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ARTICLE

Axillary shoot growth performance from different explants, varieties, culture media, and subculture methods for *in vitro* culture of *Chrysanthemum morifolium*

Crescimento de brotações axilares a partir de diferentes explantes, variedades, meios de cultura e métodos de subcultivo *in vitro* de *Chrysanthemum morifolium*

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Abstract: *Chrysanthemum morifolium* is a commercial cut flower propagated using root suckers and cuttings. In conventional methods, the suckers and cuttings that usually produce low quantity and quality planting materials, can be improved significantly using tissue culture method for achieving varied purposes. The main objective of the research was to reveal different effects of explants, varieties, culture media, and subculture methods on axillary shoot growth performances from initiation to acclimatization. Field-grown shoot tips, thirds, and fifth nodes; 'Cat Eye', and 'Pink Fiji'; initiation, enhancement, and proliferation media; random-cluster subculture methods; direct cutting of shoot tip and node for acclimatization were investigated. Shoot tips of 'Pink Fiji' cultured on MS medium containing 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA produced high axillary shoots and reduced thereafter due to lowering BAP and NAA. Reducing plant growth regulator (PGR) concentration decreased axillary shoots, but it improved the axillary shoot performances. In the fourth subculture, lowering data in all variables occurred due to substituting sucrose and gelrite to local sugar and agar. Full strength MS medium induced better axillary shoot growth than others in the proliferation stage. Yellowing leaves, decreasing shoot height, and leaf number were noted on 'Cat Eye' at fifth subculture, and 'Pink Fiji' at seventh subculture. High acclimatized plants derived from shoot tips of 'Pink Fiji' with 86% and 83% of nodes were determined on a mixture of burned rice husk, hyacinth manure, and cocopeat (1:1:1, v/v/v). Direct cutting increased the number of the qualified planting materials up to 3 – 5 folds compared to traditional method. Application of the findings can increase quantity and quality planting materials leading to high product selling prices and seed breeder incomes commercially
Keywords: axillary shoot, chrysanthemum, direct cutting, growth, performance, subculture.

Resumo: *Chrysanthemum morifolium* é uma flor de corte comercial propagada usando rebentos de raiz e estacas. O método tradicional inibe a ampliação da planta, portanto, culturas de tecidos são aplicadas. O objetivo principal da pesquisa foi revelar diferentes efeitos de explantes, variedades, meios de cultura e métodos de subcultivo no desempenho da parte aérea axilar desde a iniciação até a aclimatização. Pontas de brotos cultivadas em campo, terços e quinta nós; Olho de gato e Fiji rosa; meios de iniciação, aprimoramento e proliferação; métodos de subcultura de agrupamento aleatório; O corte direto da ponta da parte aérea e do nó para aclimatização foram investigados. Pontas de brotos de 'Pink Fiji' cultivados em meio MS contendo 2,0 mg L^{-1} BAP e 0,2 mg L^{-1} NAA produziram brotos axilares altos e reduziram posteriormente devido à redução de BAP e NAA. A redução da concentração do regulador de crescimento vegetal (PGR) diminuiu a parte aérea axilar, mas melhorou o desempenho da parte aérea axilar. No quarto subcultivo, a redução dos dados em todas as variáveis ocorreu devido à substituição de sacarose e gelita por açúcar e ágar locais. O meio MS de força total induziu melhor crescimento da parte aérea axilar do que outros no estágio de proliferação. Folhas amareladas, diminuição da altura da parte aérea e número de folhas foram observados em 'Cat Eye' na quinta subcultura e 'Pink Fiji' na sétima subcultura. Plantas altamente aclimatadas derivadas de pontas de brotos de Fiji Rosa com 86% e 83% de nós foram determinadas em uma mistura de casca de arroz queimada, esterco de jacinto e coqueiro (1:1:1, v/v/v). O corte direto aumentou o número de plantas como materiais de plantio em até 3 a 5 vezes em comparação com plântulas individuais. As descobertas podem ser aplicadas para acelerar a preparação de materiais de plantio qualificados e a comercialização do crisântemo.
Palavras-chave: corte direto, crescimento, crisântemo, desempenho e subcultura, parte aérea axilar.

Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the essential commercial cut-flowers and pot plants belonging to the Asteraceae and growing worldwide. The Chrysanthemum ranks second in the global cut flower market after rose. It has great demand and popularity because of its wide range of flower forms, colours, structures, and growth habits (Mehedi et al., 2020; Jahan et al., 2021; Hadizadeh et al., 2022). The plants are traditionally cultivated using root suckers and shoot cuttings with a low multiplication rate and low-quality plants (Beura et al., 2020; Mehedi et al., 2020; Jahan et al., 2021; Ali et al., 2023). In conventional method, one cutting produced 4 cuttings with lower root formation capacity in one month, while one *in vitro* plantlet resulted in 12 – 20 cuttings with high root formation capacity derived from tissue culture method. Utilization of the shoot cuttings regularly from mother plants used routinely and continuously increases viral infection and degeneration problem (Beura et al., 2020; Mehedi et al., 2020; Jahan et al., 2021; Ali et al., 2023). Therefore, applying micropropagation techniques due to their high potential improving plant quality, and productivity are importantly addressed.

In vitro mass propagation and production qualified planting materials for *Chrysanthemum spp*. frequently used axillary shoot proliferation method (Beura et al., 2020; Mehedi et al., 2020; Alsoufi et al., 2021; Jahan et al., 2021). The method is simple, applicable, and reliable for mass scale production and highly efficient clonal fidelity true-to-type plants of superior genotypes. This technique exploits the normal ontogenic route for branch development of axillary meristems to produce and proliferate of the shoot, less steps and difficulties (Jahan et al., 2021; Eisa et al., 2022). Success in application of the method is significantly affected by genotypes, explant sources, culture media, and PGR combination-concentration (; Imtiaz et al., 2019; Mehedi et al., 2020; Jahan et al., 2021). High compatibility of all factors applied results in optimal axillary shoot initiation, proliferation to acclimatization *in vitro* mass propagation on Chrysanthemum via axillary shoot formation were reported. MS medium fortified by 1% indole-3-acetic acid (IAA) and

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1.3 mg L^{-1} 6-Benzylaminopurine (BAP) were proved for shoot initiation and induction; however, there was no optimal culture medium findings on axillary shoot proliferation, root formation and acclimatization stage (Beura et al., 2020). New novelty was reported by Mehedi et al. (2020) by culturing shoot tip of *Chrysanthemum* 'Autumn Queen' into MS medium supplemented with 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} Kinetin (Kin) for producing maximal micro-shoot formation; half-strength MS with 0.2 mg L^{-1} indole-3-butyric acid (IBA), and 0.1 mg L^{-1} 2.4-dichlorophenoxyacetid acid (2.4-D) for root regeneration; a combination of soil, sand and rotten cow-dung (1:1:1) for plantlet acclimatization (Mehedi et al., 2020). Differed novelty was determined by Alsoufi et al. (2021) using MS medium supplemented with 4.0 mg L^{-1} Kin and 0.6 mg L^{-1} indole-3-butiryc acid (IBA) for optimal shoot formation; MS medium half the salt strength excelled and giving the highest root formation; however, there is no information dealing with the acclimatization stage. Jahan et al. (2021) established new findings compared to previous research using MS medium with 2.0 mg L^{-1} BAP to produce the maximum shoot induction; MS medium fortified with 1.5 mg L^{-1} IBA for rooting; and a mixture of soil, sand and compost (1:1:1) for optimal acclimatization of whole plantlets. The reports indicate that there is no report giving comprehensive and complete information from initiation to acclimatization. The research performed comprehensive investigations from the initiation using field-grown explants, followed by improving regeneration under reduced PGR concentration, proliferating shoots under periodical subcultures and applying new acclimatization methods to 'Cat Eye' and 'Pink Fiji'. The interesting results were discussed in detail in this paper.

Materials and Methods

Planting materials, explant preparation, disinfection, and culture incubation

Donor plants used in the experiments were *Ch. morifolium* 'Cat Eye', and 'Pink Fiji' originated from cultivars Imunk Flower, Candi village, Bandungan, Semarang District, Central Java, Indonesia (7°13′13″S 110°20′33″E and 1,050 m a.s.l.) (Fig. 5A). Young axillary shoots with six leaves, five internodes, and ± 6 cm in length were sufficiently harvested from the two donor plants for the first experiment. The leaf explants, except the young leaves, were removed then cut into 2 parts for sterilization purpose.

