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INDIRECT SHOOT REGENERATION AND MULTIPLICATION IN GERBERA (*GERBERA JAMESONII*)

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ABSTRACT

In vitro propagation in gerbera is of high commercial value. Four varieties of Gerbera viz Submarine, Danallen, Savanna and Stanza were selected for callogenesis whereas Var. Stanza was used for embryo rescue/embryo. Calli were induced from clumps following standard explant sterilization procedure. High culture sustainability rates were observed both in clumps (95 %) and seeds (99%). The earliest embryogenic calli were observed at various levels of 2, 4-D (4.39 days). The maximum calli weight (1.30 g) of leafy green color with small globular embryos was observed in Var. Stanza on MS-media modified with 2, 4-D. Less number of days were taken for callus formation in Var. Submarine (10.36) on NAA+BAP. The highest shoot induction was observed in Var. Savana (12.86 cm), while a greater number of roots (3.99) and root length (3.89cm) were recorded in Var. Stanza on various levels of IBA. The embryo rescue was achieved from seeds inoculated seeds on MS-media. The seed coats were removed at the seed coat bursting stage followed by sub-culturing of seeds. The developed plantlets were tested and multiplied on media containing combinations of BAP and kinetin. The earliest shoot induction (5.67) was recorded on MS-Medium modified with 2.5 mg/L 6-benzyleaminopurine (BAP) in Var. Stanza Red + Bi-color (Red+BC). Higher number of shoots (13.67) were recorded at 2 mg/L of BAP while enlarged shoots were observed at 3 mg/L (11.46). The findings of the present study can be used for commercial scale production of gerbera.

KEYWORDS: 6-benzyleaminopurine; gerbera; multiple shoot induction; callus; clumps; Pakistan

INTRODUCTION

Gerbera (*Gerbera jamesonii* L.) is one of the most famous and beautiful cut-flowers of tropical and subtropical climate (Gamanagatti and Patil, 2018). It is known as Transvaal daisy or Barberton daisy. The plants are used for cut flowers, bedding and corner planting for different landscaping schemes (Ahmed *et al.*, 2018). It is important due to its attractive colors, stem length, petal shape and extended vase life. Cut flower indices are the key quality attributes which makes it famous in international cut-flower markets of Europe and America. China produces gerbera in open farming conditions on 6204 hectares followed by Netherlands (169 ha), Turkey (116 ha), Japan (92 ha), Mexico (87 ha) and Taiwan (77 ha) (Deng and Bhattarai, 2018). Pakistan did not grow gerbera on significant area due to lack of hybrid seed availability for commercial purposes. Seed of elite varieties of gerbera is imported from Netherland, China and India. Pakistan 's floriculture industry is lacking in gerbera breeding and hybrid seed production at local level. The promotion of local seed or propagation material can protect investment on import of gerbera seedlings and seeds. Generally, Gerbera is propagated through division of rhizomes, seeds and

shoot tips cutting under *in vivo* conditions (Winarto and Yufdy, 2017). Various commercial varieties of gerbera are propagated through seeds, however variations in subsequent generations due to segregation did form sustainable quality cut-flower. Segregation of alleles in subsequent generations is loss of uniformity of color, number of petals and size of flower (Tung *et al.*, 2022). The division and cutting of rhizome have a low rate of success due to more time to complete the juvenility of plants with phytosanitary hazards (Cardoso and Teixeira, 2012). Commercial propagation of Gerbera through rhizomes is also not feasible due to slower and lesser rhizomes formation (Winarto and Yufdy, 2017). *In vitro* plant propagation technique facilitates mass multiplication of high value planting material (Deng and Bhattarai, 2018). It also facilitates in genetic manipulation and conservation of novel or extinct species (Deng and Bhattarai, 2018), transgenic plant development with resistance against different biotic and abiotic stresses (Partab *et al.*, 2023) and induction of autotetraploidy (Usman *et al.*, 2008; Fatima *et al.*, 2015; Wu *et al.*, 2023). In the case of gerbera, a huge amount of capital is invested to import hybrid varieties that can be propagated locally through *in vitro* techniques. Due

to high market value, a simple and cheap commercial *in vitro* protocol for Gerbera production can help in poverty reduction for low-income farming communities in rural areas of Pakistan.

In Gerbera, establishment of *in vitro* protocol has been done through various explants, amongst those, clumps, peduncle, leaf disc are commonly used for direct and indirect multiple shoot induction (Kumar *et al.*, 2023). Plant growth regulators play a key role in callogenesis and organogenesis in plant species (Usman *et al.*, 2005; Rafique *et al.*, 2013; Iqbal *et al.*, 2014; Shah *et al.*, 2021). Various types of growth regulators produce different types of calli in tissue culture environment such as friable callus is observed in Indole-3-butyric acid (IBA), friable/nodular in Naphthalene acetic acid (NAA)/2,4-Dichlorophenoxy acetic acid while compact/nodular in 6-Benzyleadenine (Nikhila *et al.*, 2023). Among these plant growth regulators, IBA has a strong role in root induction (Akhtar *et al.*, 2022 and Rafique *et al.*, 2012).

Multiplication through seeds is easy and faster compared to organogenesis from calli as surface sterilization of clumps, peduncles and shoot tips is somewhat difficult. Despite these risks, the commercialized gerbera as potted plants primarily rely on seed (Gantait and Mahanta, 2022). Moreover, in some countries, seeds of superior cultivars are used to initiate *in vitro* cultures for micro-propagation purposes (Mahanta *et al.*, 2023). The present study was carried out to observe efficacy of different explants (Clumps, callus, and seeds) for achieving multiple shoot induction for successive commercial *in vitro* propagation. Development of commercial *in vitro* protocol for producing hybrid varieties locally will help to save heavy investments on the import of hybrid seeds from abroad.

