LOW COST MEDIA FOR *IN VITRO* REGENERATION OF *Gynura procumbens* (Lour.) Merr.

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CERTIFICATE

This is to certify that the thesis entitled **"LOW COST MEDIA FOR *IN VITRO* REGENERATION OF *Gynura procumbens* (Lour.) Merr."** submitted to the Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in HORTICULTURE**, embodies the result of a piece of *bona fide* research work carried out by **MD. RASAL-MONIR,** Registration No. **17-08244** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dated: June, 2018
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The greatest gift from Allah I ever had

“I had come to the world through my Parents”

DEDECATED TO -

My Beloved Parents for instilling in me a sense of discipline and hard work. Particular dedication goes to my Honorable Supervisor **Prof. Dr. Humayun Kabir** sir for his proper guidline and inspiration to me.

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ABSTRACT

The present study was undertaken at ‘‘Horticultural Biotechnology and Stress Management Lab’’, Sher-e-Bangla Agricultural University, Dhaka.The single factor experiment was laid out in Complete Randomized Design with three replications. Nodal segment was used as explant and six types of different low cost medium (LCM) such as LCM-1 (100 ml coconut water); LCM-2 (150 ml coconut water); LCM-3 (200 ml coconut water); LCM-4 (100 ml banana pulp) ; LCM-5 (150 ml banana pulp); LCM-6 (200 ml banana pulp) and full strength of MS medium used as a control with three replication for standardization a low cost *in vitro* regeneration protocol for *Gynura procumbens*. All culture medium was supplemented with 2.0 mg/l BA + 0.5 mg/l NAA for shooting and 0.5 mg/l IBA was used for rooting. Culture media LCM-2 showed best performances in shoot initiation, shoot multiplication and root formation compared to other low cost culture medium. In terms of cost analysis, LCM-2 helps to reduce cost (78.22%) of the medium per plantlet as compare to the conventional MS medium. Acclimatization medium consisting garden soil + Vermicompost + cocodust (1:1:1, v/v) exhibited the best hardening medium with maximum survival rate (96.45%) and vigorous performances. Taken together, we can conclud that LCM-2 culture medium is a cost effective protocol and alternative to conventional MS medium for regeneration of *Gynura procumbens* aseptically.

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ABBREVIATIONS AND ACRONYMS

%	…	Percentage
µM	…	MicroMolar
^{0}C	…	Degree Celsius
100X	…	100 times
1N	…	Normal
2,4-D	…	Dichlorophneoxy acetic acid
ANOVA	….	Analysis of variances
AR	…	Agar
BA	…	Benzyl Adenin
BAP	…	6-Benzyl Amino Purin
cm	…	Cemtimeter
cm^2	...	Square centimeter
CRD	…	Completely Randomized Design
Cv	...	Cultivar
DDW	…	Double distill water
df	…	Degree of freedom
DMRT	…	Duncans's Multiple Range test
EDTA	…	Ethylene Diamine Tetra Acetate
et al.	...	and others
g	…	Gram
g/l	…	Gram per liter
GA_3	…	Gibberellic Acid
hr	…	Hour
i.e	…	In other words
IAA	…	Indole Acetic Acid
IBA	…	Indole Butyric Acid
J	…	Journal
kg	…	Kilogram
Kg	…	Kilogram
KIN	…	Kinetin
l	…	Litre
LCM	…	Low cost media
m	…	Minute
mg	...	Milligram
mg/l	…	Miligram per liter

ABBREVIATIONS AND ACRONYMS

ml	…	Milliliter
mm	…	Milimeter
MS	…	Murashige Skoog
NAA	…	Naphthalene Acetic Acid
PGRs	…	Plant growth regulators
pH	…	Hyrogen ion concentration
Psi	…	Pounds per Square Inch
SAU	…	Sher-e-Bangla Agricultural University
Sig.	…	Significance
Sp	…	Species
SPSS	…	Statistical Package for the Social Sciences
Tk	…	Taka
ug	…	Microgram
USDA	…	U.S. Department of Agriculture
UV	…	Ultra Violet

CHAPTER I
INTRODUCTION

CHAPTER I

INTRODUCTION

Gynura procumbens (Lour.) Merr. is an important medicinal plant, belongs to the family Asteraceae. The plant is considered to originate from Malaysia, Indonesia and Thailand. In Malaysia, this species has its distribution limited to the western part of peninsular Malaysia. This is not a native plant of Bangladesh. It is commonly known as 'Sambung nyawa' by the Malays that means "continuation of life" and "Bai bing ca" by the Chinese in Malaysia. This plant is also known as different names in different parts of the world. In Bangladesh it is commonly known as diabetics plants. Its habit is a scrambling or weakly climbing herb with stem up10 to 25 cm tall. This tropical herbaceous medicinal plant, is highly branched with hairy green leaves that are alternately arranged on hairy light purple stem. It produces purple, tubular and bisexual flowers (Wiart, 2002).

