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A simple cost effective method for mass propagation of *Chrysanthemum morifolium* and antibacterial activity assessment of in vitro raised plantlets

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ABSTRACT

An efficient low cost procedure for *in vitro* propagation of *Chrysanthemum morifolium* has been developed with subsequent assessment of antibacterial property of *in vitro* raised plantlets. Optimal axillary shoot multiplication was achieved on MS medium supplemented with low concentration of BAP. Psyillium husk and market sugar were standardized as suitable alternatives to the conventionally used agar and sucrose, cutting down the production cost of tissue culture raised plantlets to over six times. Optimal *in vitro* rooting was obtained on half strength MS medium containing IBA. Regenerated plantlets with well developed shoots and roots were acclimatized successfully and transferred to field conditions where they flowered. The leaves of *ex vitro* growing tissue culture raised plantlets were later assessed for activity against bacterial pathogens. The present protocol ensures minimal cost input in large scale production of a commercially important ornamental plant and opens up scope of scientific interventions directed at its allied therapeutic usage.

Abbreviations: MS: Murashige and Skoog (1962); **$HgCl_2$:** Mercuric chloride; **PGR:** Plant growth regulator, **TCR:** tissue culture raised; **BAP:** 6, Benzylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 butyric acid; **IAA**: Indole-3 acetic acid; **min:** minutes; *****:**significant at 99.9%

INTRODUCTION

Floriculture industry has emerged as one of the important and escalating commercial trades in agriculture. A big volume of cut flowers and potted plants are sold on daily basis across the world. In terms of production value Netherlands, US , Japan, Italy, Germany and Canada are the largest producers and exporters of cut flowers and plants while Germany, US, France and UK are the major consumers (Getu, 2009). In India, with over 161,00 ha land under flower cultivation (Starman *et al.*,1995), suitable climatic conditions and commercial floriculture displaying high potential per unit area than most field crops, floriculture is being perceived as a lucrative business. Chrysanthemum (commercially available as *Chrysanthemum morifolium* Ramat.) is a well known name in the global floriculture trade, just one position behind the top-ranked rose in world markets (Texeira da Silva, 2003). Exceptional foliage, attractive flowers and wide range of hues make *C. morifolium* a grower's muse while an added feature of long vase life (Ilahi, 2007) accounts for its high value as cut flower in floral arrangements. To add to it, *C. morifolium* is well recognized in traditional system of medicine for treatment of common cold, regulation of blood pressure, detoxification and improving eyesight (Lin and Harnly, 2010). In India the significance of chrysanthemum cultivation can be assessed from the fact that an average of 16.19 tons of chrysanthemums was reported to be produced in the year 2012-13 alone (www. apeda.gov.in). Since conventional propagation methods of suckers and terminal cuttings are unsuitable to support large scale production of chrysanthemums (Nhut *et al.*, 2005, Waseem *et al.*, 2009) tissue culture technology can be of much help to the growers as it promises season-independent mass production of the chosen variety. The technique has already been employed to study large scale propagation of *C. morifolium* through different regeneration pathways (Ben Jaacov and Langhans, 1972, Kaul *et al*., 1990, Lu *et al*., 1990, Fay, 1992, Khan *et al*., 1994, Ilahi *et al*., 2007, Hodson *et al*., 2008, Nalini, 2012, Karim, 2002, 2003, Shatnawi *et al*., 2010, Keresa, 2012).

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However, the technology is beset with major constraints of high production cost (minimum sale price for each chrysanthemum plant remains to be INR (Rs.) 30.00- 40.00) and problems in *ex vitro* survival of tissue culture raised (TCR) plantlets. Consequently, there is a need to develop a cost effective system for large scale production of chrysanthemum plants wherein the farmers can purchase the variety of their choice at minimal rates hence ensuring monetary gain to the growers. In addition, the potential of TCR plant parts can also be exploited to determine their medicinal significance, if any. The present study was taken up with an aim to develop a rapid and cost effective micropropagation protocol of *Chrysanthemum morifolium* and identify the antibacterial properties of tissue culture raised plantlets. The findings of the study were endeavored at having practical applications for researchers who can produce chrysanthemums of choice with minimal investments besides realizing the therapeutic potential of tissue raised plantlets and for the growers who can purchase healthy TCR plantlets at subsidized rates hence making overall chrysanthemum trade a commercially and scientifically beneficial venture.

