In vitro micro-propagation of *Chrysanthemum morifolium* Ramat. from stem nodal segments by direct morphogenesis and through callusing (organogenic and somatic embryogenic)

Alia Gul*, Mossarath Jabeen, Nosheen Shafqath***, Nadia Jabeen***, Sumera Salahodin lodhi****, Muhammad Sajid***, Fazal Hadi**, Asma*, Zenab Kainat****, Abdul Basit** Syed Hasnain Imad *******

Abstract: *Chrysanthemum morifolium* Ramat. is an exciting trade plant due its ornamental importance. Our research aimed to assess plantlet regeneration efficiency by using stem nodal segments as ex plant source through direct shoot regeneration, organogenesis, and somatic embryogenesis through callusing. Various sterilization protocols were followed to raise pathogens free plantlets. Various PGR concentrations and combinations were applied to induce, direct shoot-roots regeneration from explant, organogenic, and somatic embryogenic callusing for mass scale plantlet regeneration were induced. Optimal stem nodal segments sterilization protocol (75% alcohol for 15 s + 0.1% HgCl2 for 2 mins +washing with double distilled sterilized water 3 times) gave satisfactory results. Maximum shoot regeneration response was achieved from stem nodal segments and shoot tips when Benzyl Amino Purine (BAP) was used on MS 0.5 mg/L. The no of shoots induction was higher when MS BM was fortified with BAP with 1-2 mg/L. With fortification MS 2 mg/L of BAP shoot multiplication increased but growth was much suppressed. Further shoot growth, elongation and photosynthetic tissue formation occurred on MS BM fortified with Kn 1.0 mg/L+ IAA 0.5 mg/L. All micro shoots rooted on MS supplemented with 0.5mg/L IAA+ 0.1 mg/L Kn. Rooted plantlets growth occurred on MS BM and up to 60 plantlets per segment were obtained. From stem nodal segments and shoot tips *Organogenic* callus was induced on half MS fortified with BAP + 2,4-D 1.0 +0.5m/L, Kn + IAA, 2+1 mg/L. However, callus was also induced on MS BM supplemented with 1-2 mg/L BAP in 50% cases. Shooting occurred from the callus after 4-6 weeks of inoculation, on BM with 1-2 mg/L of BAP. The micro cuttings rooted on BM fortified with 0.1mg/L Kn + 0.5 mg /L IAA after 4.5 to 6 weeks of inoculation. However, MS BM fortified with 0.5 mg/L BAP and .1 mg/L NAA induced embryogenic callus. A light maroon embryogenic callus was formed on MS fortified with BAP and NAA minimum quantity (0.5mg/L). Embryogenesis or plantlet regeneration from the callus was induced by fortifying the MS with 1.0 mg/L BAP. Up to 60 plantlet redevelopments from small callus mass occurred after 3-4 weeks of culture. It was found that more than 95% plantlet established under *ex vitro* conditions when using polythene bags etc. for acclimatization and transferring to natural conditions. Micropropagagules were morphologically better than mother plant and flourished well, showed superior quality in morphology, color of flower, shine, texture, and early maturity compared to conventional propagules. Per flask 200 plantlets regeneration rendered somatic embryogenesis the most effective way for producing mass scale propagation of virus free plantlets. Ornamental market can take benefit of mass scale propagation in Pakistan.

Index Terms: *in vitro – Chrysanthemum morifolium, direct shoots-roots induction,* Organogenesis; Somatic embryogenesis, pathogens free plantlets regeneration

I. INTRODUCTION

Chrysanthemum are not only important as ornamental plants but also a good source of natural products [1]. Chrysanthemums are very flashy flowers that give this plant excellent ornamental value [2]. Ornamental plants particularly *Chrysanthemum* became first target for micro propagation due to its popularity and need, [3]. In floriculture trade *Chrysanthemum* is the most valuable ornamentals cut flower. The name *Chrysanthemum* derived from Greek word chrysos (golden) and anthose flowers belonging family Asteraceae. *Chrysanthemum* rank second after roses for their excellent cut flowers but grower face the pathogenic attacks caused by virus's bacteria and fungi. To overcome all these problems micropropagation is the excellent and promising tool to overcome and dominate the market ([4]*;* [5]; [6]; [7]. 1000 plant species were observed through in vitro organogenesis through empirical section of explants in 1957 under control media composition and physical environment [8]. Small group of parenchyma cells used as pioneer for organogenesis and gives a mass of cells, which yield meristematic cells. Shoot or root primordium are propagated from these meristematic cells [4]. Propagation of clones through *in vitro* culture can increase multiplication many times [9]. Now micropropagation is the alternative effective method of plant propagation leaving behind the conventional propagation's methods. Many studies are conducted on tissue culture of *Chrysanthemum* from different countries. Battacharya [10], reported leaf stem explants of C*hrysanthemum morifolium* us in mass propagation. Khan and Ahmad [11], explore that only apex of shoot tip of local cultivar can be used in mass propagation. Ben -Jaacob and Langhans [12] and Earle and Langhans [13]

explained shoot tip-initiated callus *Chrysanthemum* of can be used in micro propagation. Large scale production of *Chrysanthemum* is done through tissue culture. Amin *et al.* [14] conclude that propagation of *Chrysanthemum* is possible through axillary and adventitious buds. Embryogenesis is opposite to organogenesis; organogenesis is two step procedure (uni-polar shoot or root primordium) forming while somatic embryogenesis is one-step (producing bipolar structure with a root and shoot axis). 130 species of monocots, dicots and conifers were propagated through asexual embryogenesis. Procedure divided in to two major steps, one is induction of embryo like cells into embryo, practical use of explants, cultural environment, and media lead to process success. This is cellularly and structurally different from the zygotic embryogenesis. Somatic embryogenesis has preference over organogenesis as this is a practical mean of propagation. It bypasses the required time and cost of explants organogenesis (two steps). Subculture is not required in for clonal stock, which is time consuming for micro propagation roots. Somatic embryogenesis may introduce the excellent clones for commercial mass scale production handling of somatic embryo is easy and may facilitate biotechnical approaches for plat culture [15]. Somatic embryogenesis is a easy way to develop identical individual without manipulations required for shoot and root formation. Root and shoot axis are present in somatic embryo (bipolar) which produce these organs easily in lab (Plant science 435). Embryogenic culture produces cell masses which can be multiplied into somatic embryos. Moreover, when encapsulation technique coupled with embryo, somatic embryo can then be modified for large scale plant production [16] & [17].