Gynura procumbens potentially serves as an antihypertensive agent with Cardio protective activity due to its ability to target various mechanisms including the renin-angiotenism system and Calcium influx which are crucial players in the pathophysiology of hypertensive conditions (Hui-li Tan *et al*, 2016). It is commonly used for Diabetes treatment in traditional medicine for its hypoglycemic effects (Hamid, 2004). It has an effect on sexual and reproductive function. It significantly increase sperm count, sperm motility and reduced the percentage of sperm mortality (Sani *et al.,* 2008). *Gynura procumbens* enhance testicular lactate dehydrogenase activity (Hakim *et al.*2008). It has an anti-cancer activity mechanically inhabits the initiation phase of carcinogenesis. This activities help to prevent cancer formations (Hamid, 2009). *Gynura procumbens* has antioxidant properties probably to its high phenolic content. Root extract showed the highest antioxidant activity than other parts (Rosidah *et al.,* 2008). It was used

as a treatment of infections by pathogens such as herpes, virus and malaria parasites (Nasir *et al.,* 2015). *Gynura procumbens* has a protective effect against damage of body tissue due to its organoprotective agent which exert a regulatory effect at the level of gene expression. *G. procumbens* have been used as ethnoherbal products to threat various ailments, urinary infection and used as anti-inflammatory and anti-allergic agents (Jiratchariyakul *et al*., 2000).

G. procumbens was used for preparations of traditional Chinese medicine intended for the treatment of various ailments including uterine cancer (Liao, 2015), cervical spondylosis (Shi, 2015), and chronic skin ulcer (Yang *et al.*, 2015). Besides, it has also been used as an ingredient in special diets for patients with medical conditions such as heart (Chen *et al.*, 2013b) and liver disease (Chen *et al.*, 2013a). In the food industry, it has been incorporated into products such as tea (Hu, 2014; Liao *et al.*, 2015), coffee powder (Park, 2015), chocolate (Jang, 2014), candy (Xie, 2007c), and chewing gum (Xie, 2010). The applications of *G. procumbens* in personal care and cosmetic products have also been reported which including hand-washing solution (Xie, 2009), hand sanitizer (Xie, 2007a), oral spray (Xie, 2007b), facial masks (Yuan and She, 2014), and skin care creams (Xie, 2007d).

It is usually propagated though cutting which gives low quality seedling with low multiplication rate. This method cannot meet the increasing demand of this plant used as the raw material for the preparation products .Tissue Culture technology can be used to produce high quality seedlings instead of the traditionally used cuttings. But high production cost is an impediment to tissue culture adoption which has further limited the technology. Cost effective micro propagation would facilitate commercialization of the technology. A number of researchers influenced to find alternatives materials to substitute alternatives to gelling agents, 90% cost reduced using household sucrose Zapata *et al.,* (2001), and some medium components objectively to reduce cost in media culture preparation. At

Low cost media have been developed for the multiplication of orchids (Hossain, 1995; Pervin, 1997) by using coconut water (15%) and banana pulp (100 g/l), which effectively lowered the cost. Zapata *et al*., (2001) have tried household sugar and tap water to substitution laboratory sucrose and double distilled water used in plant tissue culture. Hossain, (1995) reported that commercial grade sugar can replace analytical grade sucrose, with no significant change in the frequency of shoot formation in banana. The cost of commercial micro propagation has to be reduced drastically without compromising on the quality of micro propagules especially in the developing countries (Kuria *et al.,* 2007). But such low cost protocol has not been developed for herbaceous plant in Bangladesh till date. Present investigation is an innovative approaches to develop a low-cost protocol for micro propagation of *Gynura* using Banana pulp, coconut water, using table sugar alternative to sucrose. The following objectives are uder taken in this experiment.

- To develop a low cost media for in vitro regeneration of *Gynura procumbens.*
- To determine the cost efficiency of regenerating *Gynura procumbens* using the alternative low cost tissue culture strategy.
- To elaborate a new micro propagation protocol for *Gynura procumbens*
- To evaluate regeneration efficiency of *Gynura procumbens* on low cost media compared to the conventional Tissue Culture media.

CHAPTER II
REVIEW AND LITERATURE

CHAPTER II

REVIEW AND LITERATURE

The present experiment was conducted to develop a low cost strategy for in vitro mass multiplications of *Gynura procumbens*. Some informative works have so far been done in home and abroad related to this research have been presented in this chapter. The relevant literature about these aspects have been reviewed and presented below.