MATERIAL AND METHODS

Micropropagation

Culture initiation

Mother plants of *Chrysanthemum morifolium* were procured from Choudhary nursery, Sahastradhara, Dehradun and grown in polyhouse of Department of Biotechnology, Graphic Era University. Nodal segments containing single axillary buds were used as the source material for micropropagation. Explants were washed with liquid detergent for 5 min (Cetrimide, Shalaks Pharmaceuticals Pvt. Ltd, India 3 - 4 drops / 100 ml) followed by washing under running tap water. Thereafter, the 2-3 cm nodal segments (with at least one node in each sector) were excised and subjected to surface sterilization with 0.1% (w/v) $HgCl_2$ (Himedia) for 4 minutes under laminar-air flow cabinet followed by thorough washing (four to five times) with autoclaved distilled water to wipe out the traces of steriliant.

Culture establishment and multiplication

For establishment of cultures, surface sterilized explants were inoculated on to full strength MS (Murashige and Skoog, 1962) basal medium containing BAP (0.0- 2.0 mg/l), sucrose (30g/l) and solidified with agar (7g/l w/v) (HiMedia) .

Single shoots were separated from established cultures and shifted to MS medium supplemented with varying concentrations and combinations of BAP (0.5 mg/l – 1.0 mg/l), IAA (0.25 mg/l – 0.5 mg/l) and NAA (0.25 mg/l - 0.5 mg/l) for *in vitro* shoot multiplication. Basal MS medium devoid of any PGR served as control. Most optimal PGR combination and concentration was used for setting up low cost experiments. In the next set of experiments solidifying agent agar was replaced with psyllium husk (commonly known as isabgol) and market sugar was used as a substitute for sucrose in the medium at a concentration range of 20-30 mg/l. Double distilled water for preparing culture medium was also replaced with RO water. MS medium fortified with 7 g/l (w/v) agar and 30g/l sucrose was used as control.

Data on average number of shoots formed and average shoot length were scored after 2 weeks and 4 weeks of culture. Routine subculturing was carried out at regular intervals of 2 weeks alternately on basal MS medium and MS medium containing 0.5 mg/l BAP.

In vitro rooting

Experiments on *in vitro* rooting were attempted with 2-3 cm long shoots having 1 or 2 nodes. The microshoots were excised and transferred to root induction medium comprising of 1/2 strength MS medium with psyllium husk or 0.6% agar and 20 g/l market sugar and fortified with different concentrations of IAA, IBA or NAA (0.25- 1.0 mg/l). Data on the number of roots per shoot and root length were collected after a period of 2 weeks and 4 weeks.

Medium and culture conditions

The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1N HCl prior to adding the solidifying agent and dispensed in conical flasks (250 ml, Borosil, India). The culture medium was autoclaved at 120 ^{0}C and 1.0 x 10^5 Pa for 15 min. All the chemicals used were of Himedia grade. Cultures in all experiments were incubated in culture room at 23 ± 2 ^{0}C and 60 - 65% relative humidity under a 16/8 hr (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 50 µmol m^{-2} s^{-1}.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to 1/4 strength MS medium devoid of carbon source and any PGR for 5 days in flasks. Thereafter, they were transferred to poly-bags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags (that were periodically removed) and kept under agronet-shade house conditions. Acclimatized plants were subsequently shifted to soil in pots/ garden soil and maintained for growth under *ex vitro* conditions.

Statistical Analysis

Data collected in the Completely Randomized Design (CRD) of experiments (Compton, 1994) was analyzed using Microsoft Excel ver. 2007 © Microsoft Technologies, USA. Experiments were repeated thrice and data represents the mean of three experiments. Each treatment consisted of minimum twelve replicates. Data was subjected to one way Analysis of Variance (ANOVA). Degree of variation was shown by Standard Error (SE), Critical Difference (CD) at 5%. The significance level was tested determined at 5% ($p <= 0.05$), 1% ($p <= 0.01$) and 0.1% ($p <= 0.001$). The significance of the data as ascertained by F-test and the CD values computed, were used for comparing differences in means of various treatments.