MATERIALS AND METHODS

Gerbera (*Gerbera jamsonii* L.) is one of the most famous and beautiful cut-flowers of tropical and subtropical climate (Gamanagatti and Patil, 2018). It is known as Transvaal daisy or Barberton daisy. The plants are used for cut flowers, bedding and corner planting for different landscaping schemes (Ahmed *et al.*, 2018). It is important due to its attractive colors, stem length, petal shape and extended vase life. Cut flower indices are the key quality attributes which makes it famous in international cut-flower markets of Europe and America. China produces gerbera in open farming conditions on 6204 hectares followed by Netherlands (169 ha), Turkey (116 ha), Japan (92 ha), Mexico (87 ha) and Taiwan (77 ha) (Deng and Bhattarai, 2018). Pakistan did not grow gerbera on significant area due to lack of hybrid seed availability for commercial purposes. Seed of elite varieties of gerbera was imported from

Netherlands, China and India. Pakistan's floriculture industry is lacking in gerbera breeding and hybrid seed production at local level. The promotion of local seed or propagation material can protect investment on import of gerbera seedlings and seeds. Generally, Gerbera is propagated through division of rhizomes, seeds and shoot tips cutting under *in vivo* conditions (Winarto and Yufdy, 2017). Various commercial varieties of gerbera are propagated through seeds, however variations in subsequent generations due to segregation did form sustainable quality cut-flower. Segregation of alleles in subsequent generations is loss of uniformity of color, number of petals and size of flower (Tung *et al.*, 2022). The division and cutting of rhizome have a low rate of success due to more time to complete the juvenility of plants with phytosanitary hazards (Cardoso and Teixeira, 2012). Commercial propagation of Gerbera through rhizomes is also not feasible due to slower and lesser rhizomes formation (Winarto and Yufdy, 2017). *In vitro* plant propagation technique facilitates mass multiplication of high value planting material (Deng and Bhattarai, 2018). It also facilitates in genetic manipulation and conservation of novel or extinct species (Deng and Bhattarai, 2018), transgenic plant development with resistance against different biotic and abiotic stresses (Partab *et al.*, 2023) and induction of autotetraploidy (Usman *et al.*, 2008; Fatima *et al.*, 2015; Wu *et al.*, 2023). In the case of gerbera, a huge amount of capital is invested to import hybrid varieties that can be propagated locally through *in vitro* techniques. Due to high market value, a simple and cheap commercial *in vitro* protocol for Gerbera production can help in poverty reduction for low-income farming communities in rural areas of Pakistan.

In Gerbera, establishment of *in vitro* protocol has been done through various explants, amongst those, clumps, peduncle, leaf disc are commonly used for direct and indirect multiple shoot induction (Kumar *et al.*, 2023). Plant growth regulators play a key role in callogenesis and organogenesis in plant species (Usman *et al.*, 2005; Rafique *et al.*, 2013; Iqbal *et al.*, 2014; Shah *et al.*, 2021). Various types of growth regulators produce different types of calli in tissue culture environment such as friable callus is observed in Indole-3-butyric acid (IBA), friable/nodular in Naphthalene acetic acid (NAA)/2,4-Dichlorophenoxy acetic acid while compact/nodular in 6-Benzyleadenine (Nikhila *et al.*, 2023). Among these plant growth regulators, IBA has a strong role in root induction (Akhtar *et al.*, 2022 and Rafique *et al.*, 2012).

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as potted plants primarily rely on seed (Gantait and Mahanta, 2022). Moreover, in some countries, seeds of superior cultivars are used to initiate *in vitro* cultures for micro-propagation purposes (Mahanta *et al.*, 2023). The present study was carried out to observe efficacy of different explants (Clumps, callus, and seeds) for achieving multiple shoot induction for successive commercial *in vitro* propagation. Development of commercial *in vitro* protocol for producing hybrid varieties locally will help to save heavy investments on the import of hybrid seeds from abroad.

RESULTS AND DISCUSSION

1-Callogenesis and shooting: The earliest calli induction was noted in Var. Danallen i.e. 5.44 days whereas other 3 varieties took more than 14 days for callus induction. However, it was observed that all 4 varieties gave better response at lower doses for callogenesis than at the higher doses. Data showed that the most responsive variety is Danallen which actively produced calli in shortest time i.e. in 4.32 days at MS-media + 1 mg of NAA formed by 2mg/L. As these doses of NAA were less at the callus formed were increased. The results ($P>0.05$) show that no callus formation was observed on MS-medium without modification in any gerbera cultivar, confirming the essential role of exogenous hormones in callus induction. Application of NAA (0.2-1.0 mg/L) significantly increased callus formation in all cultivars, with Var. Savana exhibiting the highest response (25.67% at 1.0 mg/L), followed by Var. Stanza and Var. Submarine, whereas Var. Danallen showed the lowest callusing but required the fewest days for callus initiation. All NAA-induced calli were green in color with a hard texture. The use of 2,4-D (1.0-5.0 mg/L) further enhanced callus induction, particularly in Var. Savana, which produced the maximum callus formation (30.00%) at 2.0 and 5.0 mg/L, while Var. Danallen again exhibited the fastest callus initiation. Callus color under 2,4-D ranged from light to dark green, and texture varied from hard to fluffy depending on genotype and concentration. The combined application of NAA and BAP resulted in the highest overall callus formation, with Var. Danallen producing a maximum of 39.00% callus at 0.4 mg/L NAA + 2.0 mg/L BAP, although callus initiation was comparatively delayed under these treatments. Calli obtained from NAA + BAP combinations were predominantly green to green-creamy in color and soft to fluffy in texture, indicating improved callus quality and higher regenerative potential, while significant genotypic and treatment-dependent differences were observed for all parameters studied (Table 1).

Effect of growth regulators in callus growth (g) from clumps of gerbera in different genotypes:

Different growth regulators showed variable response for callus development and growth. Modified MS-Medium with various levels of Naphthalene acetic acid (NAA) showed highest mass of callus in 1.0 mg/L in Var. Danallen (1.10). Modified MS-Medium with 0.8 mg/L of NAA produced significantly more calli in Var. Submarine (0.50). Savana (0.533) and Var. Stanza (0.66) showed better mass of callus in MS-Medium modified with 0.6 mg/L of NAA. Response of varieties for better mass of callus production was better in MS-Medium modified with 2, 4-D at 3 mg/L for Var. Danallen (1.43) and Var. Savana (1.67). Response of Var. Submarine (1.36) and Var. Stanza (1.93) showed better mass of callus in 4.0 and 5.0 mg/L of 2, 4-D. Nephthalene acetic acid (NAA) plus 6-Benzyleaminopurine showed significantly high response for calli (g) production at 1.0+5.0 mg/L in Var. Danallen (1.03) and Var. Submarine (0.47). Varieties; Savana (0.53) and Stanza (0.53) showed significantly better callus growth in MS-Medium modified with 0.4+2 mg/L of NAA+BAP. Alternate reverse dose of BAP+NAA showed better callus in Var. Danallen (0.49), Var. Submarine (0.533) and Var. Savana (0.867) at 1.0+1.0 mg/L (Table 2). The regeneration of callus was low in different levels of BA while second stage of multiplication was excessive. The results summary ($P<0.05$) showed that MS-Medium modified with 2,4-D produced better mass of callus (g) in Var. Stanza (1.30), Var. Savana (1.23) and Var. Danallen (0.99) while Var. Submarine (1.20) produced better mass of callus (g) in the combinations of NAA+BAP. The results further illustrated that for better callus growth Var. Danallen is better amongst all varieties. The least number of days were taken for callus initiation by Var. Savana (16.22) in MS-Medium modified with NAA, while for Var. Danallen (14.39) least days for callus formation were taken in various levels of 2,4-D. The Gerbera variety Submarine (10.36) and Var. Stanza (13.28) performed better for in MS-medium modified with combinations of NAA+BAP (Table 5). The callus was green and hard, with small granules (embryos). The best regeneration from calli was observed in Var. Stanza (3.50) followed by Var. Savana (2.50) and Var. Danallen (1.50) (Table 3). The results revealed that plantlets formed from the calli showed the best rooting response in MS-media modified with 1.5mg/L of IBA where a greater number of roots (16.31) were formed in Var. Savana followed by Var. Stanza (15.89) and Var. Danallen (15.81) (Table 3). The medium that produced the maximum shoot length also resulted in the highest number of shoots. Among the cultivars, the highest shoot number was recorded in Var. Submarine (16.73), followed by Var. Savana (14.34), Var. Stanza (13.56), and Var. Danallen (12.42) (Table 4).

ORGANOGENESIS FROM CALLUS OF CLUMPS OF VARIOUS HYBRID VARIETIES OF GERBERA

Table 1 Effect of growth regulators on callus formation (days), color of callus and texture of callus from clumps of Gerbera cultivars

Growth regulators	Levels of growth regulators (mg/L)	Varieties			
		Var. danallen	Var. submarine	Var. savana	Var. stanza
Naphthalene acetic Acid/NAA (mg/L)	MS-Medium	0.00±0.00g	0.00±0.00g	0.00±0.00g	0.00±0.00g
	0.2	4.33±1.33fg	12.33±0.33c-f	15.67±0.33b-e	16.00±1.00b-e
	0.4	5.67±1.20fg	14.72±1.90A	17.00±1.15a-d	16.00±1.15b-e
	0.6	6.33±0.88fg	17.00±1.53a-d	19.33±2.33abc	17.33±1.45a-d
	0.8	7.67±1.86efg	17.67±4.37a-d	19.67±2.33abc	18.33±2.03abc
	1	8.67±1.45d-g	18.33±1.20abc	25.67±2.85a	19.00±1.53abc
	No. of days to initiate calli	5.44±0.81B	23.00±1.53ab	16.22±2.02A	14.44±1.66A
	Color of calli	Green	Green	Green	Green
2, 4-Dichlorophenoxy acetic acid/2,4-D (mg/L)	MS-Medium	0.00±0.00h	0.00±0.00h	0.67±0.67h	0.67±0.67h
	1	4.00±1.00gh	18.33±2.67a-e	20.67±3.38a-d	15.33±3.33b-g
	2	4.33±0.33fgh	18.33±4.18a-e	21.78±2.63A	15.00±2.89b-g
	3	4.67±0.88e-h	15.00±4.04b-g	26.33±3.33ab	18.00±3.06a-f
	4	6.33±1.20e-h	12.00±0.58c-h	23.00±1.73abc	21.67±1.20abc
	5	7.00±1.00d-h	12.00±1.15c-h	30.00±4.51a	26.33±5.36ab
	No. of days for initiate calli	4.39±0.62C	12.61±1.76B	30.00±2.00a	16.17±2.21B
	Color of calli	Light green	Light Green	Dark Green	Dark Green
Naphthalene acetic acid (NAA) + 6-Benzylpurine (BAP) (mg/L)	MS-Medium	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00
	0.2+1	19.33±10.48a-f	15.67±2.96c-f	18.00±2.08c-f	12.00±1.53def
	0.4+2	27.33±2.67a-e	17.00±1.73c-f	19.33±2.33a-f	14.33±0.67c-f
	0.6+3	32.67±5.90abc	18.67±0.88a-f	20.67±2.33a-f	16.00±2.31c-f
	0.8+4	38.67±6.12ab	18.00±1.15c-f	21.67±2.40a-e	18.00±2.08c-f
	1+5	39.00±0.58a	19.00±3.79a-f	31.00±6.81a-d	18.33±0.88b-f
	No. of days for initiate calli	26.33±3.74A	16.17±1.43B	18.61±2.45B	13.28±1.52B
	Color of calli	Green-Creamy	Green-Light green	Green-Light Green	Green
	Texture of Calli	Soft-Fluffy	Soft-Fluffy	Soft-Fluffy	Fluffy-Soft

Means sharing similar letters in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 2 Effect of growth regulators on mass of calli (g) from clumps of Gerbera cultivars

Growth regulators	Levels of growth regulators (mg/L)	Varieties			
		Var. danallen	Var. submarine	Var. savana	Var. stanza
Naphthalene acetic acid (mg/L)	MS-Medium	0.00±0.00d	0.000±0.000d	0.000±0.000d	0.000±0.000d
	0.2	0.53±0.03bc	0.367±0.033bcd	0.267±0.067cd	0.333±0.133bcd
	0.4	0.63±0.09abc	0.400±0.100bcd	0.333±0.133bcd	0.422±0.061B
	0.6	0.63±0.09abc	0.433±0.088bcd	0.533±0.176bc	0.467±0.067bcd
	0.8	0.77±0.12ab	0.500±0.058bc	0.467±0.067bcd	0.533±0.133bc
	1	1.10±0.15a	0.533±0.067bc	0.333±0.067bcd	0.533±0.088bc
	Mass of calli (g)	0.61±0.09A	0.372±0.048B	0.322±0.054B	0.667±0.067abc
	Plant regeneration (%)	2.20±0.09	2.4±0.54	1.6±0.06	1.8±0.04
2, 4-Dichlorophenoxy acetic acid (mg/L)	MS-Medium	0.00±0.00d	0.000±0.000d	0.000±0.000d	0.000±0.000d
	1	1.00±0.10bc	0.933±0.133bc	1.267±0.067abc	1.400±0.115abc
	2	1.00±0.00bc	0.933±0.176bc	1.600±0.115ab	1.400±0.200abc
	3	1.43±0.03abc	0.800±0.115c	1.667±0.176ab	1.467±0.067abc
	4	1.40±0.10abc	1.367±0.433abc	1.400±0.115abc	1.600±0.115ab
	5	1.13±0.07bc	1.000±0.115bc	1.467±0.067abc	1.933±0.176a
	Mass of calli (g)	0.99±0.12B	0.839±0.123B	1.233±0.142A	1.300±0.154A
	Plant regeneration (%)	2.50±0.13	2.50±0.43	3.50±0.23	2.50±0.91
Naphthalene acetic acid+6-Benzylpurine	MS-Medium	0.00±0.00c	0.000±0.000c	0.000±0.000c	0.000±0.000c
	0.2+1	0.37±0.09bc	0.267±0.067bc	0.300±0.058bc	0.400±0.115bc
	0.4+2	0.50±0.06abc	0.533±0.067abc	0.533±0.176abc	0.533±0.176abc
	0.6+3	0.87±0.03ab	0.400±0.000bc	0.533±0.067abc	0.467±0.067abc
	0.8+4	0.73±0.24ab	0.467±0.067abc	0.533±0.067abc	0.467±0.176abc
	1+5	1.03±0.27a	0.467±0.067abc	0.667±0.067ab	0.467±0.176abc
	Mass of calli (g)	0.58±0.10A	0.356±0.047B	0.428±0.061AB	0.389±0.064B
	Plant regeneration (%)	1.50±0.43	1.70±0.32	2.50±0.54	1.50±0.23