Current attempt aimed to optimize protocols of various growth regulators for direct shoot regeneration, on callus induction, its propagation, organogenesis, and somatic embryogenesis of *Chrysanthemum morifolium* Ramat. *in vitro* for the success of commercial level production and clonal propagation.

II. MATERIALS AND METHODS

Site description and Sampling

Current attempt of investigations was carried out in the Department of Botany, (PTC) laboratory, University of Peshawar, Pakistan. The explant nodal segments of stem and apex of shoot were used. Ex-plants taken from the *Chrysanthemum morifolium* (mother plant) present at Agriculture University Peshawar.

Surface sterilization

The explant was properly cleaned by washing with running tap water, followed by rinsing with double distilled water. Similarly, all materials used were properly sterilized through 0.1% mercuric chloride ($HgCl_2$) in biosafety cabinet for 3 minutes.

Media preparation and culture preparation

After sterilization, the ex-plants were inoculated on half MS [18] containing 4% (w/v) sucrose and 0.7% (w/v) agar-adjusted and pH was adjusted to 5.5. The whole samples were autoclaved at 121^{o}C for 15 minutes, incubated at 25+-1^{o}C and cultures were kept for 16 hours, under photoperiod florescent tube light. Explants were cultured on MS BM (half) supplemented with various combinations of Cytokinin and Auxin.

Oranogenic callus induction

The callus was induced on ½ x MS 1.0 mg /L BAP +.5 mg/ L 2, 4-D and 2. 0 mg /L IAA+1.0 mg /L Kn. The calluses were subculture at 3-4 weeks intervals and further growth occurred on MS +1.0 mg/L BAP. The callus was placed in the shoot induction (BAP 1.0-2.0 mg /L) and rooting BM (IAA 0.5+ Kn 0 .1 mg /L) and the shoots and root emergence after 4 weeks, were measured/observed.

Somatic embryogenesis

For somatic embryogenesis explants were cultured on ½ MS BM along with varios concentration of Cytikinin-Auxin supplementations. The callus induced on MS (1/2) with 0.5mg/L BAP +along 0.1 mg/L NAA. The calluses were subcultured with 3-4 weeks intervals and was left for further growth on, 1/2 X MS +. 0.5 mg/L BAP. The callus was placed in the shoot induction (BAP 1.0-2.0 mg/L) and rooting BM, seedlings emerged after 4 weeks intervals were further inoculated on plain MS BM for growth. After 6 weeks of culture media supplemented with 0.5mg/L BAP+0.1mg/l NAA induced proliferation of callus formation from the explant (**Figure 1A**). Similarly, the same media was used for sub culturing of callus for further multiplication (**Figure 1B**). For the solidification, 0.9% agar media was used, and sucrose was used as a carbon source. For the pH adjustment up to 5.6, .1% NaOH or/and HCl was used. The BM was sterilized by autoclaving at 121°C for 15 mins at a p of 103.44 P/s. All the cultures were incubated in biotrons with 16 hr light period for 24 hours cycle at 25 ± 1°C. After acclimatization the plantlets were shifted to pots for growth in natural environment.

Statistical Analysis

Five replications of experiments were performed for both direct method of shooting rooting and callusing for organogenesis and somatic embryogenesis. Data taken was tabulated and different statistical analyses (ANOVA, Mean, standard error and standard deviation) were performed by using SPSS 21.0 software.

III. RESULTS AND DISCUSSION

Direct shoots buds' regeneration from stem nodal segments and stem tips without callus formation and with callus formation were obtained and compared. Results were obtained in three series for these various experiments for optimizing mass propagation of *Chrysanthemum.*

Table 1. The effect of cytokinin +auxinfor in vitro callus formation from the nodal and shoot tip explant of *Chrysanthemum morifolium Ramat.* ½ X MS medium.

Growth regulators for callusing			Shoot induction			Rooting			
Treatment 1	***BAP+2, 4-D.***	***%age of explant responded for callusing***	***T2***	***G .Regulaters BAP***	***No. %age of explant responded for shoot induction***	***T3***	***Kn+IAA***	***%age of explant responded***	***No.explant responded for rooting***
M1-1	0.1+0.1	1(5)	M2-1	0.5	5(25)	M3-1	0.1+0.1	1((5)	1
M1-2	0.5+0.5	5(25)	M2-2	1.0	1680)	M3-2	0.1+0.5	19(90)	19
M1-3	1.0+0.5	16(80	M2-3	2.0	16(80)	M3-3	0.5+0.5	2(10)	2
M1-4	1.0+1.0	5(25)	M2-4	3.0	10(50)	M3-4	0.5+1.0	10(50)	10
M1-5	2.0+1.0	10(50)	M2-5	4.0	5(25)	M3-5	1.0+2.0	10(50)	10

Table 1. explaining various treatments for callusing, T2, shoot induction, and T3 for rooting best combination is M3-2 provided.