The prepared explants were put under tap water for ± 30 minutes, immersed and shaken manually in 1% sunlight liquid solution for ± 30 minutes, followed by rinsing them using clean water 6 times. The prepared explants were brought and placed in the horizontal laminar air flow cabinet (Esco LHG-4DS-F8, Esco Micro Pte, Ltd, Cangi, Singapore). The explants were sterilized in 0.05% mercury chloride for 3 minutes with manual shaking, followed by dipping in 0.01% mercury chloride for 7 minutes and shaking manually, and rinsed 6 times in sterile water (@ 3 min.). The shoot tips, third and fifth nodes (Fig. 5B) were sliced using a tissue culture blade transversally, then planted directly in initiation culture medium.

All explants cultured were incubated under a 12 h photoperiod during the day from 06.00 am to 06.00 pm and a 4 h photoperiod during the night from 10.00 pm to 02.00 am under cool fluorescent lamps with 13 µmol m^{-2} s^{-1} and 23 ± 1º C in every experiment.

Axillary shoot formation and growth derived from different types of field-grown explants of two varieties on different initiation culture media in the first culture

In the experiment, exploring BAP and/or NAA effect in different combinations and concentrations for axillary shoot formation and growth was tested for different types of field-grown explants. The split-split plot (SSP) was arranged in a completely randomized design (CRD) with three replications. Each treatment consisted of 6 bottles. Each bottle was cultured with 4 explants. The main plot was 'Cat Eye' and 'Pink Fiji'. Sub-plot was (1) shoot tips (Fig. 5C), (2) third nodes, and (4) fifth nodes. The established PGR combinations and concentrations tested using MS medium as basal medium as sub-sub-plot were (1) 2.0 mg L^{-1} BAP (ICM-1), 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} α-naphthaleneacetic acid (NAA) (ICM-2), (3) 1.0 mg L^{-1} BAP (ICM-3), (4) 1.0 mg L^{-1} and 0.1 mg L^{-1} NAA (ICM-4), and (5) 0.5 mg L^{-1} BAP (ICM-5). The media were added by 30 g L^{-1} sucrose, 1.8 g L^{-1} gelrite and adjusted pH in 5.8. The media were then sterilized using a GEA 24 L steam sterilizer at 121 ºC for 20 minutes at 15 kPa.

The PGR concentration on selected medium from the first experiment was reduced to 50% and more to maintain axillary shoot formation and growth of the varieties in the first and second subculture experiment. Random as subcultured model by culturing shoot tips with 2 leaves combined with 1 - 4 nodus cultured in one bottle were applied in the first subculture (Fig. 5G). Random and clustered model (only shoot tips) (Fig. 5H) was used in the second subculture experiment. SPP in CRD with three and five replications were applied. Each treatment consisted of 3 bottles. Each bottle was planted with 10 explants.

Axillary shoot formation and growth of explants harvested from two varieties under five subculture methods on selected medium in the third subculture

Different explants from two varieties were further explored. The split plot (SP) in CRD was also utilized in the experiment. The main plot was 'Cat Eye' and 'Pink Fiji'. The subplot was five subculture methods with (1) random, (2) clustered methods using shoot tips, (3) first, (4) third, and (5) fifth nodes. MS medium containing 0.25 mg L^{-1} BAP and 0.025 mg L^{-1} NAA were used in the experiment. Each treatment consisted of 3 bottles. Each bottle was planted with 10 explants.

Axillary shoot formation and growth of two variety explants with new subculture methods on the three-proliferation media in the fourth to seventh subculture

In the stage, explants were subcultured periodically under different methods and culture once a month until degeneration. Local sugar and agar were used generally by local tissue culture laboratories for cost efficiency purposes. The SSP in CRD with five replications were used. The main plot was 'Cat Eye' and 'Pink Fiji'. The five subculture methods as the sub-plot were (1) random (R), (2) shoot tips with one node harvested from random method (R1N), (3) shoot tips with two nodes harvested from random method (R2N), (4) shoot tips with one node harvested from clustered method (C1N) (Fig. 5H), and (5) shoot tips with two node harvested clustered method (C2N). The sub-sub-plot was three proliferation media (PM) *viz*. (1) full-strength MS medium PGR-free (PM-1), (2) half-strength MS medium PGR-free (PM-2), and (3) half-strength MS medium containing 0.5 mg L^{-1} IAA (PM-3). Each treatment consisted of 3 bottles. Each bottle was planted with 10 explants.

Plantlet acclimatization

New methods for plantlet acclimatization were applied in the research without preparing rooted shoots. Shoot tips with two fully opened leaves (1.5 – 2.5 cm in length) and one internode one leaf (0.75 – 1.25 cm in length) were directly cut from 'Pink Fiji' plantlet. but for 'Cat Eye' with short internode, shoot tips with four opened leaves (0.6 – 1.1 cm in length) and two internodes two leaves (0.4 – 0.65 cm in length) were applied. Three acclimatization media tested were (1) a combination of burned rice husk and sheep organic manure (BS, 1:1, v^{-1} v), (2) burned-rice husk and hyacinth organic manure (BH, 1:1 v v^{-1}), and (3) burned-rice husk, hyacinth organic manure, and cocopeat (BHC, 1:1:1, v/v/v). In the experiment, the SSP in CRD with 6 replications were used. Two varieties were used as the main plot; shoot tips and node as sub-pot and three acclimatization media as sub-sub-plot. Each treatment consisted of 12 explants for shoot tips and 20 explants for internodes. After culturing all explants in the plastic trays, explants were covered with transparent plastic for 7 days and removed afterward (Fig. 5P). The acclimatized explants were maintained by regularly watering, then observed. Data was taken a month after acclimatization.

Variables

Variables observed in the research were (1) a percentage of explant regeneration (%) calculated using formula as follows:

$$\text{Percentage of explant regeneration} = \frac{Number\ of\ regenerated\ explant}{Number\ of\ total\ explant\ cultured} \times 100\% \quad (Eq.\ 1)$$

(2) number of shoots $explant^{-1}$, (3) height of shoots (cm), (4) stem diameter (mm), (5) number of leaves $shoot^{-1}$, (6) number of brown leaves $shoot^{-1}$, (7) percentage of brown leaves $shoot^{-1}$ (%) counted using formula as follows:

$$\text{Percentage of browning leaves shoot}^{-1} = \frac{Number\ of\ brown\ leaves\ per\ shoot}{Total\ number\ of\ leaves\ per\ shoot} \times 100\% \quad (Eq.\ 2)$$

(8) internode length (mm), (9) leaf length and width ratio, (10) number of roots $shoot^{-1}$, and (11) plant survivability (%), calculated using formula as follows:

$$\text{Plant survivability} = \frac{Number\ of\ survived\ plants}{Total\ explant\ acclimatized} \times 100\% \qquad (Eq\ .3)$$

Data was taken and measured every month after the culture of explant, except for culture initiation in one and a half months after the culture of explant.

Data analysis

The data collected derived from every experiment were analyzed using analysis of variance (ANOVA) with SmartstatXL V.3.6.5.4 with Professional license to process the data. Significant differences between means were further analyzed using the Tukey test, *p=0.05*.

Results

Two varieties showed different responses on axillary shoot growth. Height of shoots and number of leaves shoot^{-1} increased gradually in each subculture up to third subculture then lowered afterward for 'Cat Eye' and sixth subculture and reduce thereafter for 'Pink Fiji' (Fig. 1A; Fig. 1C). Internode length indicated similar trend up to the second subculture and reduced subsequently (Fig. 1B) with number of roots shoot^{-1} in irregular trend (Fig. 1D). 'Pink Fiji' had higher shoot height, longer internode length, narrower stem diameter, higher leaf length width ratio, and lower roots shoot^{-1}, while 'Cat Eye' was characterized inversely (Fig. 1A; Fig. 1B; Fig. 1D; Table 2).