2.1 Conventional and low cost tissue culture technology

Plant tissue culture refers to growing and multiplication of cells, tissues and organ on defined nutrient media under aseptice and controlled environmental conditions. the technology is used for the production of haploids, cryopreservations, propagating new plant varities, conserving rare and endangered plants, difficult to propagate plants, productions of secondary metabolites and transgenic plants (Ahloowalia *et al*., 2002). The production of high quality planting material of crop plants and fruits trees, propagated from vegetagive parts, has created new opportunities in global trade, benefited growers , farmers , nursery oweners and improved rural employment. Biotecnology offers the possibility of expanding and optimizing the use of crops by menas of genetic engineering . Howerver , in order to develop new transgenic crops, robust transformation and regeneration methods must be place (Monica *et al.*,2009). Conventional tissue culture Media contains a basal salt with major and minor mineral elements, a source of carbon (sucrose), vitamins, growth regulator, a gelling agent for semi-solid media and water. Tissue culture conditions need to be optimized for obtaining vigorous shoot growth

coupled with modifications in the nutrient medium to reduce the cost (Sood and Chauhan 2009).

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. The commercial technology was primarily based on micro propagation, in which rapid proliferation is achieved from in system cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors (Asmita *et al.*, 2017).

Low-cost tissue culture technology should adopt practices, use alternative equipment and chemicals that reduce the unit cost of micropropagule production. There are reports on tissue culture of other plant species such as banana, vetiver grass and irish potato where the conventional sources of some components of the nutrient medium have been replaced with low cost substitutes such as sucrose with table sugar (Kaur *et al.*, 2005), omission of agar (Mehrotra *et a*l., 2007) and use of sunlight and tubular skylight instead of light from fluorescent tubes (Kodym and Francisco, 2004). Cassava flour has been used as a substitute for agar (Maliro and Lameck, 2004). It has been reported that the combination of starch, semolina and potato powder or combination of starch and agar can be a low cost option for shoot induction in African violet (Sharifi et al., 2010). At Dhaka University, low cost media have been developed for the multiplication of orchids (Hossain, 1995; Pervin, 1997) based on macro-salts of any of the Vacin and Went, Phytamax and Knudson‘s C media, supplemented with 10-15 % (w/v) banana extract and 10-15 % (v/v) coconut water. Use of above media eliminated the need of micro-salts and vitamins for the micropropagation of different orchid species. Several different orchids, namely, Vanda, Dendrobium, Aerides, Acampe and Spathoglottis can be multiplied on a medium containing only peptone, inositol,banana extract and coconut water (Pervin, 1997). In many tropical orchid species, similar response

was observed on media containing coconut water (15%) and banana pulp (100 g/l), which effectively lowerd the cost.

Daud *et al*., (2011) experimented with Explants from stem segments of *Celosia sp*. were cultured on medium containing either half strength of Murashige and Skoog (MS) or without MS combined with 100 g/l of each gelling agents and 70 ml/l coconut water. The findings showed that the use of young coconut water and alternative gelling agents have response to in vitro shoots regeneration of stem segments of *Celosia sp*. Higher revenue (12.33 ± 7.27) of shoots derived from stem segments results from the cultured media containing100 g/l of potato starch, 70 ml/l coconut water, household sugar and half strength of MS (½ MS).

Ogero *et al.,* (2011) conducted an experiment with an aim of producing affordable disease-free seedlings, a low cost medium was developed and used to regenerate two sweet potato varieties (KEMB 36 and Tainurey). The conventional sources of MS nutrients were substituted with locally available fertilisers. Thirty gm per litre table sugar was used as a source of carbon. MS medium supplemented with $30gl^{-1}$ table sugar and $3gl^{-1}$ of gelrite was used as the control. The number of leaves, nodes, roots and plant height for the two cultivars were determined and compared. The low cost medium was significantly ($P<0.05$) cheaper compared to the MS medium costing 94.4% less per litre. KEMB 36 had a regeneration index of 7 nodes per plantlet on the low cost medium, while Tainurey had 3 nodes per plantlet.

Venkatasalam *et al*., (2013) noted that the composition of culture medium used for shoot regeneration has a great influence on cost and there is a potential for use of locally available low cost resources as alternatives to the conventional costly laboratory resources. In the first experiment MS medium was prepared with nine types of water viz., rain, natural, tap, aquaguard, single distilled, double distilled, Type-I (Reverse osmosis), Type-II (Electronically de-ionized) and ultra-pure

water, supplemented with sucrose at 30 g/l and solidified with agar (AR) at 7 g/l In the second experiment MS medium was prepared with double distilled water, supplemented with seven types of carbon sources viz., commercial sugar, commercial sugar (sulphur less), sucrose, fructose, dextrose, sugar cubes and galactose at 30 g/l and solidified with agar (AR) at 7 g/l. In the third experiment MS medium was prepared with double distilled water, supplemented with sucrose at 30 g/l and solidified with four types of solidifying agent's viz., agar (PT), agar (bacteriological), agar (purified) and gelrite. The quantity of solidifying agent used was at 7 gl-1except gelrite (2 g/l).