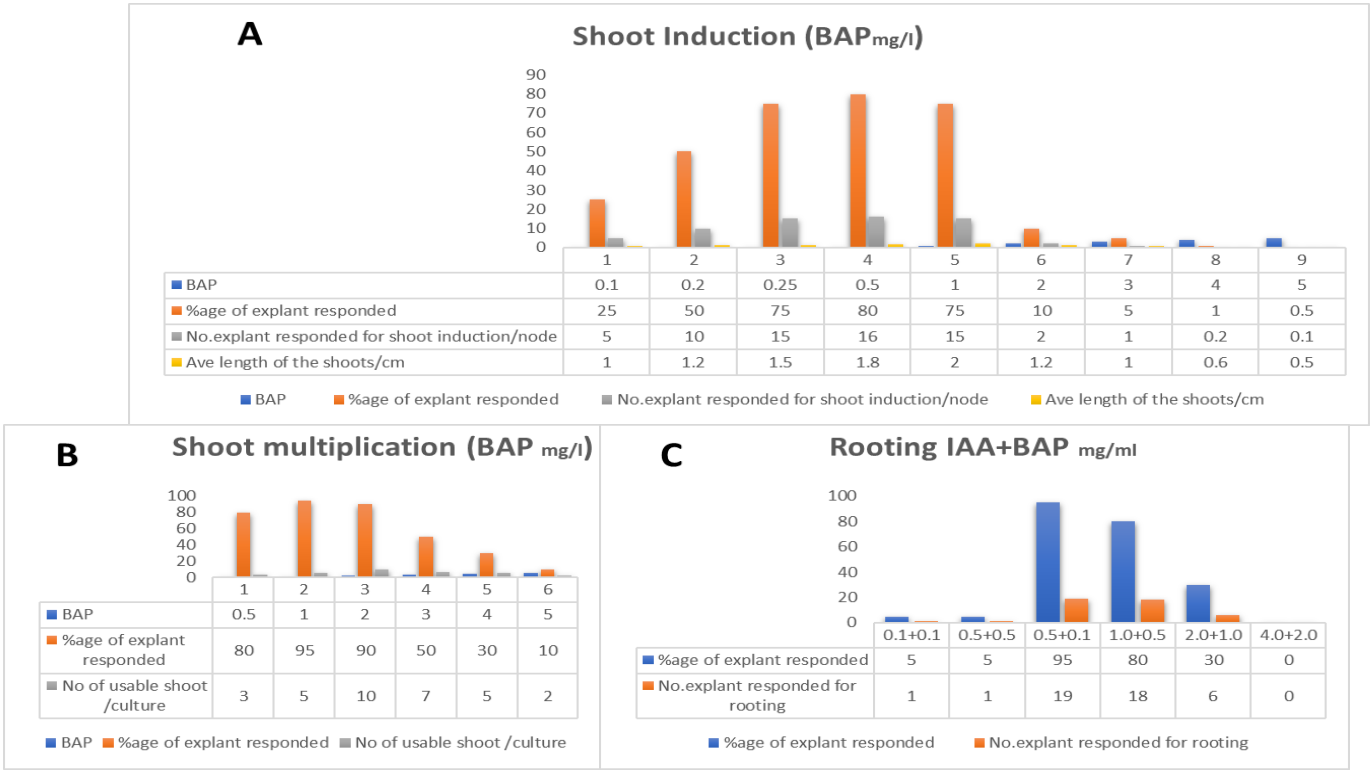
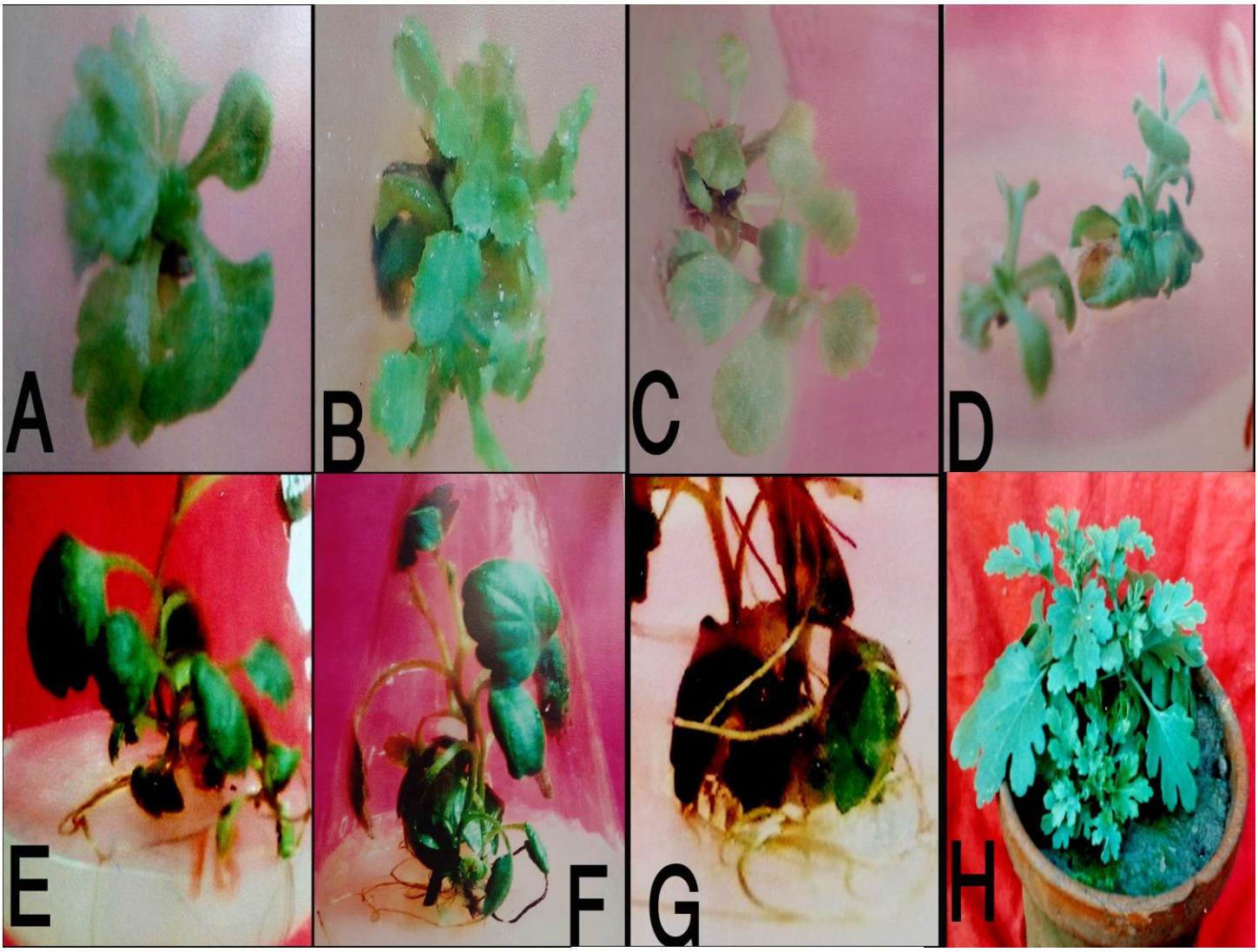


Fig.1 A, B and C Showing the effect of BAP On morphogenesis.