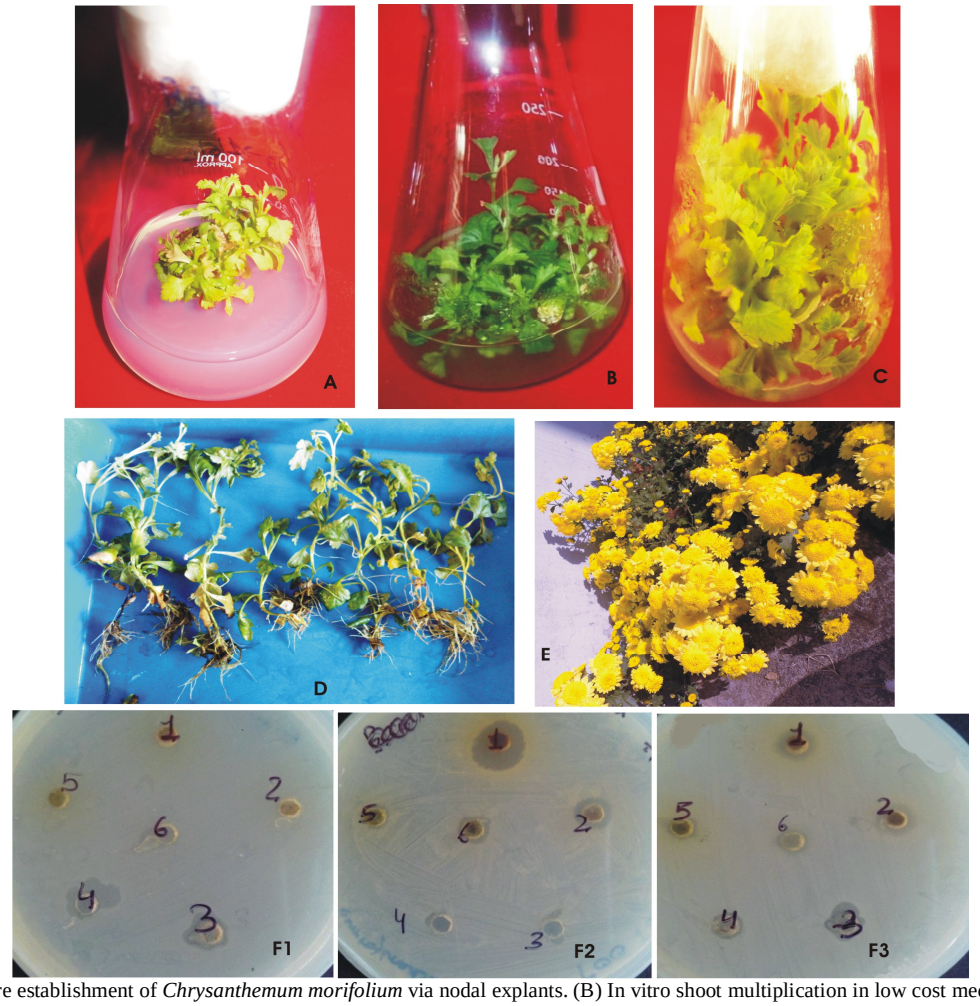


Fig. 1: (A) In vitro culture establishment of *Chrysanthemum morifolium* via nodal explants. (B) In vitro shoot multiplication in low cost medium (C) Maintenance of shoots via subculture (D) In vitro rooted plantles (E) flowering in TCR plantles (F1) Antibacterial activity of ethanolic extract against *Bacillus subtilis* (F2) Antibacterial activity of ethanolic extract against Staphylococcus aureus (F3) Antibacterial activity of ethanolic extract against *E. coli*.

Table 1: Effect of cytokinin on axillary bud induction in *C. morifolium.*

Treatment	MS + BAP (mg/l)	Average Shoot Number		Average Shoot Length (cm)	
		2 weeks	4 weeks	2 weeks	4 weeks
T1(Control)	0.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T2	0.5	1.53±0.07	2.53±0.18	2.50±0.05	4.58±0.04
T3	**1.0**	**4.73±0.07**	**6.53±0.07**	**3.65±0.04**	**5.95±0.03**
T4	1.5	2.27±0.07	3.73±0.07	2.37±0.03	3.81±0.04
T5	2.0	2.93±0.07	4.53±0.07	3.18±0.04	4.81±0.10
Significance		***	***	***	***
CD at 5%		**0.18**	**0.28**	**0.10**	**0.16**

Table 2: Effect of PGRs on *in vitro* shoot multiplication in *C. morifolium*

Treatment	MS + PGR (mg/l)			Average Shoot Number		Average Shoot Length (cm)	
	BAP	IAA	NAA	2 weeks	4 weeks	2 weeks	4 weeks
T1(Control)	0.00	0.00	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T2	0.50	0.00	0.00	10.73±0.07	17.27±0.24	6.44±0.08	8.67±0.09
T3	0.50	0.25	0.00	7.60±0.12	9.93±0.07	4.98±0.04	6.16±0.06
T4	0.50	0.00	0.25	4.67±0.18	6.40±0.12	4.85±0.04	5.74±0.06
T5	1.00	0.25	0.00	7.27±0.07	9.20±0.12	5.94±0.03	7.16±0.02
T6	1.00	0.00	0.25	6.73±0.07	9.73±0.27	5.65±0.03	7.09±0.01
T7	0.50	0.50	0.00	6.33±0.13	9.27±0.27	5.67±0.03	6.85±0.02
T8	0.50	0.00	0.50	6.13±0.07	8.60±0.12	5.91±0.01	6.98±0.02
T9	1.00	0.50	0.00	7.67±0.07	9.47±0.18	5.06±0.03	6.23±0.05
T10	1.00	0.00	0.50	4.53±0.07	6.27±0.13	4.87±0.04	5.79±0.04
	Significance			***	***	***	***
	CD at 5%			0.28	0.50	0.12	0.13

Antibacterial Assay

Sample Collection

Leaves of *in vitro* developed plantlets of *Chrysanthemum morifolium* were used to assess antibacterial activity. For the purpose eight months old TCR plants growing under *ex vitro* conditions were collected and dried under shade for 6-7 days followed by drying in hot-air oven for 4-6 hours at 50^0C to remove excess moisture.