Kuria *et al.,* (2007) was investigated the potential of cassava starch as an alternative and cheap gelling agent for potato in vitro culture micro-propagation media was investigated. A two-factor experiment in randomized complete block design was conducted. Four levels of gelling agents; 10% (w/v) cassava starch, 8% cassava starch mixed with 0.25% agar and 0.8% agar and a liquid medium, were evaluated using three selected Kenyan potato cultivars (Tigoni, Asante and Kenya Sifa). Potato transplants from the liquid medium and cassava starch gelled medium had similar ($p > 0.05$) mean number of nodes and biomass. These mean values were significantly higher compared to the transplants from the agar gelled medium. The use of 10% cassava starch reduced cost by 42.5% in comparison with use of agar.

Ogero *et al.,* (2012) reported a low cost Medium (LCM) whereby the conventional sources of four Murashige and Skoog (MS) macronutrients were replaced with locally available fertilizers was developed. Stanes Iodized Microfood® from Osho Chemical Industries in Nairobi was used as the alternative source of micronutrients. Modified conventional MS medium was used as the control. Both media were supplemented with 30 g/l of table sugar and 3 g/l of gelrite. Two cassava varieties, Muchericheri and KME 1 were regenerated on the two media.

Node, leaf and root formation patterns plus plant height were determined and compared. A reduction of 95.50% in nutrient cost was achieved.

An experiment was performed with cassava varieties by Ogero *et al.,* (2012) and concluded that a low cost protocol for cassava tissue culture was developed and used to regenerate two farmer preferred cassava varieties, KME 1 and Muchericheri. Easygro® vegetative fertilizer, a locally available foliar feed was used as an alternative source for conventional MS salts. The variety Muchericheri had a significantly higher regeneration index compared to KME 1 having produced a mean of 6.8 nodes on the low cost medium and 5.6 nodes on the conventional medium compared to KME 1 which had a mean of 5.6 nodes on the low cost medium and 4.5 nodes on the conventional medium.

Ngetich *et al.,* (2015) and found significant differences ($p<0.05$) in the shoot generation for Eddoe and wild varieties in LCM1 and LCM2 respectively compared to LCM3 and MS. Plants grown in MS media and LCM3 had the longest height compared to LCM1 and LCM2. Naphthalene Acetic Acid (NAA) and Citishooter did not show any significant differences on the number of roots. All the regenerated plants in this study were similar in morphology and vigour. Media cost was reduced by 94.7% (LCM1) and 96% for both LCM2 and LCM3.

Sharma *et al*., (204) reported that there was 85% reduction in cost of media for plant caulogenesis by using inexpensive carbon source, water source and gelling agent. Laboratory reagent grade sucrose was replaced by locally available commercial sugar (market sugar and sugar cubes) as carbon source, bacteriological grade agar by isabgol (Plantago ovata), sodium alginate, starch as gelling agent and distilled water, tap water and packaged drinking water as a water source. Based upon cost analysis the use of isabgoal as a gelling agent, use of sugar cubes and packaged drinking water for preparation of media can be used to boost the caulogenesis in Stevia rebaudiana.

Luisa *et al.,* (2013) focused on formulating culture media using low-cost materials as substitute to chemical components, such as Murashige and Skoog (MS) with the addition of 5ppm Benzyl Amino Purine (BAP) 2% sucrose and 4.5g Biolife agar. The cost of one shoot/meriplant was P0.02 using the developed media, compared to the P0.20 using the chemical formulation. Likewise, the costs of rooting one plantlet were P0.64 and P0.26 for the existing and for the developed media, respectively.

A low-cost medium for the in vitro propagation of strawberry (Fragaria ananassa cv. Chandler) from vegetative buds was developed by Kaur *et al.* (2005). The maximum shoot multiplication was obtained on MS medium supplemented with kinetin at 0.5 mg/L, BAP at 1.0 mg/l and GA3 at 2.0 mg/l with table sugar instead of sucrose. Maximum rooting was obtained on Vi MS medium supplemented with IB A at 1.0 mg/ l, charcoal at 200 mg/ l and table sugar at 20.0 g/l. Plants grown through this cost-effective protocol showed good field adaptability.

Ullah *et al.*, (2015) conducted an experiment and concluded that Protocorms were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of starches viz. isubgol (15, 20, 25 and 30g/l), cassava (15, 20, 25 and 30g/l), corn (50 and 60g/1) and potato (50 and 60g/1) used as alternative gelling agents as compared with 8g/1 agar at different stages at 35, 70 and 105 days. The MS medium supplemented with the increasing dose of isubgol, cassava, corn and potato gelled with 2g/l agar progressively increased their growth performance. The best performance on growth of shoots leaves and roots were recorded at 30g/l isubgol when isubgol used as an alternative gelling agent on agar. The use of 30g/l isubgol (500g costs US$ 10 only) was very much cheaper than conventionally used agar.