Figures: 2. Direct morphogenesis Shoots, roots regeneration ***Chrysanthemum morifolium*** **Ramat.** through *in Vitro* Culture

A. Shoot induction from stem nodal explants of *Chrysynthimum* on ½ MS +0.5 mg /L BAP after two weeks.
B. Shoots multiplicationn induction on ½ MS+ 1.0mg/l BAP after further two weeks
C. Shoots growth on ½ M S+ 1.0mg/L BAP after 3rd week.
D. Sub culturing of shoots after 4 weeks of inoculation.
E. Rooting of shoots ½ M S +0.5mg /L BAP and 0.5 mg /L IAA.
F. Plantlets further roots growth 2 weeks on ½ MS +0.5mg /L BAP and 0.5 mg /L IAA.
G. Plantlets functional roots formation, on plain media after two weeks without any growth regulators
H. Microplantlets successfully adjusted in the natural environment after one week of acclimatization.

In another set of experiments *Chrysanthemum morifolium* Ramat. stem segments and shoot tips cultured on BM fortified with various concentrations of PGR resulted in callusing.

Table. 2. Effect of PGRs on callus for organogenesis, Shooting and Shoot elongations *Chrysanthemum morifolium Ramat.* from stem nodal segments on I/2X.

Table 2. Effect of PGRs on callus for organogenesis, Shooting and Shoot elongations *Chrysanthemum morifolium Ramat.* from stem nodal segments on I/2X.

Growth regulators for callusing					**Growth regulators for Shoot elongation**			
Treatment	*IAA+Kn* (mg/L)	*No. %age of explant responded*	*T5*	*Kn* (mg/L)	*No. %age of explant responded for shoot elongation*	*T6*	*BAP* (mg/L)	*No%age.explant responded for shoot elongation*
M4-1	0.5+0.5	1(5)	M5-1	0.5,	8 (40)	M6-1	0.5,	2(10)
M4-2	1.0+0.5	2(10)	M5-2	1.0	12 (60)	M6-2	1.0	8 (40)
M4-3	1.0+1.0	2 (10)	M5-3	2.0	16 (80)	M6-3	2.0	10 (50)

M4-4	2.0+1.0	16(80)	M5-4	3.0	10 (50)	M6-4	2.5	12 (60)
M4-5	4.0+2.0	16(80)	M5-5	4.0	8 (40)	M6-5	3.0	16 (80)

Table 2. Explaining the various concentrations for callus induction and various combinations to induce morphogenesis again from subcultures.

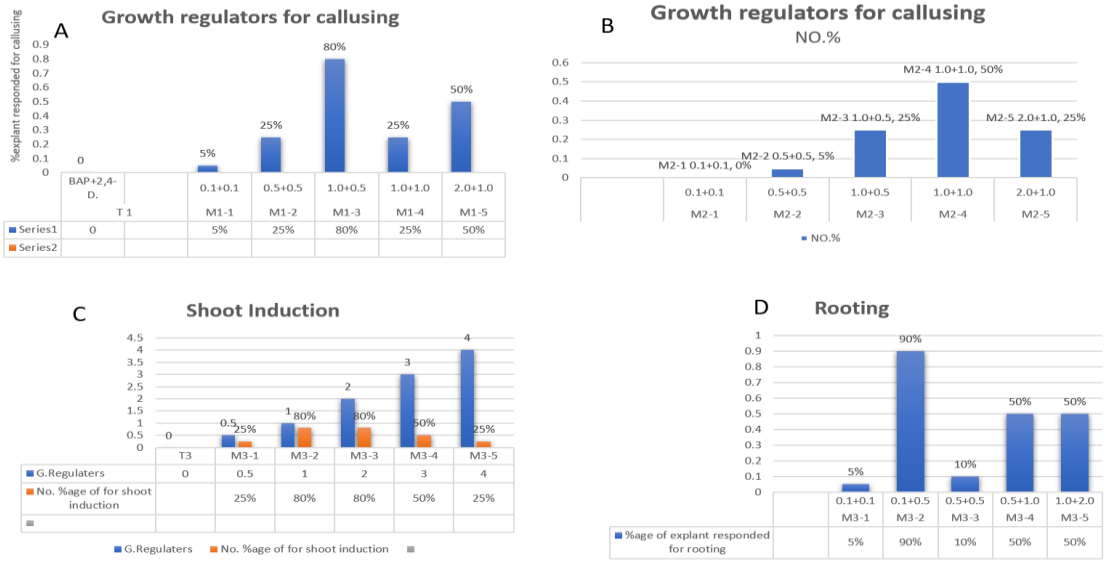


Fig 3. Explaining various treatments for callusing: The Fig. 3 explaining effect of T1 & T2 callusing, T3, Shoot induction, and T4 rooting. We used 20 explants in each treatment and the data were recorded after every 6 weeks. Different concentrations of BAP (.5, 1., 2.) +2,4-D .1,.5,1.0) & IAA 1. 2. + Kn .5, 1.0 mg / L were used for callus formation (Table.1).

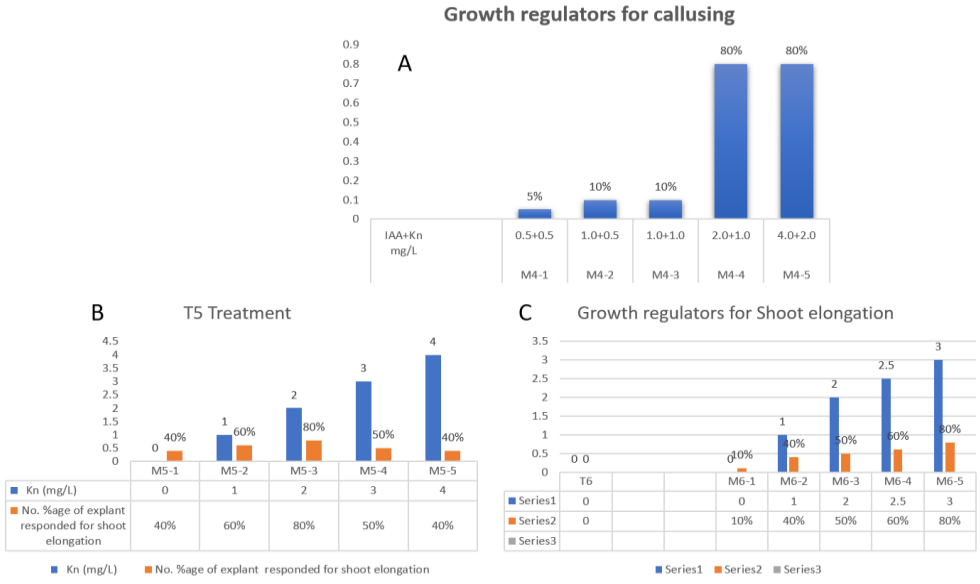


Fig 4. Show the cytokinin and auxin effect on nodal and shoot tip culture.

