2.5 cm root length was determined on half-strength MS medium 0.5 mg l^{-1} NAA and 1.5 g l^{-1} AC. Application of AC on 750 mg l^{-1} in combination with 2 mg l^{-1} NAA in MS medium with 96% root formation was reported by Kadu (2013).

Acclimatization is a critical step in *in vitro* culture of gerbera. Different results and ways in acclimatization to obtain high survivability were recorded previously. Bhargava *et al*. (2013) recorded 90% survivability of plantlets via rinsing roots under running tap water, immersing 0.1% Bavistin solution for 5 min and planting of plantlets in cocopeat for 4 weeks. Pooling out plantlets from culture vessels, planting them in peat-moose: sand: clay (1:1:1, v/v/v) covering plastic bags for 7–10 days and placing in greenhouse under 18/8 h photoperiod were applied to obtain high survival plantlets (Data not shown) (Hussein *et al*., 2008). While from the study, putting plantlet roots under running tap

water, soaking them in 1% pesticide solution (50% benomil and 20% kanamycin sulphate), planting in burned rice-husk and organic manure (1:1, v/v) and covering them with transparent plastic for ± 15 days resulted in 95% survival plantlets. Other varied results of acclimatization with no detail information of acclimatization process with 84–100% using soil (Akter *et al*., 2013; Rahman *et al*., 2014), 82.4% in sand, soil, FYM and leaf mould (Kadu, 2013), and 84% survival rate in peat + perlite mixture (2:1, v/v) (Gök *et al*., 2016).

Conclusion

From the study, it can be summarized that *in vitro* propagation protocol derived from a selection of explant types, initiation media, initial proliferation, multiplication, plantlet preparation and acclimatization was successfully established. Initiation shoots derived from shoot tips with 75% explant regeneration and 5.5 shoots per explant were determined. Initial proliferation of shoots in half-strength MS medium containing 0.25 mg l^{-1} BAP produced 7.5 shoots produced per shoot with 22.8 leaves and 0.83 leaf length, while multiplication of shoots under periodic subcultures increased a number of shoots produced per shoot gradually till the fifth subculture with 9.1 shoots and declined thereafter. The qualified shoots could be maintained till the ninth subculture. Shoots were easily rooted on half-strength MS medium supplemented with 0.5 mg l^{-1} NAA and 1.5 g l^{-1} AC. The plantlets with 95% survivability were established in a mixture of burned-rice husk and organic manure (1:1, v/v).

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Conflict of interest

We declare that there is no conflict of interest dealing with authors and Indonesian Agency for Agriculture Research and Development that facilitated and funded the research activity.

Author contributions

Budi Winarto and Muhammad Prama Yufdy plays important roles equally in designing, carrying out and analysing all data regenerating from the research till writing, editing and approving the final manuscript.

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