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MULTIVARIATE ANALYSIS, MORPHOLOGICAL CHARACTERIZATION AND *IN VITRO* ORGANOGENESIS IN ELITE VARIETIES OF CARNATION (*DIANTHUS CARYOPHYLLUS*)

SEHRISH SOMAYA[1*], MONIS HUSSAIN SHAH[1], RIAZ-UR-REHMAN[2], ZIA ULLAH[1] AND SAMIA IKRAM[1]

[1]Horticultural Research Institute for Floriculture and Landscaping, Rawalpindi, Islamabad, 45510, Pakistan
[2]Directorate of Floriculture, (T & R), Punjab, Lahore, 45000, Pakistan
**Corresponding author's email: monishussain50@gmail.com*

Abstract

Plant tissue culture techniques can be successfully adopted for rapid multiplication of the exclusive indigenous planting material. This study was conducted at the Plant Tissue Culture laboratory of Horticultural Research Institute for Floriculture and Landscaping, Islamabad, during the year 2018-2021. Three carnation varieties named Antigua, Montoyo and Tabasco were used as an explant material. Different combinations of growth regulators 6-Benzylaminopurine (BAP) and Naphthaleneacetic acid (NAA) were used to investigate the optimum concentration of both hormones for best shoot proliferation protocol. Each variety behaved differently, producing variable results against the parameters tested. In case of Antigua, the combination of 4.0 mg/L BAP + 0.5 mg/L NAA proved to be the best by producing maximum number of shoots, shoot length and shoot proliferation percentage whereas, Montoyo and Tabasco showed satisfactory results on the medium supplemented with 3.0 mg/L BAP + 1.5 mg/L NAA and 4.0 mg/L BAP + 1.5 mg/L NAA respectively. After successful standardization of shoot proliferation protocol, the plantlets were transferred to the ½ strength MS medium enriched with 0.25 mg/l Indole-3-Butyric Acid (IBA) for root initiation. The rooted plantlets were transplanted in the small cells containing mixture of soil and leaf manure (1:1) for acclimatization.

Key words: Flowers, Novel, Commercial, Trade, *In vitro* propagation.

Introduction

Carnation (*Dianthus caryophyllus* L.) belonging to the family *Caryophyllaceae*, is one of the most popular cut flower. Carnation bear flowers of Pink, Red, White, Purple and Yellow. The carnation plant is used to grow on corners, pots, baskets, beds, while carnation is prominently used as cut-flower in bouquet (Ali *et al*., 2008, Kanwar & Kumar, 2009). Carnation is the second largest produced cut-flower amongst the Rose, Chrysanthemum and Lilium. In 2023, the top exporters of Carnations were Colombia ($313M), Netherlands ($97.8M), Turkey ($52.2M), China ($32.5M), and Ecuador ($20.9M). In 2023, the top importers of Carnations were United States ($139M), Netherlands ($77.4M), Japan ($74.3M), United Kingdom ($43.5M), and Poland ($34.8M) (Anon., 2023). In 2023, Carnations were the world's 2863rd most traded product, with a total trade of $569M. Between 2022 and 2023, the exports of Carnations decreased by -2.14%, from $582M to $569M. Trade in Carnations represent 0.0025% of total world trade (Anon., 2023). In 2023, the top importers of carnations were United States ($139M), Netherlands ($77.4M), Japan ($74.3M), United Kingdom ($43.5M), and Poland ($34.8M) (Anon., 2023). Lack of awareness about the commercial importance, lack of standardization of production, high production cost and lack of recommended varieties for the indigenous climate are the main factors of carnation's low productivity in Pakistan. *In vitro* plant propagation technique facilitates mass multiplication of high value planting material. It also facilitates in genetic manipulation and conservation of novel or extinct species, transgenic plant development with resistance against different biotic and abiotic stresses (Partap *et al.*, 2023) and induction of auto tertaploidy in plant species (Wu *et al*., 2023). The carnation can also be propagated though cuttings but the propagation through cutting is not a successful method due to high rate of mortality. Moreover, propagation through cuttings, need a specific season while plant tissue culture can help for year round propagation The commercial technology is principally based on micro propagation in which quick proliferation is achieved from small stem cuttings, axillary buds etc. (Ahloowalia *et al*., 2002).