Fig 4. Explaining the cytokinin and auxin effect on nodal and shoot tip culture. Each treatment contains 20 explants and after six weeks data was recorded. Different concentrations of IAA (0.5, 1.0, 2.0) + Kn 0.1,0.5,1.0) & Kn 0.5, 1.0 mg /L, BAP (0.5, 1.0, 2.0, 2.5. 3)+ lAA were used for shoot elongation

The best treatment recorded for organogenic callus induction was M4-3 BM fortified with 2mg/L IAA along 1 mg/L Kn. However lower and higher concentrations of PGRs showed minimum response. While best treatment for shoot induction was 2mg/L Kn

induced maximum shoots elongations and responded 80% / Flask. For rooting best treatment was M3-2. Where BM, I/2X MS was fortified with Kn 0.1 mg/L with 0.5 mg/L IAA.

The best treatment recorded for organogenic callus induction was M1-3 BM fortified with 1mg/L BAP along .5 mg/L 2,4-D. However lower and higher concentrations of PGRs showed minimum response. While best treatment for shoot induction was 2mg/L BAP induced maximum shoots/ Flask. For rooting best treatment was M3-2. Where BM, I/2X MS was fortified with Kn .1 mg/L with .5 mg/L IAA

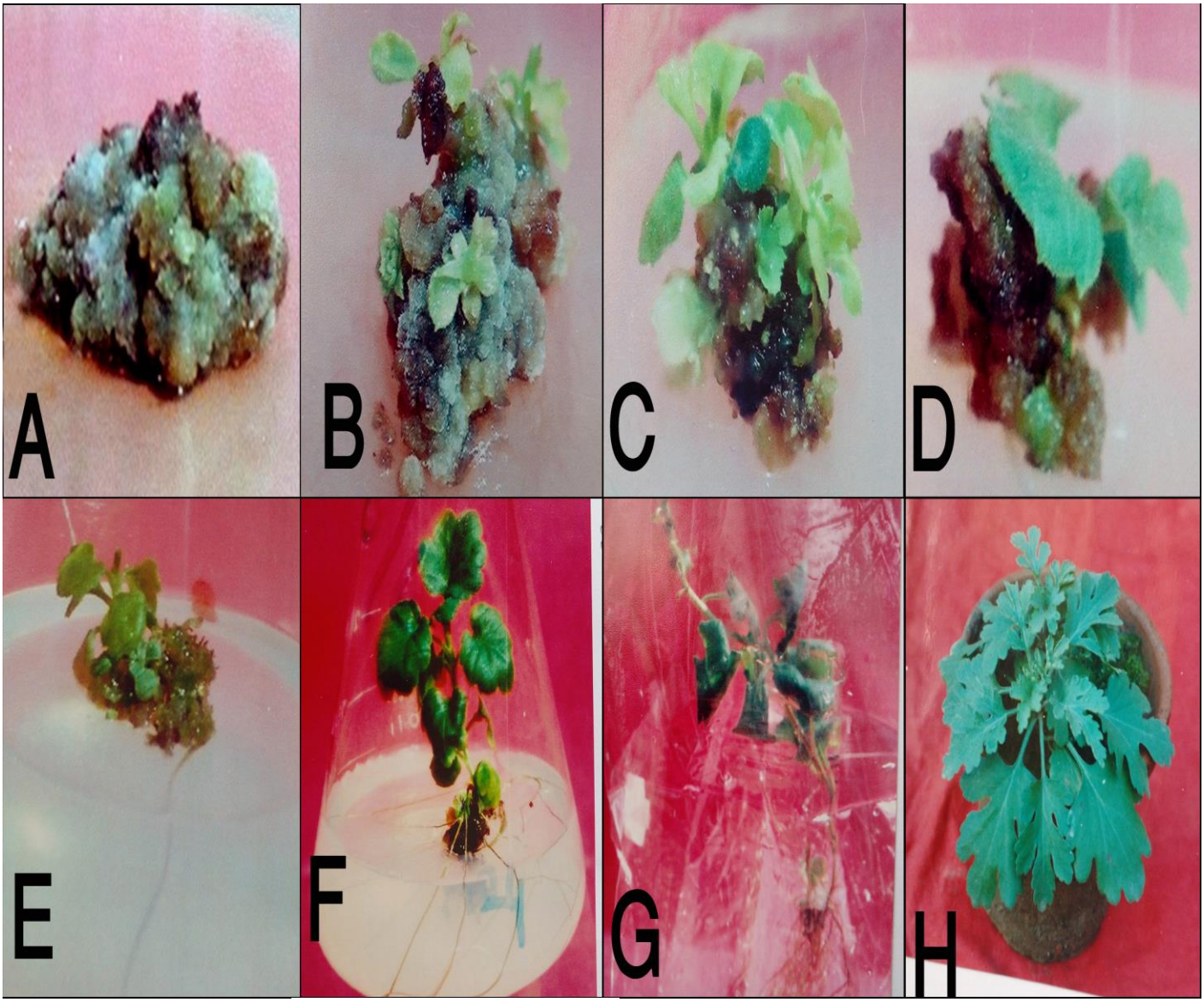


Figure 5: Organogenesis of *Chrysanthemum morifolium* Ramat. through Callusing

A. Callus induction on BM fortified with 1mg/L BAP +12,4-D after 6weeks of culture.
B. Callus proliferation after 3 weeks of culture on BM fortified with 2.0 mg/L IAA+1.0 mg/L Kn.
C. Organogenesis on BM fortified with 1.0 mg/L of BAP.
D. Shoot induction after 4 weeks of culture on BM fortified with 1.0mg /L of BAP.
E. Morphogenesis (shoots, roots) on BM fortified with 0.5 mg /L IAA+1.0 mg /L Kn.
F. Root formation on BM fortified with 0.5mg/L IAA+0.1mg /L Kn. Plantlet transferred to the MS plain medium for further functional root and plantlet growth
G. Acclimatization of plantlets at room temperature
H. After acclimatization plantlets got established in the soil showing vigorous growth.