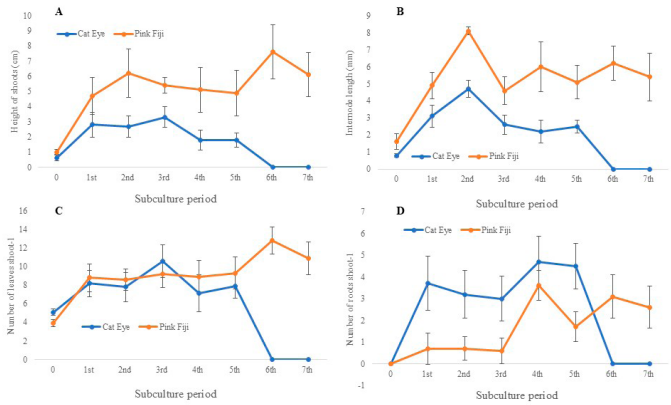

Fig. 1. Growth performances of axillary shoots derived from two different Chrysanthemum varieties under periodical subcultures. Axillary shoot performances based on (A) height of shoot (cm), (B) internode length (mm), (C) number of leaves shoot^{-1}, and (D) number of roots shoot^{-1}.

Three different culture media from initiation till proliferation, except in third subculture, gave similar trend in all variables observed. In the initiation stage, high callus formation in the bottom part of stem generally occurred (Fig. 5E) and lowered in the next subculture on proliferation media (Fig. 5I). Height of shoots and number of leaves shoot^{-1} rose gradually up to the sixth subculture and reduced thereafter (Fig. 2A; Fig. 2C). Decreasing growth performances of axillary shoots in the fourth SC was significantly affected by substituting sucrose and gelrite to local sugar and agar (Fig. 2A; Fig. 2C). Random models generally resulted in higher varied growth performances of axillary shoots than the cluster model (Fig. 3A-3D). R2N and C2N generally produced axillary shoots better performances (Fig. 1A-C).

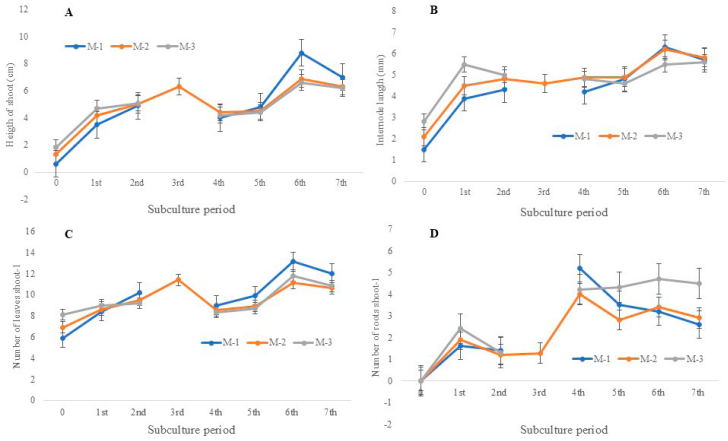

Figure 2. Growth performances of axillary shoots affected by three different culture media under periodical subcultures.. Axillary shoot performances based on (A) height of shoot (cm), (B) internode length (mm), (C) number of leaves shoot^{-1}, and (D) number of roots shoot^{-1}. From FC to third SC, MS basal medium was used with different combination and concentration of PGR. In the FC, M1-2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, M2-1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA, M3-2 mg L^{-1} BAP. In the first SC, M1-0.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA, M2-0.25 mg L^{-1} BAP and 0.025 mg L^{-1} NAA, and M3- 0.13 mg L^{-1} BAP and 0.013 mg L^{-1} NAA. In the second SC, M1-0.25 mg L^{-1} BAP and 0.025 mg L^{-1} NAA, M2-0.20 mg L^{-1} BAP and 0.020 mg L^{-1} NAA, and M3-0.15 mg L^{-1} BAP and 0.015 mg L^{-1} NAA. In the third SC, 0.25 mg L^{-1} BAP and 0.025 mg L^{-1} NAA were used. In the fourth SC till eighth SC, M1-Full strength MS medium PGR free, M2-half strength MS medium PGR free, and M3-half strength MS containing 0.5 mg L^{-1} IAA.

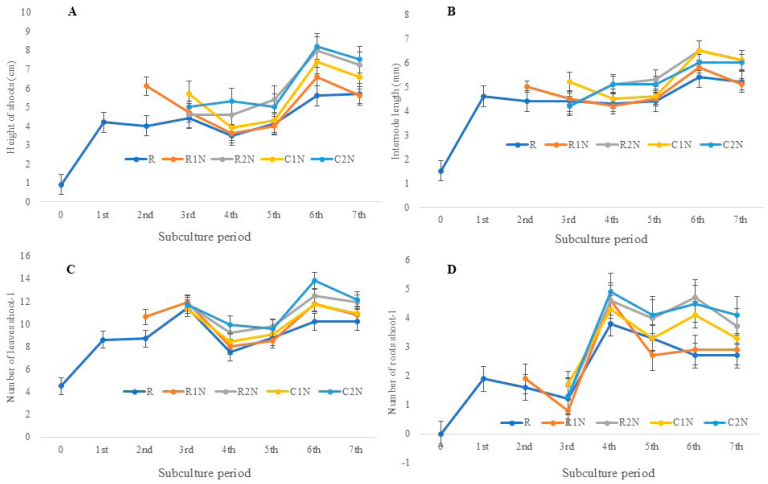

Fig. 3. Growth performances of axillary shoots affected by subculture methods under periodical subcultures. Axillary shoot performances based on (A) height of shoot (cm), (B) internode length (mm), (C) number of leaves shoot^{-1}, and (D) number of roots shoot^{-1}. R – random model, R1N - shoot tips with one node harvested from random method, R2N - shoot tips with two nodes harvested from random method, C1N - shoot tips with one node harvested from clustered method, and C2N - shoot tips with two node harvested clustered method.

Axillary shoot formation and growth derived from different types of field-grown explants on different varieties and initiation culture media in the first culture

In the first experiment, axillary shoot formation and its growth performances were significantly influenced by all treatments. Shoot tips of 'Pink Fiji' cultured on ICM-2 (MS medium supplemented with 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA) were the most suitable combination treatment in obtaining maximal axillary shoot formation. The combination stimulated 58.3% explant regeneration with 8.3 shoots explant^{-1} (Fig. 5D), 1.3 cm height of shoots, 0.87 mm stem diameter, 6.9 leaves shoot^{-1}, 2.1 mm internode length, and 1.58 leaf length and width ratio (Table 1). The shoot tip of 'Cat Eye' was optimal on ICM-1 (MS medium with 2.0 mg L^{-1} BAP) (Table 1). The lowest results were generally recorded in the third and fifth nodes. In the initiation stage, it was confirmed that shoot tips harvested from field-grown plants were better than node for axillary shoot formation. Each variety had specific initiation culture medium (Table 1).

The reduction of PGR concentration in the first and second subculture lowered the number of axillary shoots down to 66.3%. However, the reduction increased axillary shoot performances (Fig. 1A-1D; Fig. 2A-2D; Fig. 3A-1D). Higher axillary shoots were noted on 'Pink Fiji' on SR-1 (MS medium supplemented with 0.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA) and produced 2.8 axillary shoots explant^{-1} (Fig. 5F) with 3.7 cm height of shoots, 1.1 mm stem diameter, 4.9 mm internode length, and 1.32 leaf length-width ratio (Data not shown). Wider stem diameter, shorter internode length, and lower leaf length-width ratio on 'Cat Eye' were also confirmed in the stage. In the second subculture, all treatments induced interaction effect on number of leaves shoot^{-1} and leaf length-width ratio (Data not shown). The clustered method improved the maximal growth of axillary shoots (Fig. 5K) compared to random method (Fig. 5J). Axillary shoots of 'Pink Fiji' gave better axillary shoot performances on clustered model in TR-1 than other combination treatments.