Saraswathi *et al*., (2016) conducted an experiment with three banana varieties, ‘Rasthali’ (AAB–Silk), ‘Grand Naine’ (AAA–Cavendish), and ‘Udhayam’ (ABB–

Pisang Awak). Reverse osmosis water and 3% (w/v) table sugar were used as the low-cost water and carbon source, respectively. Six different gelling agent treatments were tested: sago alone (T_1), Isabgol alone (T_2), sago + agar (T_3), Isabgol + agar (T_4), sago + Isabgol (T_5), and agar alone as a control (T_6). Statistical analysis indicated that sago + Isabgol (T_7) produced the maximum number of shoots (10 per explant) in 'Udhayam' and 'Rasthali', while sago alone (T_1) produced the maximum number of shoots (6 per explant) in 'Grand Naine'.

Pant *and Jain* (2015) conducted an experiment and obtained 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.

Demo *et al.,* (2008) conducted an experiment with the three selected Kenyan potato cultivars: Tigoni, Asante and Kenya Sifa which were cultured on full strength Murashige and Skoog (MS) medium at 3% (w/v) in combination with the 3 different sugars. Plantlet survival of 100% was recorded after four subculture generations on all sugars for all the cultivars. Results also showed that table sugar not only enhanced micro-propagation but also significantly lowered the production input costs by 34 to 51% when compared with the analytical grade sucrose.

Sharma *et al.,* (2015) conducted an experiment with Aloe vera Surface sterilized shoot buds cultured on control (MS + 30g sucrose + 8g agar-agar) and low cost medium (MS + 30g table sugar + 100g tapioca pearls) supplemented with 2.0 mg/l, 0.5 mg/l and 0.2 mg/l NAA showed 81.66% and 79.33% establishment respectively. Both control as well as low cost medium was found at par with each other having 81.99% and 81.80% shoot regeneration respectively. 77.67% rooting

and 4.33 average roots per shoot were found on 1/4 MS + 30g sucrose + 8g agar-agar and 78.00% rooting and 3.67 average roots per shoot on 1/4 MS + 30g table sugar + 100g tapioca pearls.

Every crop has its specific nutrient requirements hence as a cost reduction strategy designed to produce affordable planting materials must guarantee high quality and well developed plants that can easily be adapted to green house and field conditions.

2.2 Tissue Culture of *Gynura procumbens*

2.2.1 Establishment of *Gynura procumbens* plantlet

The success of plant tissue culture depend on multiplications and establishment of plantlets which in turn are greatly influenced by number of factors.

2.2.1.1 Explant:

The type of explant, its size, positions, age, physiological state and the manner in which it is cultured, whether the culture could be successfully maintained and the morphogenesis could be induced for in vitro multiplication.

Chan *et al.,* (2009) used nodal segments of about 1.0 cm in length which derived from the one year old mature plants planted at the University Sains Malaysia campus, Penang, Malaysia.

Alizah and Nurulaishah (2014) successfully used nodal segments which was cut into 0.5-1.0 cm and used as explants for the induction of multiple shoots.

Farhana *et al., (*2014) was used shoot tip and nodal segment as explant from one year mature plant, for shoot proliferation, between two explants, shoot tips showed the best response 90%.

Sweety *et al.*, (2016) reported that the vigorous apical shoot buds or young shoot cutting of *Gynura procumbens* were collected from a mature plant growing in the Medicinal Plants Garden of the Bangladesh Forest Research Institute at Chittagong.

Shen (2017) taking leaf blade of *Gynura procumbens* (Lour.) Merr as explants material, the factors that influence the callus induction and bud differentiation of *Gynura procumbens*.

Mustafa and Muhammad (2017), used nodal and shoot tip explants of *Gynura procambens* were collected from a medicinal plant garden in Rajshahi University campus, Bangladesh. Nodal explants showed the best shoot proliferation efficiency irrespective of media type, which followed by shoot tip explants (Fig. 1). Nodal explants showed the maximum 98.21 ± 1.01% response and produced 6.22±0.51 shoots and average length of shoots 4.51±0.24 cm.

Ayu *et al.*, (2018), stem node were used as an explant which was originated from apical shoot. Stem node had a morphogenetic response to different growth regulator.

2.2.1.2 Plant Growth Regulator

Chan *et al*., (2009) reported that the nodal segments of one year old mature plants was used as the explants for the initiation of axillary branching using Murashige and Skoog (1962) medium (MS) supplemeted with 2 mg/l BA. Rapid proliferation of shoots was achieved by culturing the in vitro shoots derived from the nodal segments onto MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l NAA. An average of 18.2 ± 0.6 shoots were produced from each shoot explant. All the micro-shoots produced normal roots within two weeks of culture on the basic MS medium without any plant growth regulators

Alizah and Nurulaishah (2014) reported that the MS medium with 3 mg/l BAP gave the highest number of shoots per explant. All the micro-shoots produced normal roots within two weeks of culture on basic MS medium without any plant growth regulators.