Morphological characterization of carnation in local environmental is important against the attributes of color, stem length are crucial for export quality cut-flower production (Amsalu *et al*., 2019). The method of introducing new varieties in exotic environment, need observation of plants in that environment prior to recommendation for plantation on large area for commercial production to save the environment with invasive effects of plants (Zang *et al.*, 2018). Commercially, carnation is propagated with stem cuttings, however, plantation of cuttings have many issues that are faced by the growers such as low percentage of rooting, high susceptibility against soil borne diseases and extended pre-juvenility phase. *In vitro* plant propagation is the primary step to get great achievement in biological sciences. The *In vitro* plant propagation technique facilitates mass multiplication in plants of high price in market.

In carnation a big amount of capital is invested to import hybrid varieties that can be propagated locally through *In vitro* techniques. Commercial *In vitro* protocol development in carnation varieties can further be used for poverty elimination of middle or low income families inn Pakistan that have small landholdings with negligible conventional crop yield. Improvement in house hold economics through export of high quality cut-flower production by *In vitro* culture technique can be achieved in Pakistan like many other countries such as Kenya and

Ethiopia. The present study is conducted to check the adaptability trends of novel carnation varieties and establishment of protocol for *In vitro* propagation of carnation in Pakistan.

Material and Methods

Morphological characterization of carnation: The varieties were subjected to morphological characterization for appraisal of favorable characters for further recommendations. The attributes such as flower color, plant height, plant density, flower position compared with foliage, laterals without flower buds or flowers, number of stem, internodes etc. (Anon., 2020).

Standardization of micro-propagation protocol for elite carnation varieties: Apical and nodal segments of the three carnation varieties (Antigua, Montoyo, and Tabasco) were taken as explants source from the Germplasm Unit (GPU) of Horticultural Research Institute for Floriculture and Landscaping, Rawalpindi (HRI). Explants were rinsed in running tap water for half an hour to clean the foreign contaminants. The explant was excised with sharp scissor and sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 3 minutes followed by multiple rinsing with double autoclave distilled water. After sterilization, explants were cultured in test tubes filled with MS medium added with vitamins, 30 g l^{-1} sugar, 7 g l^{-1}agar. The pH of medium was maintained up to 5.8 prior to autoclaving. The cultures were maintained in different media supplemented with 6-Benzyleaminopurine (BAP) (3.0, 4.0 & 5.0 mg/L) and Naphthalene acetic acid (NAA) (0.5, 1.0 & 1.5 mg/L) and protocol was established. The culture conditions were maintained at 25°C constant temperature with 16-h light duration by florescent lights. After 4 weeks of inoculation, data was recorded regarding proliferation rate (%), number of shoots, shoot length (cm) per treatment. The statistical analysis was done according to the Completely Randomized Design (CRD) while Analysis of Variance (ANOVA) was carried out for the inferences (Steel *et al.*, 1996).

***In vitro* rooting and acclimatization:** Newly formed shoots were excised and transferred to half strength MS-medium having 0.25 mg/l IBA and 25 g/l sugar. Rooted shoots were then transferred to individual cells containing potting mix of 1 soil: 1 sand: leaf manure. The plants were then placed under small polyethylene tunnels and were tightly enclosed for a week to capture the moisture. Plastic covers were gradually removed over 1 month and the plants after being completely uncovered for several days, were transferred to the green house. The plants that survived acclimatization were transplanted after 5 to 10 weeks and grown to flowering.

Results

Dispersion of morphological characterization on factor bi-plot for observation of variation amongst elite varieties of carnation: The results of the morphological characterization (Fig. 1A to J) of three varieties of Antigua, Tobasco and Montoyo are present in figures-2A and 2B. There is wide range of variation in the color of flower, stem thickness and other characters as dispersed on the factor plot showing 53.76 on factor 2 while 28.46% of variation. The dispersion line showing wide dispersion on four quadrants of the factor plot showed large range of variation amongst sexual and vegetative parts of the plant. The factor plots Figure-2C and 2D represent almost 60% of variation and clear dispersion of cv. Antigua, Montoyo and Tabasco which are clearly distinguished and identified based on their morphological characters. Remaining variation in morphological characters also showed further clear dispersion of varieties on the factor plot showed that varieties were different from each other clearly on morphological level. (Fig. 2) is based on the collective analysis through PCA technique of morphological characters and varieties dispersion on the bi-plot. The variation can be clearly seen on the bi-plot as each variety is separated from other based on factors Fig. 2E & 3F).