Table 1. Interaction effect of different varieties. explant sources and initiation culture media on axillary shoot formation and growth in the first culture

Variety	Explant source	Initiation culture medium	Percentage of explant regeneration (%)	Number of shoots explant^{-1}	Height of shoot (cm)	Number of leaves shoot^{-1}	Stem diameter (mm)	Internode length (mm)	Leaf length width ratio
'Cat Eye'	Shoot tips	ICM1	25.0 ± 0.0 bcde	5.2 ± 0.8 abc	0.60 ± 0.10	5.9 ± 0.3 abc	0.82 ± 0.07 ab	1.50 ± 0.30	1.68 ± 0.32 a
		ICM2	37.5 ± 12.5 abcd	3.5 ± 0.5 bcde	0.53 ± 0.06	7.3 ± 0.3 abc	0.74 ± 0.07 ab	0.77 ± 0.21	1.38 ± 0.02 abc
		ICM3	41.7 ± 14.4 abc	4.0 ± 0.5 bcd	0.80 ± 0.10	6.8 ± 0.9 abc	0.82 ± 0.09 ab	1.07 ± 0.23	1.64 ± 0.48 a
		ICM4	41.7 ± 14.4 abc	4.0 ± 0.4 bcd	0.57 ± 0.12	7.7 ± 0.4 abc	0.75 ± 0.08 ab	0.80 ± 0.44	1.54 ± 0.24 ab
		ICM5	33.3 ± 14.4 abcd	4.0 ± 0.5 bcd	0.83 ± 0.51	7.7 ± 1.0 abc	0.73 ± 0.09 ab	1.03 ± 0.21	1.44 ± 0.21 ab
	3rd node	ICM1	8.3 ± 14.4 de	1.3 ± 2.3 cde	0.23 ± 0.40	2.8 ± 4.8 bc	0.21 ± 0.36 bc	0.23 ± 0.40	0.37 ± 0.65 abc
		ICM2	16.7 ± 14.4 cde	2.3 ± 2.1 cde	0.57 ± 0.49	5.2 ± 4.6 abc	0.53 ± 0.46 abc	0.63 ± 0.57	0.93 ± 0.81 abc
		ICM3	16.7 ± 14.4 cde	2.0 ± 1.7 cde	1.63 ± 2.57	5.7 ± 5.1 abc	0.57 ± 0.49 abc	3.03 ± 2.63	0.74 ± 0.64 abc
		ICM4	16.7 ± 14.4 cde	2.3 ± 2.1 cde	0.60 ± 0.53	5.4 ± 4.7 abc	0.52 ± 0.45 abc	0.63 ± 0.57	0.84 ± 0.77 abc
		ICM5	16.7 ± 14.4 cde	0.7 ± 0.6 de	2.23 ± 1.94	13.0 ± 11.4 a	0.83 ± 0.72 ab	1.53 ± 1.34	0.97 ± 0.85 abc
	5th node	ICM1	16.7 ± 14.4 cde	3.3 ± 2.9 bcde	0.47 ± 0.42	4.5 ± 4.0 abc	0.46 ± 0.40 abc	0.50 ± 0.44	0.86 ± 0.74 abc
		ICM2	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM3	16.7 ± 14.4 cde	2.7 ± 2.4 bcde	0.77 ± 0.68	5.2 ± 4.5 abc	0.49 ± 0.43 abc	0.93 ± 0.86	1.08 ± 0.94 abc
		ICM4	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM5	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
'Pink Fiji'	Shoot tips	ICM1	33.3 ± 14.4 abcd	6.3 ± 0.2 ab	1.40 ± 0.40	5.9 ± 0.7 abc	0.64 ± 0.04 abc	3.17 ± 0.93	1.43 ± 0.27 ab
		ICM2	58.3 ± 14.4 a	8.3 ± 1.3 a	1.27 ± 0.60	6.9 ± 1.5 abc	0.87 ± 0.07 ab	2.10 ± 0.26	1.58 ± 0.25 ab
		ICM3	41.7 ± 14.4 abc	6.3 ± 0.8 ab	1.93 ± 0.67	6.1 ± 0.4 abc	0.73 ± 0.06 ab	4.43 ± 1.19	1.53 ± 0.13 ab
		ICM4	33.3 ± 14.4 abcd	4.0 ± 0.6 bcd	1.67 ± 0.55	5.7 ± 0.7 abc	0.91 ± 0.09 ab	3.63 ± 1.35	1.53 ± 0.11 ab
		ICM5	51.4 ± 14.7 ab	4.6 ± 2.0 abcd	1.63 ± 0.25	6.0 ± 0.9 abc	0.93 ± 0.15 ab	2.80 ± 0.50	1.10 ± 0.14 abc
	3rd node	ICM1	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM2	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM3	16.7 ± 14.4 cde	1.7 ± 1.5 cde	1.53 ± 2.08	5.4 ± 6.1 abc	0.57 ± 0.50 abc	1.60 ± 1.83	1.01 ± 1.00 abc
		ICM4	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM5	8.3 ± 14.4 de	1.3 ± 2.3 cde	0.77 ± 1.33	2.8 ± 4.8 bc	0.29 ± 0.50 abc	1.17 ± 2.02	0.50 ± 0.87 abc
	5th node	ICM1	0.0 ± 0.0 e	0.0 ± 0.0 e	0.00 ± 0.00	0.0 ± 0.0 c	0.00 ± 0.00 c	0.00 ± 0.00	0.00 ± 0.00 c
		ICM2	8.3 ± 14.4 de	1.3 ± 2.3 cde	0.70 ± 1.21	2.1 ± 3.6 c	0.24 ± 0.42 bc	0.97 ± 1.67	0.24 ± 0.41 bc
		ICM3	16.7 ± 14.4 cde	2.0 ± 2.0 cde	0.60 ± 0.72	3.5 ± 3.1 bc	0.47 ± 0.41 abc	1.20 ± 1.31	1.12 ± 1.00 abc
		ICM4	25.0 ± 0.0 bcde	1.0 ± 0.0 de	2.47 ± 1.01	11.0 ± 1.0 ab	1.00 ± 0.10 a	2.03 ± 0.74	1.58 ± 0.23 ab
		ICM5	8.3 ± 14.4 de	1.3 ± 2.3 cde	0.77 ± 1.33	2.8 ± 4.8 bc	0.29 ± 0.50 abc	1.17 ± 2.02	0.51 ± 0.89 abc

The means followed by the same letter in the same column are significantly different, based on the Tukey test, $p = 0.05$. Values reflect the means and standard errors of cultured explants, with n = 24 for each treatment.

Axillary shoot formation and growth of explants harvested from two varieties under five subculture methods on selected medium in the third subculture

Exploring the different effects of five subculture methods in combination to two varieties in the third subculture did not have significant effect on axillary shoots and their growth performances. In the subculture period, significancy of axillary shoot growth was affected by simple effects of varieties (Fig. 4A, 4C, 4E, 4F, and 4G) and a part of them by simple effect of subculture methods (Fig. 4B, and 4D). Axillary shoots from Pink had better performances than 'Cat Eye'. There was a trend, explants taken from the bottom part had higher shoot height than the upper part (Fig. 4B). However, there was no interaction effect between varieties and subculture methods in all variables observed.