The type of Cytokinin and Auxin used remarkably influenced induction of organogenic callus formation from the stem nodal segments. Among different concentration best response towards callus formation occurred on I/2X MS fortified with BAP 1.0 mg /L+2,4-D 0.5mg/L (Fig.1-A) & from shoot tip occurred by 2.0 mg/L IAA +1 mg /L Kn. (Fig. 5, 1-B). The previous investigators

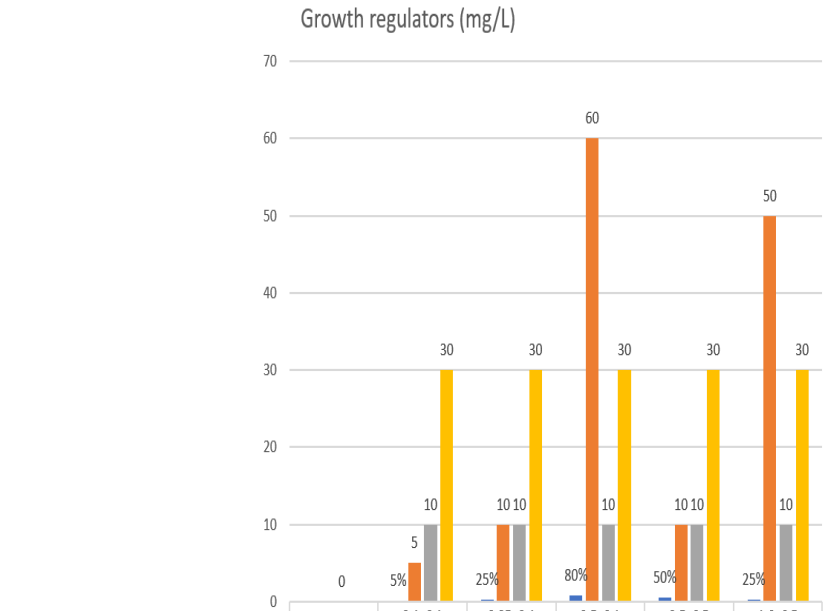
also used 2, 4 -D and Cytokinin as [19]. The results are matching with [19] as he used high 2, 4 -D /Kn ratio and in this experiment the callus was obtained by using high IAA to Kn or 2.0 mg/l IAA+1 mg/L Kn [20] also obtained callus at 0.5 mg/l IAA with low level of BAP or Kn (0.5-0.8 mg/L). We also induced callus formation by using only BAP in 1.0 – 2mg/L. [21] also reported callus formation on ½ X MS medium by providing BAP alone in the range of .5-1-2 mg/L. For shoot regeneration from the callus different concentration of BAP 1-2 mg/L showed satisfactory results (Fig.5-C, D, E) showing shoot emergence from the callus. Roest and Bolkman [22] obtained similar results on shoot regeneration when they used BAP 1.0 mg/l in the medium. These results are in conformity with the finding of Hutchinson [23] they observed that in vitro propagation of different cultivar is best when MS media supplemented with BA. Rooting of the micro shoots occurred on 0.5 mg/L IAA+ 0.1 mg/l Kn. Similiar results have been published by Khan and Ahmad [11] in the callus of bougainvillea, where shoot regeneration occurred on MS medium enriched with .5-1.0 mg /L of BAP (Fig.5-F, G). For further growth of rooted plantlets were transferred on plain I/2X MS medium (Fig.5F*).* All rooted *in vitro* raised plantlets were transferred to soil (pots) (Fig.1-H). They flourished well and showed better growth as compared to conventional propagules. Furthermore, they look greener, stout and showed good growth response in a few days.

Somatic Embryogenesis *in vitro* callus formation from the nodal explants of *Chrysanthemum morifolium* Ramat. 1/2 MS medium is presented in Table. 3.

Table. 3. Growth regulators (mg/L)

Treatment	BAP+NAA	No. %age of explant responded for callusing	No of seedlings obtained /flask	Length of plantlet shoot after 8 weeks Of culture on BM (cm)	Length of plantlet after 8 weeks of culture on BM (cm)
M3-1	0.1+0.1	1(5)	5	10	30
M3-2	0.25+0.1	5(25)	10	10	30
M3-3	0.5+0.1	16(80)	60	10	30
M3-4	0.5+0.5	10(50)	10	10	30
M3-5	1.0+0.5	5(25)	50	10	30

Table 3. Explaining the significant effect of BAP and NAA effect on callusing. The optimum results were obtained at M3-3 while further concentaration again decrease the effect in M3-5. This shows that BAP and NAA after the given concentration can totally reverse the situation.



		0.1+0.1 M3-1	0.25+0.1 M3-2	0.5+0.1 M3-3	0.5+0.5 M3-4	1.0+0.5 M3-5
No. %age of explant responded for callusing		5%	25%	80%	50%	25%
No of seedlings obtained /flask		5	10	60	10	50
Length of plantlet shoot after 8 weeks	0	10	10	10	10	10
Length of plantlet after 8 weeks of culture on BM (cm)		30	30	30	30	30

Fig. 6. *In vitro* callus formation from stem nodal segments and **shoot tips** of explants of *Chrysanthemum morifolium* Ramat. 1/2 MS medium is presented

The Fig 6. Is explaining the effect of BAP and NAA combination on embryogenic callus and the optimization of cultural conditions.

Figure 7. Somatic embryogenesis of *Chrysanthemum morifolium* Ramat. through *in vitro* culture

A. Callus formation on BM supplemented with .5 mg/ BAP .1 mg/L NAA
B. Subculturing of the callus on fresh medium after 6 weeks of culture.
C. Induction of embryogenesis on BM fortified with1. mg/L BAP
D. Plantlet regeneration on BM—1 mg/L BAP
E. Micro plantlets were grown from somatic embryos on MS fortified with 0.5 mg/L of BAP, upto 60 plantlets regenerated on BM after 6 weeks of culture.

F. Plantlets further growth and functional root formation on BM after 8 weeks of culture.
G. Plantlets got established in the soil after, acclimatization and 15 days of transfer from invitro to in vivo.

Fig.7. Explaining the various PGRs concentrations for callus formation. Embryogenic callus was formed in maroon, brown colure on MS containing BAP and NAA only. There were 20 explants in each treatment and the data were recorded after every 6 weeks. Different plant growth regulators were used for plant regeneration from the callus. After 6 weeks of culture media supplemented with .5mg /L BAP+.1mg/l NAA induced proliferation of callus formation from the explant (Fig.7-A). Callus formed was subcultured on the same medium for further proliferation, (Fig 7, 1-B).