Extraction of plant material

The dried leaves were softly crushed to prepare a powder and 40 g of powdered leaves were successively extracted with 200 ml of ethanol at 56-60^0C and ethyl acetate at 40-50^0C in Soxhlet extractor until the extract was clear. The extracted solvent was removed under reduced pressure with rotary vacuum evaporator. The sticky greenish-brown substance obtained was stored at 4°C in storage vials for experimental use (Chessbrough, 2000).

Culture of microorganisms

Gram positive (*Bacillus subtilis* (MTCC No. 121), *Staphylococcus aureus* (MTCC No. 6908)) and gram negative bacteria (*Escherichia coli* (MTCC No. 1698) and *Pseudomonas aeruginosa* (MTCC No. 4306)) were used for antibacterial assay. Pure cultures were maintained on nutrient agar slants and plates on regular basis. The cultures were streaked on sterile nutrient agar plates and kept in incubator for 24 hours at 37°C. Bacterial cultures were frequently subcultured to obtain pure cultures. Inoculum was prepared by growing the pure bacterial culture in nutrient broth over night at 37°C.

Determination of antibacterial susceptibility

Antibacterial activity was measured using disc diffusion test (Bayer *et al.*, 1966 with modification). 20ml of media was transferred aseptically into each sterile petri dish and allowed to solidify. 100µl of inoculum suspension was spread uniformly over the agar medium using sterile glass rod for uniform distribution of bacteria. The prepared sterile discs were loaded with different concentrations (50mg/ml and 100mg/ml) of plant extract. The paper discs were placed suitably apart on the medium and the culture plates were kept in an incubator at 37ºC. After 24 hours of incubation, the antibacterial activity was recorded by measuring the width of the clear inhibition zone around the disc in millimetre.