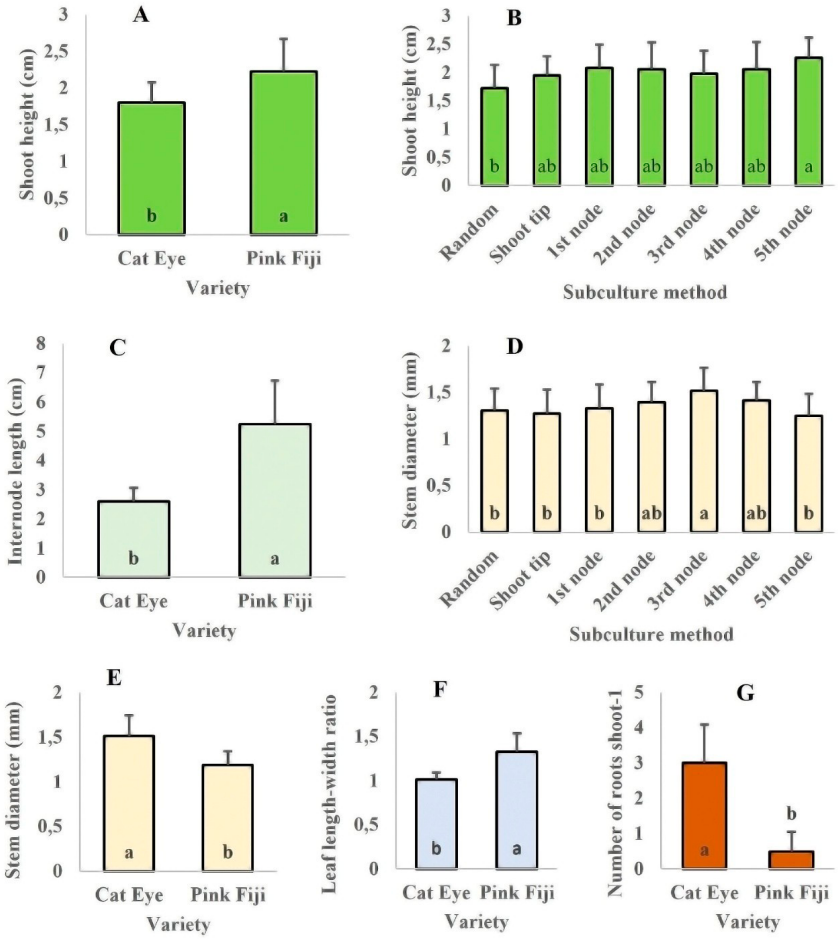

Fig. 4. Simple effects of varieties on shoot height, interned length, stem diameter, leaf length-width ratio and number of roots shoot-1; and simple effects of subculture methods on shoot height and stem diameter.

Axillary shoot formation and growth of different explants of three varieties with new subculture methods on the three-proliferation media from the fourth to eighth subculture

In the fourth till the eighth subculture, several important information was revealed. Alteration of sucrose to local sugar and gelrite to local agar brought significant effects on reducing growth performances (2.4% – 45.5%) of axillary shoots in several variables (Fig. 3A, 3C; Fig 4A, 4C). These phenomena were presumably due to the presence of impurity materials that have phytotoxicity effects and inhibit the growth of axillary shoots normally. 'Pink Fiji' explants generally performed better shoot height and leaf number (Fig. 1A, 1C); but wider stem diameter and low leaf length-width ratio was noted for 'Cat Eye' (Data not shown).

Richer nutrition, higher shoot height, wider stem diameter, and higher leaf number were noted on PM-1 (Full strength of MS medium) (Tab. 2; Fig. 4A, 4C). Explants cultured on PM-1 induced thinner and smaller roots (Fig. 5E); bigger, thicker, and hairy roots were regenerated on explants cultured on PM-3 (half-strength MS containing 0.5 mg L^{-1} IAA, Fig. 5M). Cluster models both R1N, R2N (Fig. 5N), C1N, and C2N induced better performances of axillary shoots than the random model (Fig. 3A, 3B). C2N showed optimal results for axillary shoot growth compared to others (Tab. 2; Fig. 5O).

Under explant subculture periodically, it was also revealed that degeneration had occurred in different periods for two varieties tested. In 'Cat Eye', yellow/brown leaves varied from 1 - 4 leaves $shoot^{-1}$ with 8.2% – 44.4% was recorded at fifth subculture (Tab. 2; Fig. 5L). The stage was generally taken place after peak performances of axillary shoots noted in the previous subculture (Fig. 1A, 1B, 1C). Similar situation was recorded on 'Pink Fiji' in the seventh subculture with from 0 - 3 leaves per shoot with 0% – 16.4% (Data not shown). The peak growth performances of axillary shoots were noted in the sixth subculture and declined afterwards. for (Fig. 1A, 1C).

Table 2. Interaction effect of different varieties. new subculture methods and proliferation media on axillary shoot formation and growth in the fifth subculture

Variety	Subculture methods	Proliferation medium	Height of shoots (cm)	Stem diameter (mm)	Number of leaves shoot^{-1}	Number of brown leaves shoot^{-1}	Percentage of brown leaves	Internode length (mm)	Leaf length width ratio
'Cat Eye'	R	PM-1	2.9 ± 1.5 ghij	1.48 ± 0.29	10.2 ± 3.1	1.5 ± 1.0 bc	13.7 ± 7.8 cd	2.24 ± 0.51	1.13 ± 0.30
		PM-2	2.1 ± 0.9 ijk	1.27 ± 0.16	7.8 ± 1.7	1.0 ± 0.9 cd	11.4 ± 10.2 cd	2.56 ± 0.44	1.00 ± 0.17
		PM-3	1.9 ± 0.6 jk	1.21 ± 0.13	6.8 ± 1.9	1.0 ± 0.6 cd	13.9 ± 7.5 cd	2.33 ± 0.44	1.08 ± 0.08
	R1N	PM-1	2.3 ± 0.7 hijk	1.65 ± 0.22	9.5 ± 1.5	1.7 ± 0.5 bc	18.2 ± 7.2 c	2.67 ± 0.17	1.02 ± 0.12
		PM-2	1.5 ± 0.6 k	1.31 ± 0.13	7.3 ± 1.4	1.0 ± 0.6 cd	14.1 ± 9.6 cd	2.54 ± 0.19	0.99 ± 0.03
		PM-3	1.3 ± 0.5 k	1.26 ± 0.11	6.5 ± 0.5	1.3 ± 0.5 bc	20.6 ± 8.2 bc	2.02 ± 0.37	1.08 ± 0.13
	R2N	PM-1	2.2 ± 0.7 hijk	1.50 ± 0.18	9.3 ± 2.4	1.3 ± 1.1 c	15.2 ± 15.2 cd	2.39 ± 0.46	1.04 ± 0.10
		PM-2	1.7 ± 0.7 k	1.27 ± 0.24	7.0 ± 1.3	0.8 ± 0.8 cd	11.3 ± 10.4 cd	2.38 ± 0.80	0.96 ± 0.08
		PM-3	1.4 ± 0.4 k	1.17 ± 0.08	7.3 ± 0.8	1.3 ± 0.6 c	16.9 ± 8.0 c	2.09 ± 0.31	1.06 ± 0.09
	C1N	PM-1	2.1 ± 0.4 hijk	1.35 ± 0.24	9.7 ± 1.0	0.8 ± 0.8 cd	8.2 ± 7.1 cd	2.45 ± 0.43	1.04 ± 0.13
		PM-2	1.4 ± 0.4 k	1.40 ± 0.24	7.3 ± 1.4	1.0 ± 0.9 cd	13.4 ± 12.1 cd	2.18 ± 0.35	0.99 ± 0.13
		PM-3	2.0 ± 0.4 ijk	1.37 ± 0.26	7.0 ± 0.6	1.0 ± 0.9 cd	14.5 ± 13.3 cd	2.58 ± 0.31	1.11 ± 0.24
	C2N	PM-1	2.1 ± 0.6 ijk	1.47 ± 0.15	8.7 ± 1.0	1.3 ± 0.6 c	14.3 ± 6.1 cd	2.43 ± 0.46	1.03 ± 0.09
		PM-2	1.3 ± 0.3 k	1.28 ± 0.13	7.3 ± 1.2	2.5 ± 0.5 ab	34.9 ± 9.9 ab	1.84 ± 0.15	1.15 ± 0.12
		PM-3	1.5 ± 0.5 k	1.40 ± 0.27	7.0 ± 0.9	3.2 ± 1.0 a	44.4 ± 9.7 a	2.09 ± 0.34	1.10 ± 0.10
'Pink Fiji'	R	PM-1	3.2 ± 1.9 fghi	1.27 ± 0.19	7.7 ± 1.5	0.0 ± 0.0 d	0.0 ± 0.0 d	3.89 ± 1.61	1.20 ± 0.03
		PM-2	3.6 ± 2.2 efg	1.17 ± 0.18	7.5 ± 2.3	0.0 ± 0.0 d	0.0 ± 0.0 d	4.58 ± 2.24	1.16 ± 0.08
		PM-3	2.9 ± 1.7 ghij	1.28 ± 0.13	8.0 ± 3.2	0.0 ± 0.0 d	0.0 ± 0.0 d	3.13 ± 0.92	1.22 ± 0.13
	R1N	PM-1	4.6 ± 0.6 cde	1.04 ± 0.09	8.0 ± 0.9	0.0 ± 0.0 d	0.0 ± 0.0 d	5.29 ± 1.04	1.17 ± 0.11
		PM-2	4.0 ± 0.8 efg	1.17 ± 0.15	7.2 ± 1.0	0.0 ± 0.0 d	0.0 ± 0.0 d	5.18 ± 1.07	1.14 ± 0.09
		PM-3	4.1 ± 0.6 defg	1.24 ± 0.08	8.3 ± 0.5	0.0 ± 0.0 d	0.0 ± 0.0 d	4.38 ± 0.45	1.25 ± 0.06
	R2N	PM-1	6.5 ± 0.8 ab	1.10 ± 0.09	10.5 ± 0.5	0.0 ± 0.0 d	0.0 ± 0.0 d	6.59 ± 1.36	1.22 ± 0.08
		PM-2	7.8 ± 0.7 a	1.13 ± 0.15	11.3 ± 1.2	0.0 ± 0.0 d	0.0 ± 0.0 d	7.25 ± 1.55	1.16 ± 0.04
		PM-3	6.5 ± 0.7 b	1.31 ± 0.18	11.3 ± 1.5	0.0 ± 0.0 d	0.0 ± 0.0 d	5.49 ± 0.56	1.19 ± 0.08
	C1N	PM-1	4.5 ± 0.6 de	1.19 ± 0.17	9.7 ± 1.0	0.0 ± 0.0 d	0.0 ± 0.0 d	4.84 ± 1.08	1.19 ± 0.05
		PM-2	4.4 ± 0.6 def	1.17 ± 0.19	8.7 ± 1.0	0.0 ± 0.0 d	0.0 ± 0.0 d	4.94 ± 0.47	1.21 ± 0.08
		PM-3	3.3 ± 0.6 efgh	1.18 ± 0.21	8.7 ± 0.8	0.0 ± 0.0 d	0.0 ± 0.0 d	3.61 ± 0.65	1.20 ± 0.04
	C2N	PM-1	5.8 ± 1.0 bc	1.13 ± 0.08	10.5 ± 1.4	0.0 ± 0.0 d	0.0 ± 0.0 d	4.99 ± 0.78	1.18 ± 0.09
		PM-2	5.3 ± 0.8 bcd	1.14 ± 0.13	9.2 ± 1.2	0.0 ± 0.0 d	0.0 ± 0.0 d	5.25 ± 0.66	1.22 ± 0.04
		PM-3	4.5 ± 0.6 de	1.26 ± 0.07	9.8 ± 0.8	0.0 ± 0.0 d	0.0 ± 0.0 d	4.18 ± 0.28	1.21 ± 0.11