Cytokinin in the form of BAP was used for plant regeneration from the callus. At this concentration, little growth of the callus but rapid plantlets regeneration occurred (Fig.7-C) Altaf *et al* [24] they observed *Citrus reticulata* on MS medium that embryogenesis in the callus produced with low level of BAP. The embryos developed into plantlets when transferred to the basal medium (Fig.7-D). Embryogenesis was also demonstrated in *Coffea arabica* species when the callus obtained at NAA and Kn was transferred to a medium provided with 0.5-0.9 mg/l NAA [25] & [26]. Findings of current study fully agreed with the results of Nazneen *et al.*, [27].

Proliferation of somatic embryos into plantlets

For further growth plantlets from cytokinin BAP transferred on ½ MS medium. As result complete plantlets are developed from seedlings within three weeks. Basal end of micro plantlets continues to grow embryogenic callus without effecting plantlet growth in flask which is interesting.

Regeneration media with 0.5mg BAP and 60 was used for growth of these callus masses and 60 plantlets were obtained. Similar findings again obtained i.e., development of embryo followed by micro plantlet and callus formation. It was fascinating that embryogenic callus have ability for more micro plantlets regenerations more than 10 subculture, therefore we obtained numerous plantlets and my these results are in agreements with Nazneen [27], as she also induced somatic embryogenesis in *Chrysanthemum morifolium* Ramat. by using the same combination of cytokinin and auxin concentration in 1/2XMS BM. plantlet further growth occurred on MS BM(Fig.7 E, F).Plantlet acclimatized successfully and got established in the soil (Fig.7- G).

IV. CONCLUSIONS

The results of the present studies indicate that using plant shoot tips or stem nodal segments of *Chrysanthemum morifolium* Ramat. for mass scale propagation by using MS, BM with BAP 2mg/L, Kn 2.0 mg/L for maximum shoots length while, Kn &I AA 0 .5mg/L for rooting on ½ MS for direct shoots and roots induction. The best treatment recorded for organogenic callus induction was M1-3 BM fortified with 1 mg/L BAP along .5 mg/L 2,4-D. Best treatment for shooting was observed with BM, BAP 1 mg/L +2,4-D 0.5mg/l. While best rooting observed with IAA 2mg/L+Kn 1.0 mg/L.

The best callusing for somatic embryogenesis was recorded on BM fortified with BAP 0.5 mg/L and NAA 0.1 mg/L followed by BAP 0.5 mg/L, NAA,0.5 mg/L concentration while, minimum response was observed at BAP, .1 and NAA 0.1% The concentration above BAP 1.0 mg/L with combination of NAA .5 mg/L responded the minimum callusing (50%) and only 25% explants responded for callusing.

Organogenesis and embryogenesis play a significant role in mass propagation *in vitro* for pathogens free plants adding commercial value to this ornamental plant. Current study findings show that more suitable method for micro propagation of *Chrysanthemum* is embryogenic callusing and one step seedling formation.

Plants develop from embryogenesis proved superb morphology of flower i.e., color and size and more no of plantlets raised through this practice excellently render them better for floriculture trade. This protocol can be used for commercial level propagation of *Chrysanthemum morifolium* Ramat. to earn foreign exchange and to meet the local demands

V. ABBREVIATIONS

2,4-D 2,4-dichlorophenoxyacetic acid,
GA3 gibberellic acid IAA indole-3-acetic acid IBA indole-3-butyric acid
Kn, Knetin, 6-Furfuyl amino purine
BAP, Benzyl aminopurine
MS Murashige and Skoog (1962) medium
NAA α-naphthaleneacetic acid
PGRs plant growth regulators
T, Treatment
½ MS Half Basal media
MS full medium
BM Basal medium
PGR, Plant growth regulators

ACKNOWLEDGEMENTS: The authors are highly grateful to the Department of Botany, University of Peshawar for providing lab facilities during this research.

AUTHORS DECLARATION: All authors read the final version of paper and agree to submit for publications. In addition, it is declared that the current work is original work of the author, and no part is submitted anywhere else for publications.

CONFLICT OF INTEREST: the authors declared that no conflict of interest exists.

Author Contributions: AG conducted the experimental work. MJ supervised and helped in overall work, MS Checked the technical aspects of the written work. NJ and NS assisted the writing and statistical analysis. AB, SHI, ZK assisted in Nursery care and Photo editing.

REFERENCES

[1]. Nasri F, Zakizadeh H, Vafaee Y and Mozafari AA (2018) Callus Induction and Plant Regeneration of Chrysanthemum morifolium and C. coccineum via Direct and Indirect Organogenesis and Genetic Fidelity Analysis Using IRAP, ISSR and SCoT Molecular Markers. J. Ornamen. Plants. 8(4): 265-284. http://jornamental.iaurasht.ac.ir/article_ 545325.html

[2]. Erler, R. and I. Siegmund, 1986. Year Book of the International Horticultural Statistics, USA, pp:44

[3]. Levin, R., V. Gaha, B. Tal, S. Hirsch, D. Denola and I. Vasil, 1988. Automated plant tissue culture for mass propagation. Biotechnol., 6: 1035-1040.

[4]. Zakaria, A.; F. Akhtar, M. M. Sahamsulhaq, H. Banu, M. M. Rehman and A.K. Farruqzaman2001. Novel propagation system. Online Journal of Biological Sciences 1(11): 1106-1111, 2001.

[5]. Lim KB, Kwon SJ, Lee SI, Hwang YJ and Naing AH (2012) Influence of genotype, explant source, and gelling agent on in vitro shoot regeneration of chrysanthemum. Horticult. Environ. Biotechnol. 53(4): 329-335. https://doi.org/10.1007/ s13580-012-0063-x

[6]. Naing AH, Jeon SM, Han JS, Lim SH, Lim KB and Kim CK (2014) Factors influencing in vitro shoot regeneration from leaf segments of Chrysanthemum. Comptes Rendus – Biologies. 337(6): 383–390. https://doi.org/10.1016/j.crvi.2014.03.005

[7]. Samala S, Kongton K, Saisaard K, Nupan B, Thongyai K and Taweerodjanakarn S (2017) Effects of plant growth regulators on multiple shoots regeneration of in vitro Chrysanthemum morifolium Ramat. Acta Hortic. 1167(1): 143-148. https://doi.org/10.17660/ActaHortic. 2017. 1167.22

[8]. Thorpe, T. A., I. S. Harry and P.P.K Umar, 1990.Application of micropropagation to forestory. In: Debergh P.C Zimmmerman, (edi), Micropropagation. Kluwer Acaemic Publ., Dordrechi, Netherlands.