Farhana *et al.,* (2014) reported that 1.0 mg/l BAP produced shoots in each explants supplemented with MS media. In vitro derived shoots were subcultured on the similar medium and it gave similar production with healthy shoot. 100% rooting was observed on full strengthen MS medium containing NAA 0.5mg/l.

Sweety *et al.,* (2016), highest rate (100%) of shoot induction was observed on MS medium supplemented with 1.5 mg/l 6-benzylaminopurine (BAP) and 1.0 mg/l α-naphtalene acetic Acid (NAA) from nodal explants. The highest elongation of shoot buds (8.36 cm) was obtained on MS basal medium fortified with 1.0mg/l 6-benzylaminopurine (BAP) and 0.25 mg/l α-naphtalene acetic acid (NAA).The maximum number of roots (6.91±0.50) and root length (7.60cm) was observed in half strength MS medium supplemented with 0.5 mg/l indole-3-butyric acid (IBA).

Shen (2017), the results showed that the initial medium for the callus induction was MS+2,4-D 1 mg/l; the best medium for adventitious was MS+6-BA 1 mg/l + NAA 0.4mg/l.The best subculture media for shoot proliferation was 1/2 MS + NAA 0.2 mg/l + IBA 0.3mg/l.

Mustafa and Muhammad (2017), *in vitro* response of the explants to multiple shoot regeneration varied greatly with the position of the explanting branch on the donor plant. Highest frequency 98.21% of shoot formation, maximum number 6.2 of shoots per explant and average 4.5 cm length of shoot were obtained on MS medium supplemented with 4.0 µM 6-Benzylaminopurine (BAP). Shoot multiplication and growth were significantly affected by level of concentration of sucrose. Optimum sucrose concentration was also estimated for in vitro shoot regeneration from nodal explant in MS medium with different concentrations of

sucrose. The highest rate (97.31%) of shoot production was achieved in a shootregenerating medium with 30 gm/l sucrose. For rooting, the in vitro proliferated and elongated shoots were excised into 2–4 cm long microcuttings, which were planted individually on a root-induction MS medium containing 4.0 µM Indole-3-butyric acid (IBA). Within 4 weeks of transfer to the rooting medium, all the cultured microcuttings produced 2–6 roots.

2.2.1.3 Hardening media

Alizah and Nurulaishah (2014), rooted plantlets were removed from medium, washed thoroughly and placed in a mixture of sterilized vermiculite and sterilized soil (1:1), before acclimatized in greenhouse. The plantlets was transplanted into bigger earthen pot with 85% survival.

Farhana *et al.*, (2014) reported that after hardening, the plant were transferred to larger pot filled with soil containing organic manure for further growth. About 100% of plantlets survival was observed after hardening of the regarded gynura procumbens.

Sweety *et al.*, (2016), reported that for acclimatization, well rooted plantlets were gently removed from the test tubes and thoroughly washed with running tap water to remove traces of medium and transferred to plastic pots having soil and compost (1:1). The pots were kept under semi controlled temperature 30±2°C and light (3000 lux) in a growth chamber with 80-85% humidity. Then the plants were transferred to earthen pots filled with soil containing organic manure for further growth. Through this process of acclimatization, regenerated plantlets were established under ex vitro conditions. About 97% of plantlets survival under ex vitro environment was observed after hardening of *G. procumbent*.

Mustafa and Muhammad (2017), after successful rooting of microshoots, attempts were taken to establish regenerated plantlets onto soil. Plantlets had been

transferred to small plastic pots containing soil mix (garden soil: compost: sand, 2:1:1) and maintained under humid ex vitro condition in the growth room. The *in vitro* derived plantlets acclimated better under ex vitro condition when they were maintained in growth room for 20 days before transferring them to outdoor condition. Finally, 95% transplanted plantlets were survived and acclimated well under ex vitro condition after 25 days of transplantation.

2.2 Surface Sterilization

Alizah and Nurulaishah (2014), nodal segments were collected from the grown plants and washed with detergent and rinsed under running tap water. They were surface sterilized with 95% (v/v) ethanol for 30 s and further surfaced sterilized with 20% Clorox® for 15 min, and rinsed three times with sterile distilled water. They were again surface sterilized second time with 5% Clorox® and rinsed again with sterile distilled water.

Farhana et al., (2014), the explant was washed thoroughly in the running tap water for 30min , followed by treatment with a solution of tween-80 for 10min and then after washed three times with sterile distilled water . Then explant were washed with 70% ethanol, 0.1% $HgCl_2$ for 6min and rinsed with sterile distilled water for four times. The shoot tips and nodal segment were trimmed at both end (1-1.5cm) prior to the inoculation on culture media.