Organogenesis in elite verities of carnation in modified MS-medium: The overall results of shoot length (cm), number of shoots and proliferation percentage of the three carnation varieties (Fig. 1K to P) are summarized (Table 1). It can be inferred from the results that the highest shoot length (cm) was observed in MS-Medium modified with 4.0 BAP + 0.5 NAA for variety Antigua (10.40), whereas Montoyo and Tabasco performed well in the MS-Medium modified with 3.0 BAP + 1.5 NAA and 4.0 BAP + 1.5 NAA producing 9.60 and 13.90, respectively. It was noted that with increase in the concentration or the absence of growth hormones, the shoot length decreased significantly for all the three varieties. The maximum number of shoots were produced in the MS-Medium modified with 4.0 BAP + 1.5 NAA in case of Tabasco (8.33), MS-Medium modified with 4.0 BAP + 0.5 NAA in Antigua (6.32) and 5.76 number of shoots in variety Montoyo in case of MS-Medium modified with 3.0 BAP + 1.5 NAA. Whereas the minimum results were found in control and MS-Medium modified with 5.0 + 1.5 for all of the three varieties. The results depicted (Table 1) showed that the maximum shoot proliferation percentage was seen in variety Tabasco (71.46), Montoyo (63.07) and Antigua (57.53) in MS-Medium modified with 4.0 BAP + 1.5 NAA, 3.0 BAP + 1.5 NAA and 4.0 BAP + 0.5 NAA respectively. Again, control and MS-Medium modified with 5.0 BAP + 1.5 NAA depicted minimum results for the three varieties.

This study showed that different concentrations of BAP and NAA when combined have positive results towards the shoot multiplication. With an increase in the concentration of BAP, the shoot length, number of shoots and shoot proliferation percentage increased significantly (Fig. 1K & L). However, the combination with the highest concentration used in this study showed minimal results in terms of parameters discussed previously. This shows that the optimum concentration of BAP will be effective for rapid shoot multiplication and when the level reaches beyond the optimal concentration, the results could be negative. The large population was developed by this protocol. The plants were shifted to green house for sale after acclimatization and hardening process (Fig. 1Q, R & S).

Fig. 1. Showing morphological and *In vitro* response of Elite varieties of Carnation where A: Complete flower of elite varieties, B: Complete flowers with flower stem and maximum size that attained by the stem in Islamabad, C: Flower Petal shape and size compared with each other. F-G complete flower size close-up, color of flower along with whorls of the flowers. H: is Gynoecium portion of the flower of three varieties, I-J: Calyx portion of three varieties. Micro-propagation portion starts from K: small plantlets from inoculation vessel. M-P: Culture vessels, R: Mature plants from plant tissue culture lab., S: plants are ready for sale.