Means sharing similar letters in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 3 Effect of BAP on shoot induction and growth (cm) in Gerbera

MS media + BAP (mg/L)	No of shoots				Shoot length (cm)			
	Var. danallen	Var. submarine	Var. savana	Var. stanza	Var. danallen	Var. submarine	Var. savana	Var. stanza
MS-Medium	00±00d	00±00d	00±00d	00±00d	00±00d	00±00d	00±00d	00±00d
0.5	12.31±2.31cd	8.56±1.61cd	9.87±2.34cd	10.63±2.34bcd	11.3±3.21ab	9.98±2.32bcd	11.67±2.34bcd	12.42±2.12b
1.0	10.23±1.56d	9.76±2.34bc	11.23±3.21bc	9.78±2.49cd	9.81±3.88cd	11.23±3.42cd	13.89±3.22ab	10.23±1.23bc
1.5	15.81±2.42a	12.23±2.12a	16.31±2.34a	15.89±3.53a	12.42±3.21a	16.73±2.54a	14.34±4.32a	13.56±4.32a
2.0	14.3±3.21b	8.78±2.43d	13.22±3.21b	13.23±4.32b	11.67±2.98b	8.34±2.89cd	13.41±2.35ab	8.91±1.87c
2.5	12.2±2.34ab	9.56±3.21bc	10.23±2.56cd	12.67±3.22ab	9.56±3.89cd	13.41±2.81b	12.98±2.90cd	7.81±1.23cd
3.0	13.4±2.45c	11.56±3.24b	9.93±3.24bcd	8.45±3.42bc	8.79±2.45cd	16.3±2.13ab	11.45±2.12acd	10.21±2.54bcd

*Means sharing similar letters in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 4 Effect of IBA root induction and growth (cm)

MS media + IBA (mg/L)	No of roots				Root length (cm)			
	Var. danallen	Var. submarine	Var. savana	Var. stanza	Var. danallen	Var. submarine	Var. savana	Var. stanza
MS-Medium	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.000±0.00d	0.00±0.00d	0.00±0.00d
0.5	1.32±0.34c	2.12±0.54c	2.39±0.42cd	4.31±0.21bc	4.93±1.21c	3.42±1.33c	1.23±1.84cd	3.21±1.34ab
1.0	2.23±0.54cd	1.43±1.23bc	1.34±0.12c	4.42±0.32b	3.65±1.43ab	4.21±1.36bc	2.43±1.85cd	4.31±1.67ac
1.5	4.31±0.23a	4.56±0.45a	3.94±0.31a	5.32±1.34a	4.34±1.56ab	4.35±1.45acd	3.54±1.78a	5.63±1.45a
2.0	1.92±0.45bc	2.12±0.91bc	2.45±0.91bc	4.67±1.23bc	3.94±1.23bc	3.89±1.32ab	1.75±1.49ab	3.42±1.56cd
2.5	1.46±0.94c	2.72±0.37bc	2.98±0.73bcd	4.91±1.67c	3.21±1.34bcd	4.32±1.23c	2.34±1.61abc	4.21±1.91acd
3.0	1.78±0.56bcd	1.71±0.23bcd	2.44±0.21bc	4.34±1.23bcd	5.23±1.93a	5.12±1.87a	3.21±1.98cd	6.43±2.12bc

*Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 5-Summary of mean responses of Gerbera cultivars for callus induction in MS-medium modified with PGRs for callus formation from clump explants

Explants response	Levels of growth regulators (mg/L)	Var. danallen	Var. submarine	Var. savana	Var. stanza	Mean
Mass of Callus (g)	NAA (0.2-1.0)	0.61±0.23ab	0.43±0.32c	0.32±0.42c	0.42±0.34bc	0.45±0.03C
	2,4-D (1.0-5.0)	0.99±0.12a	0.87±0.42b	1.23±0.23a	1.30±0.43a	1.10±0.04A
	NAA+BAP (0.2+ 1+5)	0.58±0.32b	1.20±0.23a	0.63±0.12bc	0.39±0.32c	0.70±0.08B
	BAP+NAA (0.2+5-1+1)	0.49±0.23ac	0.40±0.12bc	0.63±0.23b	0.43±0.22ab	0.49±0.02BC
	Mean	0.67±0.32BC	0.73±0.21A	0.71±0.12AB	0.64±0.32BC	
No. of days for callus induction	NAA (0.2-1.0)	15.44±2.31bc	14.72±2.31a	16.22±3.45c	14.44±3.45ab	15.21±0.17AB
	2,4-D (1.0-5.0)	14.39±2.92c	12.61±3.21ab	21.78±4.12a	16.17±5.34a	16.24±0.87AB
	NAA+BAP (0.2+ 1+5)	27.44±3.11a	10.36±3.24c	20.78±2.64ab	13.28±2.54c	15.47±2.53AB
	BAP+NAA (0.2+5-1+1)	19.72±3.21ab	13.89±4.21ab	20.18±2.34ab	14.94±2.34bc	17.33±0.75A
	Mean	19.25±2.31AB	10.40±3.12BC	19.89±3.21A	14.71±3.33BC	
No of shoots	BA (0.5-3.0)	12.78±0.54a	9.89±0.45c	11.67±0.42ab	11.71±0.60ab	11.51±0.36A
Length of shoot (cm)	BA (0.5-3.0)	10.56±0.32c	12.25±1.03ab	12.86±0.03a	10.52±0.47bc	11.55±1.12A
No. of roots	IBA (0.5-3.0)	1.86±0.39bc	2.09±0.45c	2.22±0.24b	3.99±0.95a	2.54±0.76A
Length of roots (cm.)	IBA (0.5-3.0)	3.61±0.54ab	3.62±0.74b	2.07±0.56c	3.89±0.67a	3.78±0.45B
Color of callus after 28 days of inoculation		Dark green	Green	Green	Green	Green
% shoot induction		1.50±0.12cd	1.50±0.54cd	2.50±0.45ab	3.50±0.21a	2.25±1.27B

Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

The clumps of gerbera are previously reported for Followed by the callus formation and multiple shoot regeneration (Mahanta *et al.*, 2023, Tyagi and Kothari, 2004 and Ray *et al.*, 2005). The sterilization of clumps is easy compared to leaves and shoot tip for multiple shoot induction and callus formation. The sterilization protocols used in present study are practiced internationally as 0.1% of Mercuric chloride (HgCl_2) for 3-5 minutes was used for surface sterilization of clumps by Son *et al.* (2011) while 70% ethanol also been used for surface sterilization followed by ionic dissociation of fungal and bacterial spores by Ibrahim and Yassin, (2020). Sodium hypochlorite with 2-3 drops of tween-20 is also evident by Kumar *et al.*, (2021) (Figure 1, A, B). While this method of plant propagation

is relevantly non-destructive for plants as only a few flowers can be used for callus formation and further work for shoot induction as well (Pasternak *et al.*, 2024). Multiple shoot induction from clumps is dependent on various factors such as flowers color and growth stage (Farooq *et al.*, 2021). It is reported that yellow, orange and pink cultivars showed better response in half strength of Modified MS-medium with various levels of 6-Benzyleaminopurine/6-Benzyleadenine (BAP/BA). Various stages of flower clumps of young and mature are used for callus formation in various international known varieties e.g. Pastourelle and Mardi Gras (Shaheen *et al.*, 2022). Young clumps of gerbera can also be utilized for multiple shoot induction followed by callus formation as well in modified MS-medium with

ORGANOGENESIS AND MULTIPLE SHOOT INDUCITION IN HYBRID CULTIVARS OF VAR. STANZA THROUGH EMBRYO RESCUE**Table 6 Effect of growth regulators on mean Number of days to start shoot induction (Nos.), number of shoots (Nos.) and shoot length (cm) for Var. Stanza (Red+BC)**

BAP	days to start shoot induction (Nos.)	Shoots (Nos.)	Shoot length (cm)	KIN	days to start shoot induction (Nos.)	Shoots (Nos.)	Shoot length (cm)	BAP+Kin	days to start shoot induction (Nos.)	Shoots (Nos.)	Shoot length (cm)
Control	5.78±0.22c	6.25±0.32	6.50±0.36	Control	4.57±0.56	5.89±0.12	5.89±0.89	Control	5.45±0.89	6.85±0.54	6.78±0.23
0.5	12.33±1.12ab	8.33±0.99bc	10.16±0.61ab	0.5	13.12±0.96ab	10.33±0.27bc	9.53±0.56ac	0.5+0.5	12.76±1.22a	10.34±0.27ab	8.77±0.53abc
1	14.12±0.83a	9.21±0.96cd	8.8±0.67bc	1	14.33±0.55a	13.12±0.48ab	9.71±0.89ab	1+1	12.43±1.14ab	10.21±0.49ab	9.03±0.65ab
1.5	10.67±2.10ab	11±0.83ab	9.4±1.34bc	1.5	9.21±1.73bc	12.32±1.44bc	9.96±1.61bc	1.5+1.5	7.23±0.48cd	10.42±1.27ab	7.91±0.95abc
2	8.33±1.21b	13.67±1.81a	10.37±0.62a	2	8.33±0.28bc	13.34±2.17a	9.67±0.29a	2+2	10.1±1.03cd	13.33±2.16a	9.93±0.57a
2.5	6.33±1.21c	11.33±0.55ab	9.23±2.07bc	2.5	6.66±0.55cd	10.36±0.74cd	6.93±1.04cd	2.5+2.5	7.67±0.73acd	10.21±0.83cd	7.43±1.55bc
3	5.67±1.13bc	7.67±0.73 cd	11.46±3.18cd	3	5.21±0.48d	7.39±0.56d	7.33±0.43d	3+3	6.68±1.23d	9.67±0.27cd	7.72±0.53bcd
Mean	9.57±2.13	10.22±1.98	9.90±1.89	Mean	9.48±3.21	11.14±2.12	8.82±1.79		9.41±2.32	10.69±2.16	8.46±2.19

*Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 7 Effect of IBA on root induction and growth (cm) in Var. Stanza (Red+BC)

MS media + IBA (mg/L)	No. of roots	Root length (cm)
0	0.00±0.00d	0.00±0.00d
0.5	3.42±0.98c	2.45±0.23ab
1.0	5.42±1.54a	5.54±1.23a
1.5	5.01±2.31ab	5.12±0.53c
2.0	4.53±1.54bc	4.34±1.31bc
2.5	4.21±1.34cd	4.12±1.13bcd
3.0	2.79±1.32bcd	4.72±1.66bcd

*Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

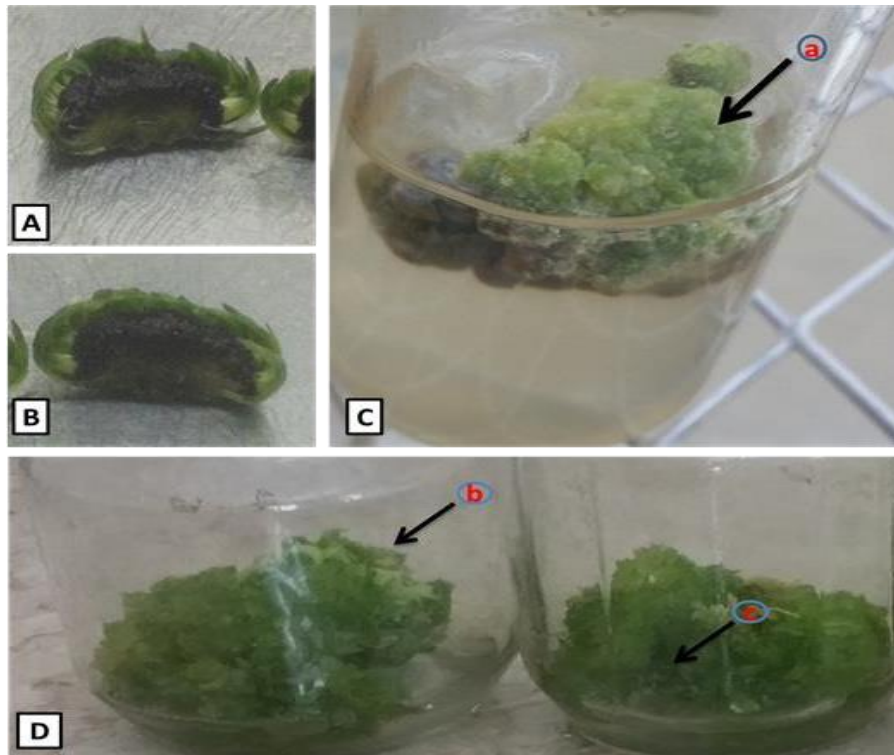


Fig. 1: clump of gerbera of cv. Savanna after excision in the laminar flow cabinet (A and B), a green embryogenic calli in test tube where embryo are shown with the sign of arrow (C), the shifted calli (a) in the jars for multiplication where the shoot formation was observed in calli multiplication media (b and c) as shown in panel D

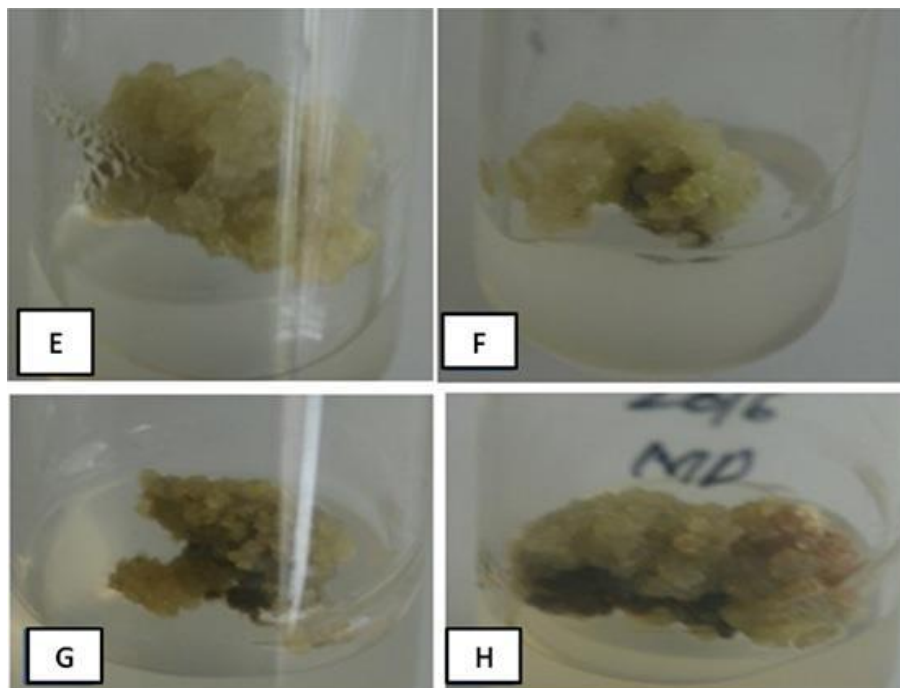


Fig. 2: E to H: the non-regenerative calli of gerbera of cv. Stanza. The calli of this type are made in combination of PGRs of NAA+BA due to rapid and excessive amount of calli. The calli have embryo but did not give plantlets on MS-medium modified with different levels of BA for shoot regeneration. (The arrow sign shows the embryo in Greenish calli.)

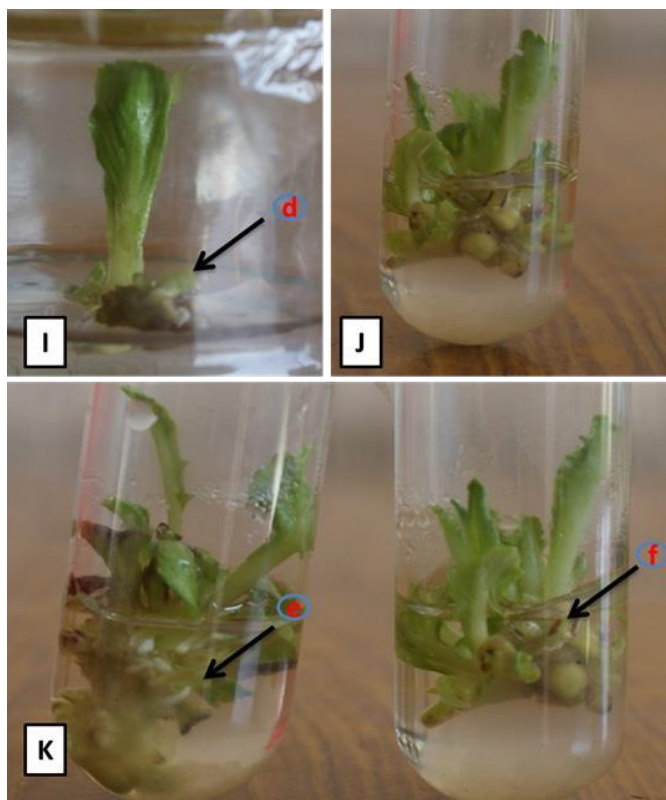


Fig. 3: plant regeneration from calli (d) panel I, gerbera mass multiplication from calli in cv. Savana (J), plantlets of cv. Savanna are multiplying in MS-medium where new leaves and shoots are emerged from downside the shoot base isolated from calli (e), the globular calli in the base and shoot multiplication as well (f) as shown in panel K.