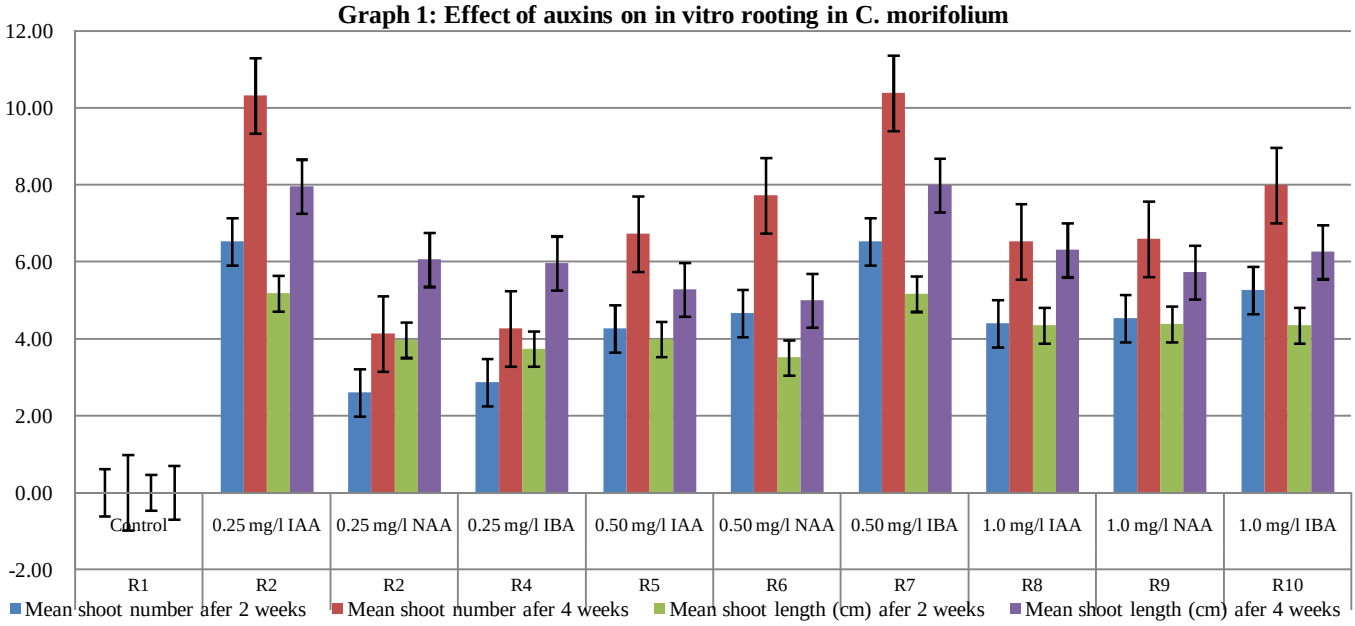
RESULTS

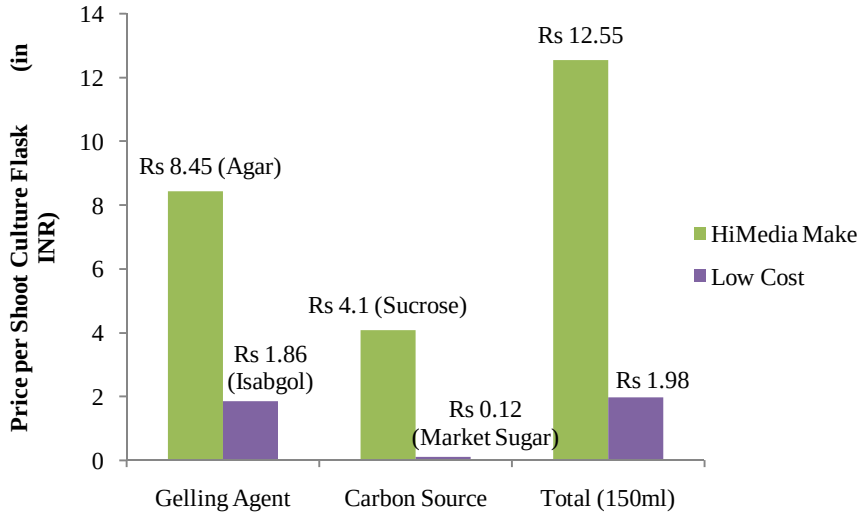
Initiation and multiplication of shoot cultures

In the present study, axillary bud break in nodal explants was observed within two weeks of culture in all media combinations tried except the control. BAP at a concentration of 1.0 mg/l proved to be most efficient in initiating bud induction giving an average of 4.73±0.07shoots per explant after 2weeks and 6.53±0.07 shoots after 4 weeks (Table 1). The regenerated shoots were transferred onto fresh medium of same composition to establish an initial stock of shoots (Figure 1A). This stock was further used for conducting experiments to assess the effect of various PGRs on shoot multiplication summarized in Table2. Significant differences were observed in the effectiveness of PGRs and their levels in MS medium. In control, the shoots showed necrosis within a week of transfer. For all the parameters studied, it was noted that treatment T2 proved to be most optimal giving a mean number of 10.73±0.07shoots (mean shoot length 6.44 cm) after 2 weeks and 17.27±0.24shoots (mean shoot length 8.67cm) after 4 weeks. Synergistic effect of cytokinin and auxin proved inefficient in inducing optimal shoot multiplication. Media combinations containing auxins (IAA/NAA) at a concentration of 0.5 mg/l along with cytokinin BAP, callus initiation was observed at the base of shoots towards third week of culture. With a concept of developing a system for cost effective large scale production of chrysanthemums, a media formulation comprising of full strength MS medium (prepared with RO water), supplemented with BAP(0.5 mg/l), psyllium husk (as solidifying agent) and market sugar (as carbon source) was standardized in our study (Table 3). In experiments conducted to determine the best concentration of psyllium husk and market sugar for shoot multiplication, MS medium fortified with 0.5 mg/l BAP (Hi Media, INR 700.00/5gm), 7.0 g/l agar (HiMedia @INR 4,024.00/500 gm) and 30 g/l sucrose (Himedia @INR 455.00/500gm) served as control in which the most optimal results were observed. However, of all other treatments tried, medium containing 25 g/l psyllium husk (Sidhpur Sat Isabgol @INR 310.00/500gm) and 20g/l market sugar (INR 455.00/500gm) proved to be the best (Figure1B) wherein a good number of healthy shoots developed. In this combination 10.53±0.07shoots with mean shoot length 6.36±0.07 cm and 16.87±0.18 shoots with mean shoot length 8.62±0.05 cm were obtained after 2 weeks and 4 weeks, respectively. It was observed that on increasing the concentration of psyllium husk in medium from 25 g/l to 30 g/l, shoot multiplication declined while a still higher concentration of 30 g/l resulted in extremely hard media making shoot inoculation difficult and damaged the shoots while being removed for subsequent subculture. Replacing sucrose with market sugar proved to be an economical choice without any negative affect on *in vitro* shoot development. Henceforth, T3 was standardized to be most favorable alternative to the high cost medium for shoot multiplication. For long term maintenance of cultures, *in vitro* multiplied shoots were periodically subcultured at an interval of 2 weeks. The subculture routine involved alternate subcultures on basal MS medium and MS medium containing 0.5 mg/l BAP. By this procedure, over 50 sub cultures have already been carried out without any declination in multiplication rate over a period of two years of maintenance of cultures (Figure1C). The modified protocol resulted in deduction in production cost by over 6 times. Figure 2 depicts a comparison in cost of a single flask of chrysanthemum culture containing over 100 root-able shoots. The input cost could also be reduced by using a Reverse Osmosis (RO) water assembly (approx INR 8,000.00/ unit) for water supply for media preparation instead of a double distillation unit (Borosil, @ 31,455.00/unit) as the use of RO water did not intervene with the growth characters *in vitro*.