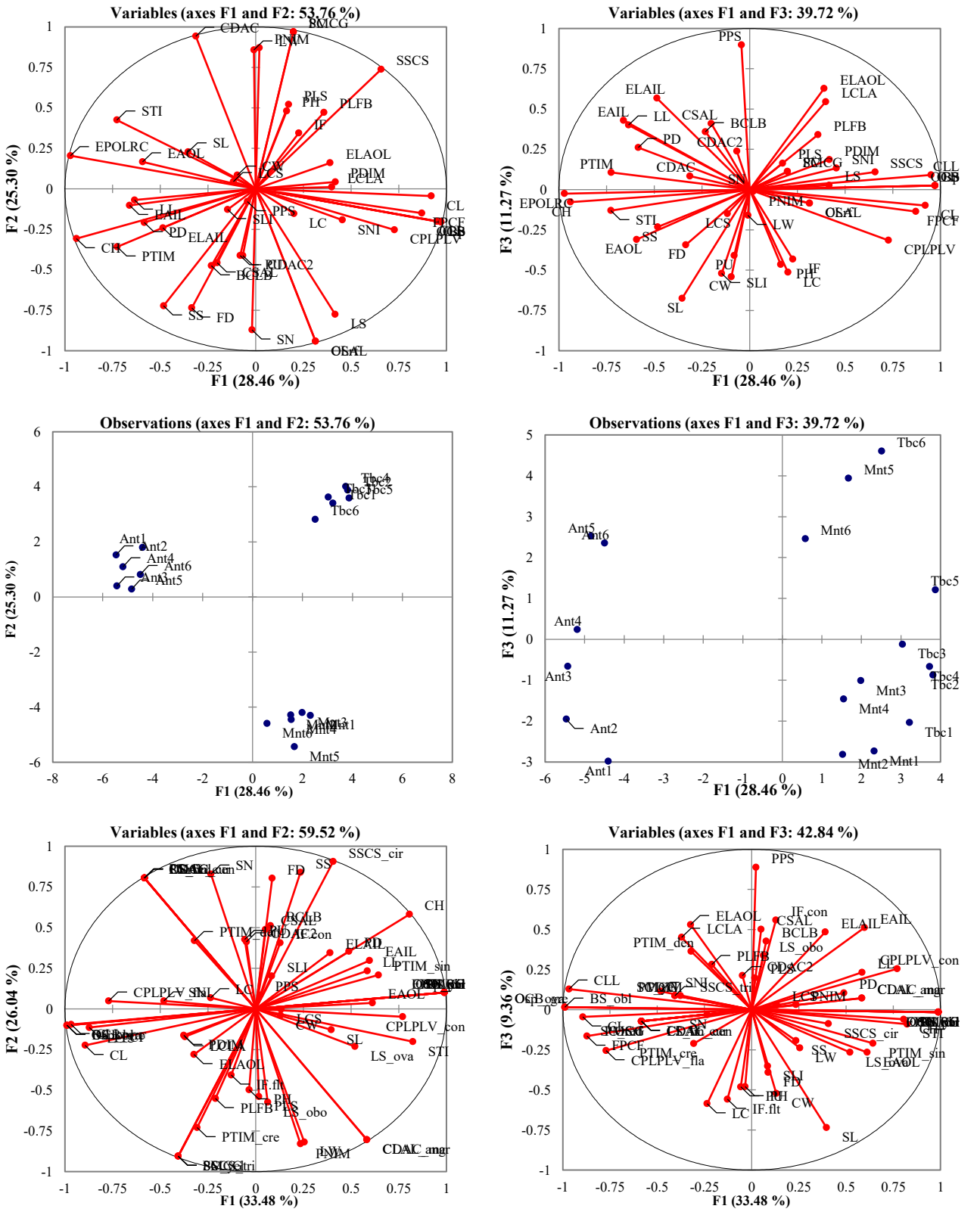

Fig. 2. Labels: A, B and E**:** Plant: Length of stem: PLS, Plant: Height: PG, Plant: Density: PD, Flower position compared to foliage: FPCF, Stem: Number of internodes: SNI, Plant: Laterals with flower buds or flowers of second order: PLFB, Plant: Clustering on lateral branches: BCLB, Inflorescence: form: IF, Stem: Length of internode: SLI, Stem thickness of internode: STI, Stem shape in cross section: SSCS, Leaf Shape: LS, Leaf length: LL, Leaf width: LW, Leaf: curvature of longitudinal axis: LCLA, Leaf Cross Section: LCS, Leaf color: LC, Bud: shape: BS, Epicalyx: Position of outer lobes in relation calyx: EPOLRC, Epicalyx: apex of outer lobes: EAOL, Epicalyx: apex of inner lobes: EAIL, Epicalyx: Length of apex of inner lobes: ELAIL, Calyx: Length: CL, Calyx: width: CW, Calyx: Longitudinal axis of lobes: CLAL, Calyx: Distribution of Anthocyanin coloration: CDAC, Calyx: Distribution of Anthocyanin coloration: CDAC2, Calyx: shape of apex of lobes: CSAL, Calyx: Length of lobes, Flower: Diameter: FD, Corolla Height: CH, Corolla: Profile of lower part in lateral view: CPLPLV, Petals: Predominant shape: PPS, Petals undulation: PU, Petals: Number of incisions of margin: PNIM, Petal: type of incisions of margin: PTIM, Petals: Depth of incision of margins: PDIM, Petal: Main color Group: PMCG, Ovary shape: Osp, Ovary: color of base: OCB, Ovary Surface: OSrf, Style: Number: SN, Style: Length:SL Style: Shoulder: SS, Stigma: color: SC. **C, D and F:** Ant: Antigua, Mnt: Montoyo and Tbc: Tabasco.

Table 1. Effect of different concentrations of BAP and NAA on shoot length (cm).