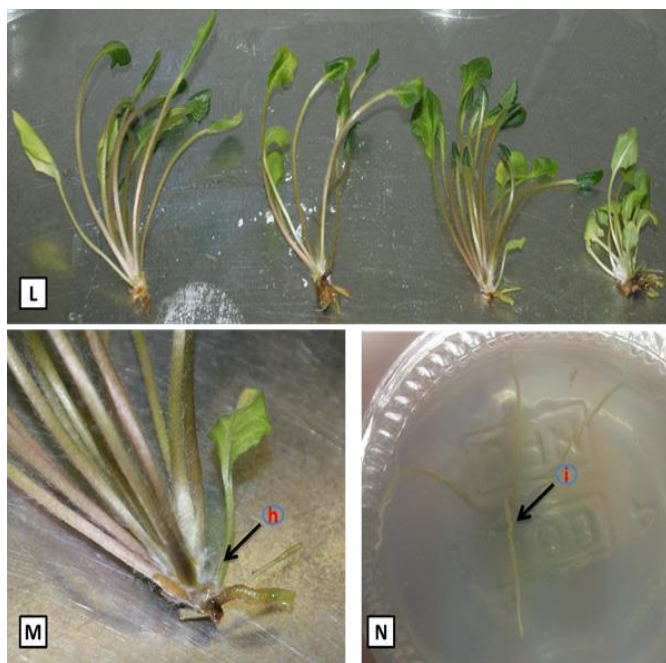


Fig. 4: Fully grown plantlets of cv. Stanza for multiplication (L), grown adventitious roots in shoot multiplication media towards completion of maturity (M), fully developed roots in culture vessel (N)



Fig. 5: Isolated plant for development with mass of calli attached (O g), fully developed plantlet (P) and fully matured with small roots of cv. Savana (Q)

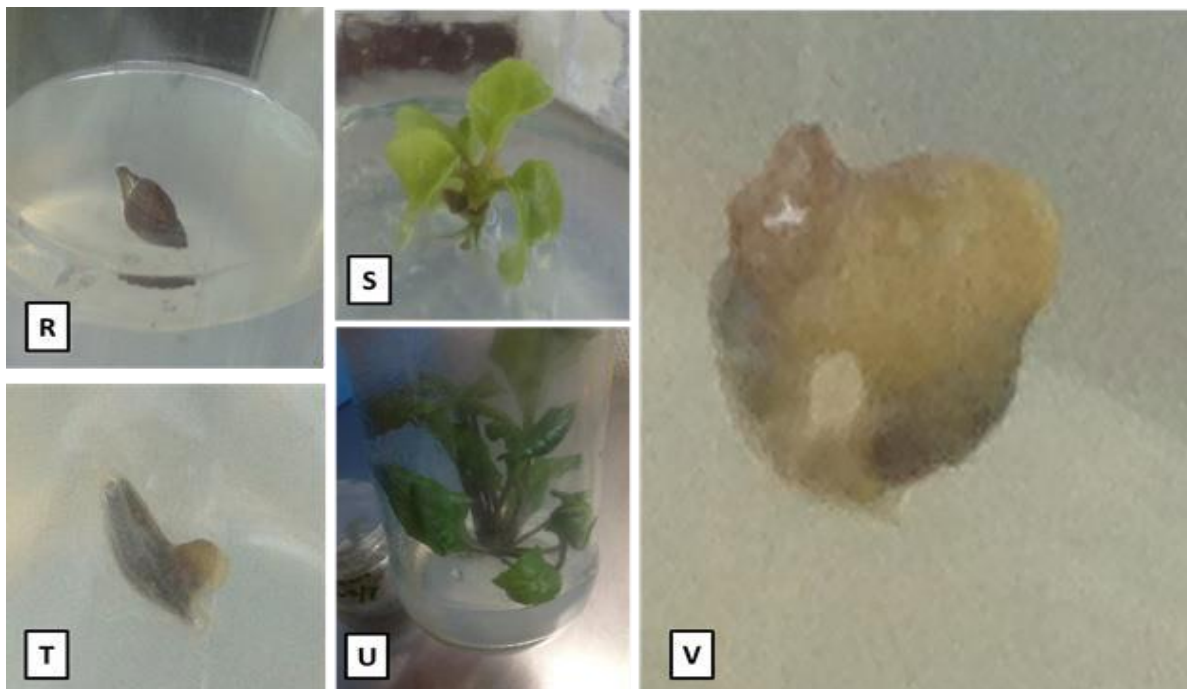


Fig. 6: Inoculation of seeds for initiation of embryo rescue (R), the critical stage of rescuing the embryo (T), embryo rescue in hybrid varieties is necessary as the growth pattern is of such kind that plant leaves become calli with media contact (V), fully developed plantlets as product of embryo rescue technique (S and U)

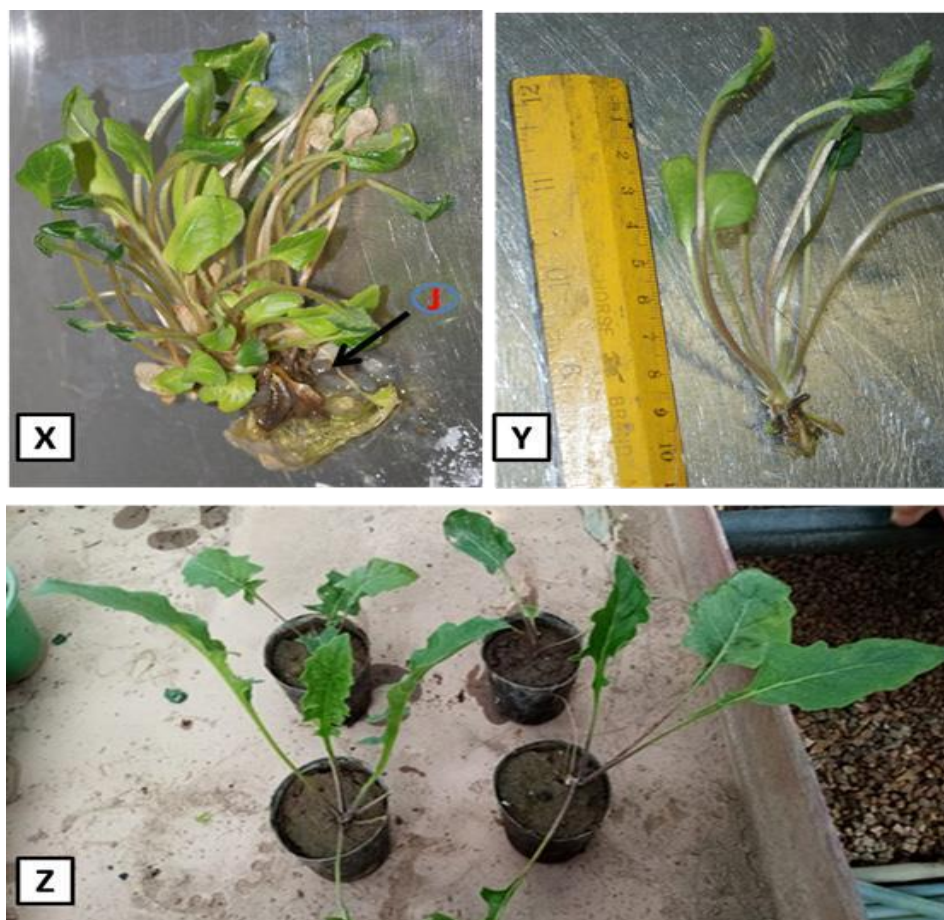


Fig. 7 Fully grown plantlets of cv. Stanza for multiplication (X), grown adventitious roots in shoot multiplication media towards completion of maturity (Y), fully developed roots in culture vessel (Z)