Table 3: Effect of solidifying agent and carbon source on *in vitro* multiplication of regenerated shoots of *C. morifolium*.

Treatment	MS + BAP (0.5 mg/l)		Average Shoot Number		Average Shoot Length(cm)	
	Agar (g/l)	Sucrose(g/l)	2 weeks	4 weeks	2 weeks	4 weeks
T1	7	30	10.67±0.07	17.07±0.27	6.37±0.09	8.57±0.07
	Isabgol (g/l)	Sugar (g/l)				
T2	20	20	8.73±0.07	14.40±0.12	5.91±0.05	7.97±0.06
T3	25	20	10.53±0.07	16.87±0.18	6.36±0.07	8.62±0.05
T4	30	20	5.40±0.12	6.87±0.18	4.95±0.03	5.81±0.03
T5	20	25	8.20±0.12	12.80±0.31	5.67±0.04	7.81±0.02
T6	25	25	7.60±0.12	9.53±0.18	5.03±0.05	6.29±0.08
T7	30	25	7.67±0.07	9.47±0.18	5.07±0.02	6.31±0.09
T8	20	30	4.73±0.07	6.53±0.18	4.88±0.03	5.85±0.06
T9	25	30	4.67±0.18	6.27±0.07	4.85±0.03	5.81±0.02
T10	30	30	5.47±0.07	6.87±0.18	5.08±0.03	6.31±0.09
Significance			***	***	***	***
CD at 5%			**0.29**	**0.57**	**0.15**	**0.18**





Make	Components	Cost (in INR)
HiMedia	Agar	4,024.00 (per 500gm)
	Sucrose	455.00 (per 500gm)
Low cost	Isabgol	310.00 (per 500gm)
	Market sugar	20.00 (per 500gm)

Fig. 2: Comparative analysis of production cost in conventional tissue culture method versus low cost method (Total 150 ml depicts the amount of medium required for production of over 100 rootable size shoots)

In vitro rooting

In the present study, shoots cultured in auxin-free medium (control) failed to form roots while a varied effect of auxins (IAA, NAA or IBA) was observed by incorporating them in half-strength MS medium at different concentrations (Graph 1). Consistent rooting was noted in all the three auxins tried within 15 days of culture. Emergence of root primordia was observed within 10 days of culture following which rapid root development occurred. Overall, treatment R7 (0.50 mg/l IBA (Hi Media, INR 664.00/ 5gm)) was most optimal in all rooting parameters and significantly better than all the treatments tested. In this combination an average number of 6.53 roots per shoot after 2 weeks, 10.40 roots after 4 weeks and average root length 5.17 after 2 weeks and 8.00 after 4 weeks was observed. The roots so formed were long and healthy. NAA proved to be comparatively less efficient in inducing root formation while the roots developed on IAA supplemented medium were long but very thin.

The optimized PGR concentration was subsequently used in half strength MS medium supplemented with 0.6% agar and 20 g/l market sugar for *in vitro* root formation (Figure 1D). This medium resulted in formation of a healthy and viable root system. Replacing agar with psyllium husk in rooting medium proved to be detrimental for safe removal of rooted shoots from culture tubes. Also, psyllium husk stuck to the roots and could not be properly washed off. Contrastingly, lower concentrations of psyllium husk resulted in liquefied medium that caused shoot hyperhydration.

Hardening, acclimatization and field transfer

Plantlets developed via axillary bud culture were hardened *in-vitro* by placing them on liquid 1/4 MS medium without PGR and carbon source for 5 days (or more in case of climate constraints for shifting of plantlets to *ex vitro* conditions). These plantlets were subsequently transferred to a rooting mixture of soil: sand: manure (1:1:1) and maintained in net house for next 4-5 weeks where they exhibited enhanced growth. When transferred to soil in pots and garden soil, over 96% survival rate was obtained. These plantlets were maintained in areas with plenty of air circulation, water drainage and optimal sunlight. The plants were periodically checked for any kind of insect or fungal infection. The tissue culture raised plantlets exhibited flowering in the month of October (Figure 1E) proving the developed protocol to be a successful cost effective package.