Sweety et al., (2016), after removing the leaves, the shoot tips and nodal segments with dormant axillary buds, were washed thoroughly under running tap water for 30 minutes, followed by treated with liquid detergent (Tween 20) for 10 minutes and dipping in 5% (v/v) savlon solution for 10 minutes. The materials were then washed 6-7 times with distilled water. After rinsing with 70% ethanol for less than 60 seconds, they were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 minutes and washed with sterile double distilled water 4-5 times after each surface disinfection

treatment under aseptic conditions. The surface sterilized explants were cut into small pieces (0.5-1.0cm) with a sterilized surgical blade and then inoculated onto the culture media.

Mustafa and Muhammad (2017), explants were washed thoroughly under running tap water for 15 minutes and then washed with continuous agitation in a few drops Savlon containing water for 15 minutes. The washed explants were then treated with 0.1% HgCl2 for 10minutes under laminar air flow cabinet to disinfect them. Finally, explants were washed 3 to 5 times with sterile distilled water and were placed in culture tubes (25 × 150 mm) containing 4.0 µM BAP supplemented MS (Murashige and Skoog, 1962) medium prepared with 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Co. USA). The pH of the medium was adjusted to 5.7 ± 1 before autoclaving at 121°C for 20 minutes at 1.2 kg/cm^2 pressure.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHOD

3.1 Time and Location of the Experiment

The study was carried out at Horticultural Biotechnology and Stress Management Lab, Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka-1207 during March, 2018 to February, 2018 to develop a low cost media for *in vitro* mass multiplication of *Gynura procumbens*.

3.2 Experimental materials

3.2.1 Sources of materials

The explant was collected from Gerplasm center of Sher-e-Bangla Agricultural University.

3.2.2 Types of Materials

Nodal segment (Plate 1) was used as an explant of about 1.0 cm in length which derived from the one year old mature plants.

3.3 Sterilant and surfactant

- $HgCl_2$ (0.1%)
- Savlon (3% w/v) marketed by ACI Pharma in Bangladesh
- and Tween-20 were used as surfactant cum detergent.

3.4 Gelling and solidifying agents

Agar 0.8% was used for solidifying of media and it was the product of merk india.

3.5 Carbon source

Sucrose and table sugar at 3% was used for media preparation**.**

3.6 Basal nutrient salts

Micronutrients:	**Macronutrients:**
❖ KI ❖ H_3BO_3 ❖ $MnS0_4.H_2O$ ❖ $ZnSO_4.7H_2O$ ❖ $NaMoO_4.2H_2O$ ❖ $CuSO_4.5\ H_2O$ ❖ $CoCl_2.6H_2O$ ❖ $FeSO_4.7H_2O$ ❖ And Na_2 EDTA.$2H_2O$	❖ $NH_4\ NO_3$ ❖ KNO_3 ❖ $CaCl_2.2H_2O$ ❖ $MgSO_4.H_2O$ ❖ KH_2PO_4 ❖ and $NaH_2\ P0_4$ **Vitamins:** ❖ Myo- Inositol ❖ Nicotinicacid ❖ Pyridoxin-HQ ❖ Thiamine-HCl

3.7 Other essential chemicals and reagents

- ❖ NaOH
- ❖ HCl
- ❖ Buffer Solutions
- ❖ Ethanol

3.8 Laboratory equipment

3.8.1 Major equipment (Plate 3 & 4)

- ❖ Distilled water plant
- ❖ Autoclave machine
- ❖ Air purifier
- ❖ Analitical Balance

- Electric balance up to 0.00lg precision
- Hot plate with magnetic stirrer
- pH meter
- Hot air oven
- Micro oven
- Refrigerator
- Thermometer
- Laminar air flow cabinet etc

3.8.2 Small instruments

For basic works different types of small instruments were used in laboratory, such as needles, forceps, spatula, knives, scissor, scalpel blades, spirit lamp, fire box, gloves, micropore, micropipettes, pipette holder, stirring rod, measuring tape, drying rake, trolley, marker glass marker etc.

3.8.3 Glassware and plastic wares

Only heat tolerant glass wares and plastic wares was used in tissue culture experiment. Different types of glass wares and plastic wares were also used for media preparation and for aseptic transformation of explants into prepared culture media. For example test tube, beaker, culture bottle or vial, conical flask, volumetric flask, measuring cylinder, petridishes, grauated pipette, micropipettes, wash bottle, basket screw-cap bottle, plastic racks and brushes etc.

3.9 Distilled water

Distillation is most effective in removing heavy metals, nitrates and minerals, and the boiling process kills the vast majority of microorganisms. Distilled water was autoclaved with 15 psi at 121°C for 30 minutes and then it was treated as double distilled water. It was used as an important composition of media and double

distilled water was used for preparation of PGRs solutions, buffer solutions, $HgCl_2$, NaOH and other some work.