6-Benzyleaminopurine/6-Benzyleadenine (BAP/BA) and Indole acetic acid (IAA) (Zhao *et al.*, 2023). The callus formation is initiated from different points from the point of excision in the beginning. Later stages of callus development are swelling of surface cells individually and form embryo (Figure 1, C-a, D-b, c). The various kind and textured callus formed from clumps which can be categorized into soft, hard, fluffy and granulated (Figure 1 C-a) (Zhao *et al.*, 2023; Hasbullah *et al.*, 2012; Shabbir *et al.*, 2012). The granulated callus is the embryo-genic callus which forms by combining different kinds of embryo stages such as globular, heart, torpedo and cotyledon. The various stages of embryos are difficult to isolate and separate (Zhao *et al.*, 2023; Hasbullah *et al.*, 2012). Handling the calli at this stage is very critical as callus growth is very rapid. Overgrowth can lead to the release of Reactive oxygen species in the system (ROS). To control the overgrowth and ROS production MS-Media amendment with 1% of lactose lower the overgrowth (Figure 2 E, F, G & H) (Mosqueda-Frómata, *et al.*, 2024). Multiple shoot

induction was in abundance form; each type of callus as observed in various percentages and ratios (Zhao *et al.*, 2023).

It may be due to the non-suitability of the cultivar for tissue culture and optimum strength of callus formation due to is low organogenic capacity of varieties (Figure 2, E-H). The unmodified MS-medium did not initiate the calli formation during the study and it is necessary to modify the MS-media with different Auxins (2,4-Dichlorophenoxy acetic acid) and Cytokinins (BAP/BA). The growth hormones toxicity can also be an issue for non-regeneration of the shoots from callus. It is also possible that clumps have age at the time of collection, usually a 10-day old clump is considered suitable for shoot regeneration while 7- or 14-days old clumps did not show good shoot induction (Pasternak and Steinmacher, 2024). In the present study Var. Danallen showed best response for callus formation in MS-media modified with 1.0 mg/L of 2, 4-D, these results are confirmed where Hasbullah *et al.* 2011 showed the callus formation at 1.0 mg/L of

2, 4-D (Figure 1, C). Combination of PGRs for better calli induction was also applied during the present study, however these combinations did not have better response for calli formation and other attributes. Hasbullah *et al.*, (2011) used BAP+NAA combination for calli induction where 0.5 mg/L of BA and 1.0 mg/L of NAA was used for better callus induction. Callus of friable texture with creamy yellow color was formed, that form of calli is used for cell suspension culture while no record found that how many days it was taken to form the calli. The present study showed that combination and cross combinations of BA+NAA produces the calli of granulated and creamy colored, however it did not respond in MS-media modified with BA. Moreover, the explants took a longer time to form the calli. Plant growth regulators (PGRs) are necessary to stimulate the epigenetic changes that induce organogenesis (Figure 3, I, J, K). However, some media additives are also used for shoot induction and multiplication. Glutamine (100mg/L), Adenine sulphate (ADS: 20mg/L) and high concentrations of sucrose (30g/L) are helpful in transition from callogenesis phase to organogenesis; however, during the present study standard MS-medium was used (Rabori and Ghazvini, 2009; Rafique *et al.*, 2013). The root development (Figure 3, K-e) and shoot elongation is generally achieved simultaneously in one phase as shown in Figure 3 & 5, I-d, K-f (Cardoso and Teixeira De-Silva, 2012) also come up with somewhat similar results. In the present study more roots and root elongation were achieved in MS-Medium supplemented with 1.5 mg/L of IBA (Figure 3). These results are correlated with Shabbier *et al.*, 2012, who used MS-medium supplemented with 1 mg/L to 3 mg/L of IBA. There are different factors that affect rooting in gerbera reported such as genotypes and PGRs and system used for rooting. Generally, plant physiology acts as auto responder of rooting in culture vessels, however addition of rooting hormones such as low level of auxins promotes early establishment of adventitious roots (Figure 4, M-h, N-i). Sousa *et al.*, 2006 reported that quarter strength of MS-medium also enhances the root formation in plantlets regenerates from calli. Low temperature exposure is also a strategy for root development. In the low temperature exposure gerbera cultures were stored at 4°C for 2 weeks in darkness and up to 2 weeks in light due to which vegetative growth was stopped, and root elongation were observed without any harm to the plants. The use of PGRs (IBA) was only strategy for rooting in the present study as performed by Rafique *et al.* (2012) for in vitro propagation.

CONCLUSION

Among varieties, Danallen produced calli in a smaller number of days with better mass (g) and significantly

higher regeneration percentage. The Var. Stanza showed 3.5% shoot regeneration from calli multiplying media. The newly formed plantlets showed higher multiplication rate in Var. Savana (16.31) on MS-media modified with 1.5 mg/L of BA as more shoots emergence was observed. Var. Submarine has more shoot length (16.73 cm) compared to other varieties and performed better for *in vitro* hybrid population establishment due to better shooting, rooting and multiplication attributes. The better shoot multiplication for Var. Stanza showed better response in terms of early callus initiation (5.21 days) in MS-media modified with 3 mg/L of ??, a greater number of shoots were observed in Var. Stanza in MS-medium modified with 2 mg/L of Kinetin. More number of roots (5.42) with a higher root length (5.54cm) was observed in MS-media modified with 1.0 mg/L of IBA. It is concluded that Var. Stanza is the best hybrid variety with a higher propagation potential through embryo rescue compared with other varieties and could be used for commercial propagation using tissue culture tools.

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CONTRIBUTION OF AUTHORS

Sr. No.	Authors' name	Contribution	Signature
1.	Monis Hussain Shah	Conducted the research and collected data	
2.	Riaz Ur Rehman	Proof read the manuscript	
3.	Sehrish Somaya	Wrote up the manuscript	
4.	Muhammad Usman	Reviewed the manuscript	