Treatment/Parameters	Composition MS (BAP + NAA) mg/L	Variety			Over all means
		Antigua	Montoyo	Tabasco	
Shoot length (cm)	Control	1.230 ^{n}	1.420 ^{n}	1.410 ^{n}	1.3533 ^{g}
	3.0 + 0.5	3.247 ^{jk}	3.233 ^{kl}	3.283 ^{jk}	3.2544 ^{e}
	3.0 + 1.0	5.690 ^{g}	5.700 ^{g}	5.157 ^{gh}	5.5156 ^{c}
	3.0 + 1.5	7.550 ^{e}	9.600 ^{c}	7.513 ^{e}	8.2211 ^{a}
	4.0 + 0.5	10.400 ^{b}	6.477 ^{f}	8.700 ^{d}	8.5256 ^{a}
	4.0 + 1.0	6.813 ^{f}	4.100 ^{i}	9.697 ^{c}	6.8700 ^{b}
	4.0 + 1.5	3.833 ^{ij}	2.747 ^{klm}	13.900 ^{a}	6.8267 ^{b}
	5.0 + 0.5	2.353 ^{m}	1.670 ^{n}	6.967 ^{ef}	3.6633 ^{d}
	5.0 + 1.0	1.567 ^{n}	1.190 ^{n}	4.710 ^{h}	2.4889 ^{f}
	5.0 + 1.5	0.513 ^{o}	0.563 ^{o}	2.643 ^{lm}	1.2400 ^{g}
	Means	4.3197 ^{b}	3.6700 ^{c}	6.3980 ^{a}	
Number of shoots	Control	1.4000 ^{n}	1.6000 ^{mn}	1.4000 ^{n}	1.4667 ^{e}
	3.0 + 0.5	2.3667 ^{ijkl}	1.9667 ^{klmn}	2.1000 ^{klm}	2.1444 ^{d}
	3.0 + 1.0	3.3133 ^{efgh}	2.9667 ^{ghi}	2.8000 ^{hij}	3.0267 ^{c}
	3.0 + 1.5	3.2667 ^{fgh}	5.7667 ^{c}	3.9333 ^{e}	4.3222 ^{b}
	4.0 + 0.5	6.3267 ^{bc}	4.7000 ^{d}	5.9333 ^{bc}	5.6533 ^{a}
	4.0 + 1.0	3.6400 ^{ef}	3.4667 ^{efg}	6.4333 ^{b}	4.5133 ^{b}
	4.0 + 1.5	2.4867 ^{ijk}	2.8333 ^{ghij}	8.3333 ^{a}	4.5511 ^{b}
	5.0 + 0.5	2.3667 ^{ijkl}	2.2000 ^{jklm}	4.8000 ^{d}	3.1222 ^{c}
	5.0 + 1.0	1.8667 ^{klmn}	1.8333 ^{lmn}	2.8333 ^{ghij}	2.1778 ^{d}
	5.0 + 1.5	1.5667 ^{mn}	1.6000 ^{mn}	2.2667 ^{jkl}	1.8111 ^{de}
	Mean	2.8600 ^{b}	2.8933 ^{b}	4.0833 ^{a}	
Shoot proliferation % age	Control	15.500 ^{n}	16.633 ^{n}	15.500 ^{n}	15.878 ^{h}
	3.0 + 0.5	18.033 ^{mn}	23.167 ^{jk}	18.467 ^{lmn}	19.889 ^{g}
	3.0 + 1.0	24.667 ^{ij}	32.500 ^{fg}	22.600 ^{jkl}	26.589 ^{e}
	3.0 + 1.5	39.300 ^{e}	63.067 ^{b}	29.500 ^{gh}	43.956 ^{c}
	4.0 + 0.5	57.533 ^{c}	44.433 ^{d}	42.500 ^{de}	48.156 ^{a}
	4.0 + 1.0	44.933 ^{d}	38.467 ^{e}	55.967 ^{c}	46.456 ^{ab}
	4.0 + 1.5	27.867 ^{hi}	34.300 ^{f}	71.467 ^{a}	44.544 ^{bc}
	5.0 + 0.5	23.133 ^{jk}	32.400 ^{fg}	59.933 ^{bc}	38.489 ^{d}
	5.0 + 1.0	18.100 ^{mn}	19.600 ^{klmn}	30.700 ^{fgh}	22.800 ^{f}
	5.0 + 1.5	16.400 ^{n}	16.567 ^{n}	21.700 ^{jklm}	18.222 ^{gh}
	Mean	28.547 ^{c}	32.113 ^{b}	36.833 ^{a}	