[9]. Sauvaire, D. and R. Galgy, 1978. Multiplication of vegetative dela Cannal a sourse par bounturage *in vitro* CRACad D Sci. Se., 287: 446-470.

[10]. Battacharya, P., B. Dey, N. Das and B.C. Bhacttacharya, 1990 Rapid mass propagation of Chrysanthemum morifolium by callus derived from stem and leaf explants.Plant Cell Reports,9: 439-442.

[11]. Khan, M, and Ahmad, 1994. Micropropagation of horticultural crops. Pak.J. Agri. Res.15:1,202-206.

[12]. Ben-Jaacov, J. and R.W. Langhans, 1972. Rapid multiplications of *Chrysanthemum* plant by stem tip proliferation. Hort. Sci., 7: 289-290.

[13]. Earle, E.D. and W.R. Langhans, 1973. Propagation *of Chrysanthemum in vitro*.1. Multiple plantlets from shoot tips and yhe establishedment of tissue culture. J. Hort. Sci., 99: 128-132

[14]. Amin, M.N., M.A.K. Azad, F. Begum and M.N. Islam, 1997. Micropropagation of *Chrysanthemum morifolium* through axillary bud and leaf-derived callous culture. Plant Tissue Cult. Conf. (IPSA, Gazipur, Dhaka. Dec.10), pp: 5.

[15]. Merkle, S.A.1994. Hard wood propagation and gene transfer via somatic embryogenesis. In International Symposium on Applications of Biotechnology to Tree Culture, Protection and Utilization.St.Paul, Minnesota, pp.143-152.

[16]. Gray, D.J., and A.Purohit.1991. Somatic embryogenesis and the development of synthetic seed technology.Crit Rev Plant Sci.10:33-61.

[17]. Huang M, Ma X, Zhong Y, Hu Q, Fu M and Han Y (2018) Callus induction and plant regeneration of Spirodela polyrhiza. Plant Cell, Tissue and Organ Cult. 135(3): 445–453. https://doi.org/ 10.1007/s11240-018-1477-7.

[18]. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473-497. *OnLine J. Biol. Sci., 3 (6): 553-560, 2003* 560.

[19]. Ravishanker, G.A.T.Rajasekaran, K.S.Sharma and L.V.Venkatarman.1989. Production of pyrethrums in cultured tissue of Pyrethrum *(Chrysanthemum cinarifolium*). Pyrethrum-post17: 2,66-69.

[20]. Dikhshit, P, R.Kumar and V.N.Maurya.1997.Regeneration of *Chrysanthemum* through tissue culture. Recent Hort.4: 85-88. DOI:10.1055/s-0029-1234451

[21]. Haque,I.,J.Khan,M.Alam and M.S.Khattak.1998.*In vitro* culture of *Chrysathemum*. S.J. of Agric.14: 3,211-13.

[22]. Roest, S. and G.S.Bolkmann.1975. Vegetative Propagation of *Chrysanthemum morifolium* Ramat *in vitro*.Sci.Hort.3, 317-330.

[23]. Hutchinson, J.F., 1981. Tissue culture propagation of fruit trees. *In*: Proc. Symp. on Tissue culture of Economically Important Plants, A.N. Rao (Ed.)Singapore, pp: 113-120.

[24]. Altaf N., A. Tabassum, and M.S.Ahmad.1985. Plant regeneration by organogenesis and Somatic embryogenesis from tissue culture of *Citrus reticulata* Blanco.I.Ilahi: edi.Uni.Pesh.pp.1-7.

[25]. Sondhal, M.R., T.L.Salibury and W.R.Shap.1979a.Characterization of embryogenesis in *Coffea arabica* callus by scaning electron microscope. Z.Pflenzen Physiol 94.185-188

[26]. Sondhal, M.R., D.A.Spahlinger, W.R.Shap.1979b.Ahistorical study of high frequency induction of somatic embryogenesis in cultured leaf explants of *Coffea arabica* L.Z.Pflenzen Physiol 94,101-108.

[27]. Nazneen.S.2000. *In vitro* regeneration of Ornamentals through Tissue culture M.Phill Thesis University of Peshawar.

Authors
1st Author: Dr. Alia Gul Lecturer Department of Botany, Hazara University, Mansehra, 21300, Khyber Puhtunkhwa, Pakistan, aliagul@hu.edu.pk, aliagulbotanist@gmail.com, https://orcid.org/0000-0002-5493-5130?lang=en,
*Correspondence: aliagulbotanist@gmail.com
2nd Author: Dr. Mussarath Jabeen, Department of Botany, University of Peshawar, 25000, Pakistan
3rd Author: Nosheen Shafqath: Department of Agriculture, Hazara University, Mansehra, 21300, Khyber Puhtunkhwa, Pakistan, noshinshafqat@gmail.com
4th Author: Nadia Jabeen Department of Agriculture, Hazara University, Mansehra, 21300, Khyber Puhtunkhwa, Pakistan, ORCID id 0000-0001-6617-8301: nadiakhan909090@yahoo.com
5th Dr. Sumaira Salahuddin Lodhi, Department of Biochemistry, Hazara University, Mansehra, sumaira.bch@gmail.com
6th Author: Dr. Muhammad Sajid, Department of Agriculture, Hazara University, Mansehra, 21300, Khyber Puhtunkhwa, Pakistan, drsajid_1@yahoo.com
7th Author Dr. Fazal Hadi, Department of Botany, University of Peshawar, Pakistan. Email: hadibotany@uop.edu.pk
8th Author Asma, Department of Botany, Hazara University, Mansehra, 21300, Asma.faheem84@gmail.com
9th Author Zenab Kainat, MPhil Department of Biochemistry, Hazara University, Mansehra, 21300, zenabkainat@gmail.com
10th Author; Abdul Basit, Department of Botany, University of Peshawar, 25000 abdulbasitraza440@gmail.com
11th Sayed Hasnain Imad, BS Institute of Chemical Sciences, University of Peshawar, Pakistan, 25000 Email: sayyedhasnain661@gmail.com