Antibacterial study

The current research work ethanol and ethyl acetate extracts of *C. morifolium* leaves were prepared and antimicrobial activity was assayed against bacterial pathogens by disc diffusion method. As per data obtained (Table 4, 5), the ethanolic extract showed minimum degree of inhibition against *Bacillus subtilis* (Figure1: F1) but effective degree of inhibition against *Staphylococcus aureus* (Figure1: F2) and *E. coli* (Figure1: F3). The ethyl acetate extract showed minimum degree of inhibition against *Bacillus subtilis* (only at high concentration) and moderate degree of inhibition against *Staphylococcus aureus*. This indicated ethanol to be a better solvent for extracting antimicrobial substances from the leaves of *C. morifolium* than ethyl acetate. No zone was observed against *Pseudomonas aeruginosa* in either of the extracts.

Table 4: Antibacterial activity of ethanol extract of *in vitro* raised *C. morifolium* leaves

S. No	Microorganism	Zone of inhibition by Ethanol Extract (mm)	
		50 mg/ml	100 mg/ml
1.	*Bacillus subtilis*	4	5
2.	*Staphylococcus aureus*	6	9
3.	*Escherichia coli*	5	7
4.	*Pseudomonas aeruginosa*	NZ	NZ

NZ- No Zone

DISCUSSION

With an aim to integrate scientific know-hows with societal benefits, the present study was taken up to develop a protocol for low cost micropropagation of a commercially significant ornamental plant-*Chrysanthemum morifolium* and also assess the antibacterial property of its leaves. The aim was to deliver a cost effective package of plant production that will result in maximal production with minimal expenditure and also assess the probable role of otherwise unused plant parts in therapeutics.

Culture of nodal segments carrying axillary buds involves the exploitation of buds already present on the parent stock plant, hence providing an efficient means of rapid clonal propagation allowing production of genetically stable and true-to-parent type progeny (Hu and Wang, 1983). In the present study, best results of axillary bud induction from nodal explants were obtained on MS medium supplemented with 1.0 mg/l BAP. The results are in corroboration with previous findings on micropropagation of *C. morifolium* wherein efficacy of BAP for axillary shoot induction was established (Khan *et al.,* 1994, Haq *et al.,* 1998, Karim *et al.,* 2002, 2003). This is in support of the fact that plant tissues metabolize BAP more readily than other synthetic growth regulators and has also been known to induce production of natural hormones as zeatin within the tissue (Malik *et al.,* 2005). Contrastingly, BAP in combination with IAA (Waseem *et al.,* 2011) and BAP with GA_3 (Keresa *et al.,* 2012) have also been reported to give maximal shoot induction in *C. morifolium.*

In vitro shoot multiplication is the major criterion for successful commercial micropropagation. In our study, BAP at a concentration of 0.5 mg/l gave most optimal results of shoot multiplication while a combination of cytokinin and auxin proved to be comparatively less efficient for the same. Superiority of BAP in axillary shoot multiplication has also been reported in *C. cinerariaefolium* (Lindiro *et al.,* 2013). These observations on efficacy of low concentrations of BAP alone in inducing shoot proliferation/ multiplication and calli formation in presence of auxins reflect high levels of endogenous hormones in the mother plants. In the present study, use of psyllium husk and market sugar was standardized as suitable substitutes to agar and sucrose in culture medium with an aim to lacerate the overall production cost

of TCR plantlets. Psyllium husk is a mucilaginous and sticky material that can be used as a gelling agent in culture media (Bhattacharya and Bhattacharya, 1994, Babbar *et al.,* 1998, Tyagi *et al.,* 2007, Atici *et al.,* 2008). In our study, psyllium husk at a concentration of 2% proved to be the best while higher concentrations resulted in decline in shoot multiplication which could be due to ineffective diffusion of media nutrients at high concentration of solidifying agent. Our results are in consonance with earlier studies reporting positive effect of psyllium husk on *in vitro* shoot induction, multiplication and long-term survival of cultures (Fischer, 2004, Yusuf *et al.,* 2011).

Market sugar contains 96-97% sucrose and 0.7-1% reducing sugar while sucrose is made up of 99.98% sucrose and 0.01% reducing sugar (Sahu and Sahu, 2013). Considering this similarity, market sugar was tried as an alternative to sucrose which turned out to be a beneficial choice. In our study, 2% market sugar with 2% psyllium husk gave the best results of shoot multiplication. A higher sucrose concentration hampered shoot development which could be attributed to destabilization of osmosis in plant cells at elevated sugar levels. Role of sugar in *in vitro* propagation have also been confirmed in some other plants (Kaur *et al.,* 2005, Goel *et al.,* 2007, Agarwal *et al.,* 2010).