The means followed by the same letter in the same column and means not followed by letter are not significantly different based on the Tukey test, $p = 0.05$. Values reflect the means and standard errors of cultured explants, with n = 30 for each treatment.

Plantlet acclimatization

The highest survivability of acclimatized explants up to 86% was reported on shoot tip explants of 'Pink Fiji' planted on BHC (1:1:1, v/v/v), followed by their node explants in a similar medium (Table 3; Fig. 5Q). Acclimatization using direct cutting of shoot tips and nodes generally rooted in 7 - 9 days and could be harvested 11 – 13 days after planting. Shoot tips gave a better result than nodus (Fig. 5R, 5S), having higher shoot height, number of leaves, and roots plant^{-1}. By direct cutting, the number of acclimatized plants increased significantly up to 3 – 5 folds compared to general method with a single plantlet (7 - 9 cm in height and 7 - 11 leaves per plantlet).

Table 3. Interaction effect of varieties. media and acclimatized plantlet sources on acclimatization and growth of plants

Variety	Acclimatization of media	Acclimatized plantlet sources	Percentage of survivability (%)	Height of plants (cm)	Number of leaves plant^{-1}	Number of roots plant^{-1}
'Cat Eye'	BS	Shoot tips	70.0 ± 10.0 ab	15.5 ± 2.4	5.7 ± 1.0	3.3 ± 1.0
		Nodus	44.4 ± 7.7 bc	13.5 ± 2.3	3.8 ± 0.9	3.9 ± 0.9
	BH	Shoot tips	66.7 ± 5.8 ab	24.9 ± 1.6	8.3 ± 1.0	5.6 ± 0.5
		Nodus	24.4 ± 10.2 c	20.3 ± 4.0	6.0 ± 1.7	4.6 ± 1.9
	BHC	Shoot tips	76.7 ± 15.3 a	21.1 ± 3.1	6.7 ± 0.8	4.8 ± 0.7
		Nodus	80.0 ± 6.7 a	17.3 ± 2.3	5.4 ± 1.2	3.7 ± 0.7
'Pink Fiji'	BS	Shoot tips	73.3 ± 11.5 ab	17.8 ± 0.1	6.0 ± 0.7	4.1 ± 0.2
		Nodus	23.3 ± 12.1 c	17.5 ± 6.4	3.8 ± 2.8	3.0 ± 2.7
	BH	Shoot tips	75.0 ± 8.3 ab	27.0 ± 6.9	7.1 ± 0.9	3.7 ± 0.7
		Nodus	60.0 ± 15.0 ab	23.2 ± 1.0	5.0 ± 0.4	2.7 ± 0.8
	BHC	Shoot tips	86.1 ± 4.8 a	28.5 ± 2.9	6.3 ± 0.2	4.2 ± 0.5
		Nodus	83.3 ± 10.4 a	22.1 ± 3.7	4.3 ± 0.3	2.7 ± 0.3

Means not followed by letter in the same column are not significantly different (Tukey test, $p = 0.05$) Values reflect the means and standard errors of cultured explants, with n = 36 - 60 for each treatment.