Discussion

Use of morphological traits for selection of desirable plant population in breeding programs of carnation: Morphological characterization is an essential tool for the identification, classification, and selection of desirable traits in carnation breeding. The morphological characterization is an excellent tool to distinguish the plant population at various levels of breeding such as differentiation amongst wild and commercial varieties. The morphological traits such as flower color and petal shape, are highly significant in distinguishing between different populations and could be used in conjunction with molecular markers to improve the accuracy of population identification (Koutroumpa *et al*., 2021 and Xu *et al*., 2020). Moreover, morphological characterization has also been used to select desirable traits in various stages of carnation breeding against various stresses such as salt and disease tolerance. Certain morphological characteristics, such as leaf size and stem diameter, were highly correlated with salt tolerance and could be used to select tolerant cultivars (Liu *et al*., 2020). During the present study the morphological characterization was used to observe the variation amongst three varieties of cv. Antigua, Montoyo and cv. Tobasco. In total 52 traits were studied that were clearly different from each other; especially in flower colors, stem heights, volume of flowers and thickness of stem were different in cv. Antigua, Montoyo and tobacco as F1 and F2 showed 53.76% variation in overall characters of plants of three varieties (Fig. 2). The Principle component analysis can be used to distinguish the varieties from one another (Taghizadeh, & Khadivi, 2023).

Nodal explants are used for the mass multiplication of carnation in *In vitro* conditions (Ahmad *et al*., 2020). BAP (6-Benzylaminopurine) is a synthetic Cytokinin which is commonly used in *In vitro* propagation of carnation. It is a plant growth regulator which promotes cell division and shoot proliferation. These properties of BAP are important tool for producing large numbers of uniform plantlets in a short period of time in *In vitro* conditions. Media modification with BAP significantly increased shoot

proliferation and improved the growth and development of carnation plantlets (Liu *et al*., 2021). Culture media modification with BAP significantly promote shoot proliferation and promote the development of healthy and vigorous plantlets in *In vitro* environment (Ahmad *et al.*, 2020). This phenomenon is observed in the present study and significant shoot proliferation was observed in MS-media modified with BAP+NAA (4.00+0.500 mg/L) and observed the highest average No. of Shoots (5.563 Nos.). In addition to promoting shoot proliferation, BAP has also been shown to influence other morphological characteristics of carnation plantlets. The addition of BAP to the culture medium significantly increased the number of leaves and the chlorophyll content in carnation plantlets (Kaur *et al*., 2020). The BAP promotes shoot proliferation and other morphological characteristics, allowing for the efficient and rapid production of more numbers of uniform plants in *In vitro* conditions. BAP showed a significant tool in carnation tissue culture and is likely to continue to play a significant role in future research and breeding efforts.

NAA (Naphhalene acetic acid) is a synthetic Auxin that is commonly used in *In vitro* propagation of carnation. It is a plant growth regulator that promotes root formation and is essential for the successful acclimatization of plantlets after transfer to soil. Several recent studies have highlighted the importance of NAA in *In vitro* propagation of carnation. The Naphthalene acetic acid (NAA) in combination with other plant growth regulators optimize the *In vitro* rooting of carnation plantlets. The authors found that the addition of NAA to the culture medium significantly increased the number and length of roots in carnation plantlets and improved their survival rates after transfer to soil (Guo *et al.*, 2021). Naphthalene acetic acid (NAA) to develop an efficient protocol for *In vitro* propagation of carnation using shoot tip explants. The authors found that the addition of NAA to the culture medium significantly increased root induction and enhanced the growth and development of carnation plantlets (Mehboob *et al.*, 2020). In addition to promoting root formation, NAA has also been shown to influence other morphological characteristics of carnation plantlets. The addition of NAA to the culture medium significantly increased the number of leaves and the biomass of carnation plantlets (Dhiman *et al.*, 2020). Overall, these recent studies demonstrate the importance of NAA in *In vitro* propagation of carnation. NAA promotes root formation and other morphological characteristics, allowing for the efficient and rapid production of healthy plantlets with high survival rates. NAA remains an important tool in carnation tissue culture and is likely to continue to play a significant role in future research and breeding efforts.

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Conclusions

Pakistan is a low per capita income country fighting for economic stability and food security. The conventional crops are not economically better for prosperous future due to high cost of production with low returns. The present study deals with the morphological characterization of carnation varieties and development of *In vitro* micro-propagation protocol. Each year, Pakistan imports large number of fresh carnation flowers from various regions of the world such as Japan, Malaysia and Thailand. The present study will depict better varieties for cultivation for earning good amount of capital compared with conventional crops. This is the first study on this aspect as farmers in Pothwar region could be used for commercial cultivation of carnation in winters in frost free protective conditions in Islamabad. In normal season each stem of carnation sells at Rs. 300 and out of season in Rs. 600 in Karachi and Islamabad in reputed stores. The carnations can be successfully grown in Kotli Sattian and Ghora Galli area after winter season is over in Islamabad to obtain optimal stem length and growth. The present study could provide a platform for commercial production and export of carnation to capture new markets thus, earning capital for the nation.

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