Increased levels of BAP in the medium have been reported to increase the number of shoots but suppress their growth (Arora, 1990). Henceforth, *in vitro* multiplied shoots were maintained by periodic subculturing alternately at regular intervals of 2 weeks each on basal MS media in alternation with MS media with 0.5 mg/l BAP. The process resulted in development of healthy shoots without any basal callusing or decline in multiplication rate, a condition often observed in case of excess PGR provided to the cultured tissue.

The system so developed resulted in slashing down major production cost of tissue culture plantlets by over six times the expense in conventional micropropagation protocols.

Present study on *in vitro* rooting suggested efficacy of IBA supplemented half strength MS medium. Reduced macroutrient concentration is known to be favorable for *in vitro* rooting considering the fact that nitrogen ions concentration requirement for root formation is much lower than of shoot formation (Driver and Suttle, 1987). Half strength medium for rooting in *C. morifolium* has already been reported (Kaul *et al.,* 1990, Waseem *et al.,* 2009). In our study, IBA at a concentration of 0.25 mg/l proved to be most optimal for *in vitro* rooting. The results are in agreement with previous studies on chrysanthemum reporting superiority of IBA over other auxins (Khan *et al*., 1994, Singh and Arora, 1995, Hoque and Fatima, 1995, Karim *et al*., 2002, Waseem *et al*., 2009, Waseem *et al*., 2011, Nalini, 2012, Lindiro *et al*., 2013). Contrastingly, basal MS medium has also been reported to be efficient for *in vitro* rooting in *C. morofolium* (Vantu *et al.,* 2005).

Plants growing under tissue culture conditions are semi-autotrophic (Hazarika, 2003) and leaves may be photosynthetically incompetent. Besides, micropropagated plants are also generally susceptible to transplantation shock due to delicate root system, reduced amount of epicuticular wax and reduced or abnormal stomata. This can result in excessive dehydration, poor control of gaseous exchange and also poor absorption of nutrients by the root system. In the present study, a decrease in medium strength (one-fourth) for *in vitro* hardening was done to create favourable stress conditions to enable the TCR plantlets perform well under field conditions. The plantlets so developed when raised under *ex vitro* conditions showed healthy growth and blossomed in flowering season.

The present research work also dealt with the evaluation of two different extracts of *in vitro* grown leaves of *C. morifolium* for antibacterial susceptibility. Previous reports on phytochemical screening of *C. morifolium* suggested presence of flavonoids, sesquiterpenoids, chlorogenic acids, vitamins, and amino acids (Zhang and Zhang 2007, Zhao *et al.,* 2009) and subsequently led to exploitation of plant parts to assess their antimicrobial property. However, most of the research on the antimicrobial assay of *C. morifolium* has been focussed on the flower extracts. Such studies indicated enhanced antimicrobial activity of yellow flowers as compared to purple and white flowers (Urzua and Mendoza, 2003, Voon *et al.,* 2011). Methanolic extract of *C. morifolium* inflorescence has also been reported to show antimicrobial activity against *B. cereus, L. monocytogenes, E. coli* and *Salmonella anatum* (Shan *et al.,*2007) while petroleum ether and ethyl acetate extracts were found to be effiicient against *S. aureus* and methicillin resistant *S. aureus* (MRSA) (Zhao *et al.,*2009).

Ethanolic leaf extract of *Chrysanthemum indicum* have been reported to exhibit antimicrobial activity against *K. pneumonia, E. coli, S. mutans, P. aeruginosa* and *B. subtilis* (Rajalakshmi *et al.,* 2013). Besides, other studies have also reported efficiency of ethanolic extracts of *Chrysanthemum* sps (Sassi *et al*., 2008, Pitinidhipat and Yasurin, 2012). These reports support of our findings wherein superority of antibacterial activity of ethanolic extracts of *C. morifolium* leaves against Gram negative (*E. coli*) and Gram positive (*B. subtilis* and *S. aureus*) bacteria was established. The results are suggestive of broad spectrum antibiotic compounds in the leaves of tissue culture raised *C. morifolium* that need to be isolated and identified for scientific confirmation of the findings.

CONCLUSION

The findings of this research overcome the major hurdles in application of tissue culture technology to the field i.e. high production cost and failure to survive under *ex vitro* conditions. The protocol is a low cost strategy for mass production of a commercial flower variety *C. morifolium* that ensured successful flowering in TCR plantlets after shifting to field conditions besides confirming the antibacterial properties in field grown plants. The study is therefore a complete package for large scale production of chrysanthemum plantlets at subsidized cost that can be easily and equally adopted by both farmers and researchers. The findings also open up scope for appraisal of chrysanthemum leaves as a potential component in medicinal preparations.

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