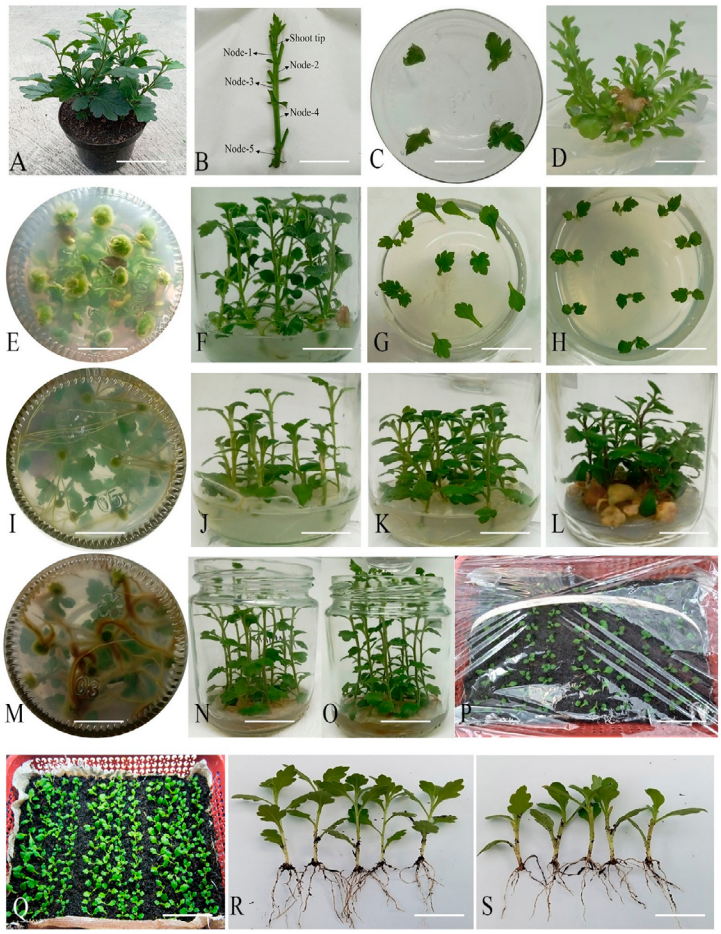

Fig. 5. Micropropagation of *Chrysanthemum morifolium*: initiation to acclimatization. A. Mother plants of 'Cat Eye', B. Shoot tip, third, and fifth node used in the first culture, C. Shoot tip explants cultured in the first culture, D. Axillary shoots from shoot tip of 'Pink Fiji' 1.5 months after culture on MS medium with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, E. High regenerated callus of 'Pink Fiji' (++++) at wounded stem area 30 days after culture on ICM-2 in the first subculture, F. Regenerated shoots in the first subculture of shoots 30 days after culture on MS medium added 0.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA, G. Random model in initial culture, H. Shoots cultured in clustered model, I. Smaller and thinner roots of 'Pink Fiji' with less callus (+) in full strength MS medium 20 days after culture, J. Varied shoot height of 'Pink Fiji' under random model on MS medium 0.25 mg L^{-1} BAP and 0.025 mg L^{-1} NAA 15 days after culture, K. Almost similar shoot height under clustered model (C1N) in the similar age and culture medium, L. Yellow leaves on regenerated shoots of 'Cat Eye' under C2N model on half-strength MS containing 0.5 mg L^{-1} IAA 30 days after culture, M. Thicker and hairy root performances of regenerated roots on half-strength MS supplemented with 0.5 mg L^{-1} IAA 20 days after culture, N. 'Pink Fiji' shoots on R2N model cultured on a half-strength MS medium added 0.5 mg L^{-1} IAA at 30 days after culture, O. 'Pink Fiji' well shoot on C2N model in full strength MS medium 30 days after culture in the sixth subculture, P. Shoot tips and nodes of 'Pink Fiji' cut directly then cultured on the burned rice husk, hyacinth organic manure and cocopeat (BHC, 1:1:1, v/v/v) and covered by transparent plastic for 7 days in initial culture, Q. Well growth of 'Pink Fiji' shoots tips and nodes in BHC after 30 days after acclimatization, R. Rooted shoot tips of 'Pink Fiji' after 45 days after acclimatization, S. Rooted nodes of 'Pink Fiji' after 45 days after acclimatization. Bars. A = 4.37 cm; B = 1.37 cm; C, E, F, I, J, K, and M = 2.04 cm; G. and H = 2.67 cm; L = 2.40 cm; N. and O = 3.2 cm; P = 7.74 cm; Q = 8.57 cm; R and S = 3.75 cm.

Discussion

The axillary bud proliferation as the most used due to genetic stability regenerants, easy and efficient method were affected by genotype or varieties used (Beura et al., 2020; Eisa et al., 2022). 'Pink Fiji' was better on axillary shoot initiation and performances up to 8.2 shoots than 'Cat Eye' (5.2 shoots). The 'Cat Eye' had shorter shoot height, internode length, lower leaf length-width ratio, and higher root number, but 'Pink Fiji' indicated inversely. In different studies, 5.4 shoots were performed by Snow White, then 5.1 shoots on Baltica White, and 4.4 on Neznakomka (Borodulina et al., 2019); 9.5 shoots on Urban Red than 5.9 on New man, 5.2 on Blooming Beauty White (Prathyusha et al., 2021). Though less research has been done to explore variety effects on axillary shoot formation and proliferation, these findings confirmed that each variety has a specific capacity and performance on axillary shoot initiation and proliferation.

In the axillary method, the existence of PGR, especially BAP individually and/or in combination with auxins, has an important role to activate and promote cell division, shoot initiation, differentiation, and sprout dormant axillary bud and its multiplication (Beura et al., 2020; Alsoufi et al., 2021; Mekonen et al., 2021). Balance combination-concentration of BAP-NAA increased axillary shoot growth and MS medium with 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA induced 8.3 shoots $explant^{-1}$ for 'Pink Fiji', and 5.2 shoots on 2.0 mg L^{-1} BAP for 'Cat Eye'. In the previous report, 21.7 shoots $culture^{-1}$ on 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA , and 11 shoots on 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA (Imtiaz et al., 2019), 3.2 shoots $explant^{-1}$ on 0.5 mg L^{-1} NAA and 2 mg L^{-1} BAP (Alsoufi et al., 2021)

In plant tissue culture, sucrose, agar and their quality play and give significant effect in photosynthesis, plant branching, morphogenesis and development, maintain the osmotic potential and serve as energy in shoot proliferation (Mohamed et al., 2021; Doidy et al., 2024; Pasternak and Steinmacher, 2024; Sinurat et al. 2024). High quality carbon sources and agar such as gelrite stimulated better morphogenesis of explant. Declining growth performances were from 2.4% – 45.5% noted on the height of shoots, stem diameter, number of leaves $shoot^{-1}$, leaf length, and width ratio. The performance reduction was presumably due to the existence of impurity and toxic materials such as arsenic, cadmium, chromium, copper,

mercury, selenium, etcetera (Mello et al., 2022; Sinurat et al., 2024). So far, there is no reports comparing sucrose and local sugar in axillary formation, while application agar declined shoot induction down to 34.3% and 18.4% number of shoots varied varieties of Chrysanthemum. These results confirmed that low quality sugar and agar reduced the growth performances of shoots.

Subculture is one of *in vitro* activities aiming to improve multiplication rate, reduce hyperhydricity problems, increase vigor, and cellular activity (Winarto et al., 2019; Jan et al., 2020). In the research, a higher number of axillary shoots (8.3 shoots) with low performance were noted in the first culture, then increased gradually till peak of growth in sixth subculture and reduced thereafter for 'Pink Fiji'. In other research, 7.2 MR of *Gerbera jamesonii* 'Carambola' in the fourth subculture and reduced thereafter (Winarto et al., 2019), 12.8 MR of *Amaranthus viridis* in the fifth subculture and declined thereafter (Jan et al., 2020). All results above showed that each genotype had different proliferation capacities under periodical subcultures.

Senescence is the final growth stage of plants indicated by degenerative events, causes drastic physiological, biochemical, and metabolic changes, functional transition from nutrient assimilation to nutrient remobilization, and immersing yellowing leaves as leaf senescence indicator taken place due to the breakdown of chlorophylls in chloroplasts (Guo et al., 2021). In the research, 1 - 4 yellowing leaves (8.2% – 44.4% from total leaves) of 'Cat Eye' were easily observed in the fifth subculture, and 'Pink Fiji' in the seventh subculture with 0-3 yellowing leaves (0% - 16.4% from total leaves). So far, there is no research report in *Chrysanthemum.* In the *in vitro* culture of Rosa hybrida 'Baby Love', 30% of yellowing leaves were recorded at rooting stage (Ha et al., 2020), 23.3% of yellowing leaves were noted on multiplication step of shoots and 20.0% on rooting stage in micropropagation of Gerbera (Tung et al., 2022), and 45% leaves on *in vitro* culture of Prunus rootstock (Thakur et al., 2023)

The acclimatization of plantlets is a critical stage due to altering their morphology, anatomy, and physiology, having high transpiration rates, low absorption rate, abnormal stomata, low photosynthesis capacity, and high mortality during the acclimatization process. Therefore, establishing optimal process and media is important to be established. Utilization of healthy and complete plantlets with mature roots were transferred to small pots containing 1:1:1 non-sterilized garden soil, sand, and coco-peat and gradually adapted to field conditions producing 90% survival plantlets of *C. morifolium* to natural conditions (Jahan et al., 2021). Application of sterilized cocopeat sprayed with ½ strength of Hoagland's solution weekly that increased high capacity of plantlets to adapt ex vitro condition progressively, gave a 100% survival rate of complete plantlets (Ali et al., 2023). In the research, direct cutting of shoot tips (2.5 cm in height with 2 - 3 leaves) and nodus (1 - 2 nodes with 1 - 2 leaves) followed by direct planting in BHC (1:1:1, v/v/v) medium, covering transparent plastic for 7 days, placing in a shady area, and watering regularly stimulated high survivability of 'Pink Fiji' plants up to 86% and 83%, respectively. Covering the acclimatized cuttings with the transparent plastic for 7 days, placing them in shady area, and watering regularly could significantly support them having high capacity to adapt *ex vitro* environment for their natural growth. Due to the optimal condition, high survivability of the cuttings was proved. With this method, the number of planting materials increased up to 3 - 5 folds compared to the complete plantlet method.

Conclusions

MS medium supplemented with 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA was optimal for 'Pink Fiji' and 2.0 mg L^{-1} BAP for 'Cat Eye'. Reduction of the PGR concentration let to lowering axillary shoot production. Utilization of local sugar and agar decreased axillary shoot growth and performances. MS full strength induced better axillary shoot growth than others for proliferation. Immersing yellow leaves as senescence indicator was noted in the fifth subculture for 'Cat Eye' and seventh subculture for 'Pink Fiji'. The highest survivability and well growth of shoot tips of 'Pink Fiji' up to 86% was noted on BHC medium. Direct cutting of shoot and node explants in acclimatization increased the number of planting materials up to 3 – 5 folds. The findings can be applied to explore other varieties.

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Author Contribution

BW: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Supervision, Validation, Visualization, Writing – original draft, Writing – Review & Editing. **AS:** Investigation, Formal Analysis, Writing – Review & Editing. **JAB:** Data Curation, Investigation, Validation, Writing – Original Draft. **S:** Investigation, Writing – Review & Editing. **YH:** Data Curation, Writing – Review & Editing. **SJ:** Investigation, Writing – Review & Editing. **AS:** Investigation, Data Curation, Writing – Review & Editing. **JK:** Conceptualization. Supervision, Investigation, Writing – Review & Editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

All the research data is contained in the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

References

ALI, S.; RAZA, S.; SHAHZAD, S.; BATOOL, T.S.; MANZOOR, A. Regeneration of chrysanthemum (*Chrysanthemum morifolium*) via somatic embryogenesis and screening of clones for agronomic traits', **Plant Cell, Tissue and Organ Culture**, v.153, n.3, p. 657–667, 2023. https://doi.org/10.1007/s11240-023-02506-6

ALSOUFI, A.S.M.; AHMED, Z.S; SALIM, A.M. The efficiency of interaction between cytokines and auxins in micropropagation of Chrysanthemum plant (*Chrysanthemum indicum* L.). **2nd Virtual International Scientific Agricultural Conference, IOP Conference Series: Earth and Environmental Science**, v.735, p. 1-7. https://doi.org/10.1088/1755-1315/735/1/012048

BEURA, R.; PANDA, M.; BEURA, S.; BAHIRA, S. *In vitro* propagation of Chrysanthemum morifolium using shoot tip and nodal segment as explant. **Global Journal for Pharma and Allied Sciences,** v.1, n.2, p. 12-17, 2020.

BORODULINA, I.D.; TRUFANOVA, A.; SHEVCHENKO, G.; SOKOLOVA, G.; PLAKSINA, T. Optimization of clonal micropropagation of Chrysanthemum. **Ukrainian Journal of Ecology**, v.9, n.4, p. 676–678, 2019. https://doi.org/10.15421/2019_809

EISA, E.A.; TILLY-MANDY, A.; HONFI, P.; SHALA, A.Y.; GURURANI, A.; *Chrysanthemum*: A Comprehensive Review on Recent Developments on *In Vitro* Regeneration. **Biology**, v.11, p. 1774-1800, 2022. https://doi.org/10.3390/biology11121774

GUO, Y.; REN, G.; ZHANG, K.; LI, Z.; MIAO, Y.; GUO, H. Leaf senescence: progression, regulation, and application. **Molecular Horticulture**, v.1-5, p. 1-25. 2021. https://doi.org/10.1186/s43897-021-00006-9

HA, N.T.M., DO, C.M., HOANG, T.T., NGO, N.D., BUI, L.V., NHUT, D.T. The effect of cobalt and silver nanoparticles on overcoming leaf abscission and enhanced growth of rose (*Rosa hybrida* L. 'Baby Love') plantlets cultured in vitro. **Plant Cell, Tissue and Organ Culture**, v.141, p. 393-405, 2020. https://doi.org/10.1007/s11240-020-01796-4

HADIZADEH, H.; SAMEEI, L.; SHAKERI, A. Chrysanthemum, an ornamental genus with considrable medicinal value: A comprehensive review. **South African Journal of Botany**, v.144, p. 23-43, 2022. https://doi.org/10.1016/j.sajb.2021.09.007

IMTIAZ, M.; KHATTAK, A.M.; KHAN, M.A.; JALAL, F.; HUSSAIN,

S.; SAID, F.; BO, H. Rapid in-vitro propagation of *Chrysanthemum morifolium* through shoot bud explants. **Pakistan Journal of Botany**, v.51, n.3, p. 1093–1098, 2019. https://doi.org/10.30848/PJB2019-3(11)

JAHAN, M.; ISLAM, M.R.; ISLAM, S.A.M.S.; DAS, P.; ISLAM, M.D.M.; KABIR, M.H.; MAMUN, A.N.K. Clonal propagation of *Chrysanthemum morifolium* Ramat using various explants obtained from field grown plants. **GSC Biological and Pharmaceutical Sciences**, v.16, n.2, p. 087–093, 2021. https://doi.org/10.30574/gscbps.2021.16.2.0231

MEKONEN, G.; EGIGU, M.C.; MUTHSUWAMY, M. *In vitro* Propagation of Banana (*Musa paradisiaca* L.*)* Plant Using Shoot Tip Explant. **Turkish Journal of Agriculture-Food Science and Technology**, v.9, n.12, p. 2339-2346, 2021. https://doi.org/10.24925/turjaf.v9i12.2339-2346.2883.

JAN, T.; ULLAH, I.; MUHAMMAD, B.; TARIQ; MANSOOR, A.; ULLAH, Z.; NAWAZ, M.A. Efficient in vitro propagation of *Amaranthus viridis* L. Using node explants. **Acta Scientiarum Polonorum, Hortorum Cultus**, v.19, n.4, p. 41–51, 2020. https://doi.org/10.24326/asphc.2020.4.4.

MEHEDI, M.N.H.; MITU, N.; ROBBANI, M.; SUKHI, K.F.N.; RAHMAN, M.H.S.; AL-NOOR, M.M. Impact of Different Explants and Growth Regulators on *In vitro* Regeneration of Chrysanthemum. **Asian Journal of Biochemistry, Genetics and Molecular Biology**, v.4, n.4, p. 10–18, 2020. https://doi.org/10.9734/ajbgmb/2020/v4i430112

MELLO, M.L.; ZANETTI BARROS, N.; SPERANÇA, M.A.; PEREIRA, F.M.V. Impurities in Raw Sugarcane Before and After Biorefinery Processing. **Food Analytical Methods**, v.15, n.1, p. 96–103, 2022. https://doi.org/10.1007/s12161-021-02105-1.

MOHAMED, G.G.; AMER, A.M.; OSMAN, N.H.; SEDIKC, M.Z.; HUSSEIN, M.H. Effects of different gelling agents on different stages of rice regeneration in two rice cultivars. **Saudi Journal of Biological Sciences**, v.28, p. 5738-5744, 2021. https://doi.org/10.1016/j.sjbs.2021.06.003

PARK, J.S.; NAING, A.H.; KIM, C.K. Effects of ethylene on shoot initiation, leaf yellowing, and shoot tip necrosis in roses. **Plant Cell, Tissue and Organ Culture**, v.127, n.2, p. 425–431, 2016. https://doi.org/10.1007/s11240-016-1066-6

PATERNAK, T.P.; STEINMACHER, D. Plant Growth Regulation in Cell and Tissue Culture *In Vitro*. **Plants**, v.13, p. 327-350, 2024. https://doi.org/10.3390/plants13020327

PRATHYUSHA, N.; DORAJEERAO, A.V.D.; RAVINDRAKUMAR, K.; APARNA, D.; SALOMI SUNETHA, D.R. *In vitro* propagation of chrysanthemum (*Chrysanthemum morifolium*) cultivars from ray floret explants. **The Pharma Innovation**, v.10, n.10, p. 415-417, 2021.

SINURAT, E.; FRANSISKA, D.; UTOMO, B.S.B.; SUBARYONO, S.; NURHAYATI, N. Characteristics of powder agar extracted from different seaweeds species and locations in Indonesia. **Journal of Applied Phycology**, v36, p. 675-684, 2024. https://doi.org/10.1007/s10811-023-03084-y

THAKUR, A.; SIDHU, G.S.; SINGH, H. *In vitro* multiplication of peach rootstocks. **Indian Journal of Horticulture,** v80, n.3, p. 251-257, 2023. https://doi.org/10.58993/ijh/2023.80.3.4

WINARTO, B.; YUNIARTO, K.; WEGADARA, M. Axillary Shoots Derived from Thin Cell Layer and Adenine Sulphate Application in *in vitro* Mass Propagation of Gerbera [*Gerbera jamesonii* (H. Bolus ex Bolus f.)]. **Notulae Scientia Biologiae**, v.11, n.1, p. 63-76, 2019. https://doi.org/10.15835/nsb11110351