3.10 Growth Chamber

The culture was stored in a growth chamber to enhance micro shoot and micro root formation. The growth room maintained at 16 hour photoperiod of 3000 Lux light provided by cool white florescent and controlled temperature 25±1°C maintained with air cooler (Plate 3).

3.11 Culture Media

For carried out this reasrch we used MS medium (Murashinge and skoog, 1962) with out any plant growth regulator as a control and Different low cost media.

3.11.1 Preparation of stock solutions

The preparation of stock solutions was the first step in the preparation of culture medium. Various constitutions of the respective nutrient medium were prepared in to stock solutions for ready use during the preparation of the media for different experiments. As different constitutions were required in different concentrations, stock solutions for macronutrients, micronutrients, organic compounds (vitamins and amino acids) etc. were prepared separately as mentioned below:

3.11.1.1 Preparation of stock solution-I (Macronutrient)

Stock solution of macronutrients was prepared with 10 times that of the required concentration. Required amount of all the macro-salt components prescribed a particular medium formulation was weighed accurately with electronic balance and dissolved separately in substantial volume of double distilled water (DDW). The solution was sequentially poured in to a 1liter volumetric flask. Final volume

of the solution was made up to 1L by adding sufficient amount of DDW. Special care was taken during dissolving calcium chloride ($CaCl_2$). The solution was filtered through Whatman No.1 filter paper to remove all solids contaminations like dust, cotton etc. and was poured into a clean glass bottle. The bottle was then labeled properly and stored in refrigerator at 4°C temperature for use.

3.11.1.2 Preparation of stock solution-II (Micronutrient)

It was made 100 times the final strength of the medium in 1 L of distilled water as described earlier for the stock solution-I. Although 100 ml stock solutions of $CuSO_4.5H_2O$ and $COCI_2.6H_2O$ was prepared separately and then the appropriate volume was added into the main stock solution. It was then filtered, labeled and stored in a refrigerator at 4°C for further use.

3.11.1.3 Preparation of stock solution-Ill (Iron-EDTA)

It was made 100 times of the final strength of the medium in one liter distilled water. Two constituents, Na_2EDTA and $FeS0_4.7H_20$, were dissolved in 750 ml of distilled water in a conical flask. After the complete dilution the volume was made up to 1litre by further addition of distilled water. This stock was also filtered and stored in refrigerator at 4°C for further use.

3.11.1.4 Preparation of stock solution-IV (Vitamins and amino acids)

All of the desired ingredients except myo-inosital was taken at 100 times (100X) of their final strength in a measuring cylinder and dissolved in 600 ml of distilled water. Then the final volume was made up to 1000ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4°C for later use. But the myo-inosital were made separately 100 times (100X) of

the final strength of the medium in 1000ml of distilled water. This stock solution was also filtered and stored in a refrigerator at 4°C for further use.

Table 1. Composition of conventional MS media

Stock I	**Major Salts(10X)**	mg/l	g/l
	KNO_3	1900	19
	NH_4NO_3	1650	16.5
	$MaSO_4.7H_2O$	370	3.7
	$CaCl_2.7H_2O$	440	4.40
	KH2PO4	170	1.70
Stock II	**Micro salts (100X)**	mg/l	g/L
	KI	83	.83
	H BO3	620	6.20
	MnSO4.4H2O	2230	22.3
	ZnSO4.7H20	860	8.6
	NA2MOO4.2H2O	25	0.25
	CUSO4.5H2O	2.5	0.025
	CACL2	2.5	0.025
Stock III	**Iron EDTA Sol^n(100X)**	mg/l	g/l
	FeSO4.7H2O	27.8	2.78
	Na2EDTA.2H2O	37.3	3.73
Stock IV	**Organics (100x)**	mg/l	g/L
	Myo- Inositol	1000	100
	Nicotinicacid	50	0.5
	Pyridoxin-HQ	50	0.5
	Thiamine-HCl.	10	0.1
	Glycine	200	20

Note: FeS04.7H20 and $Na_2EDTA.2H_20$ were dissolved separately. Heat was applied whenever necessary. The two solutions were combined and made to the final volume.

3.11.2 Preparation of Hormonal Stock solution

Two Hormone was used for regerneration of gynura procumbens

- BA for Shoot regernation
- IBA for rooting

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below

Hormone (Solute)	Solvents used
BA	1 N NaOH
IBA	70% ethanol
NAA	70% ethanol

Separate Stock of hormone was prepared. In present experiment, the stock solution of hormones was prepared by the following procedure. 100 mg of powder was placed in a small beaker and then dissolved with few drops of 70% ethyl alcohol or 1 (N) NaOH solvent. Finally, the volume was made upto 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4±1 ^{0}C for use up to two month. Growth regulators were purchased from Sigma, USA

3.12 Preparation of MS media

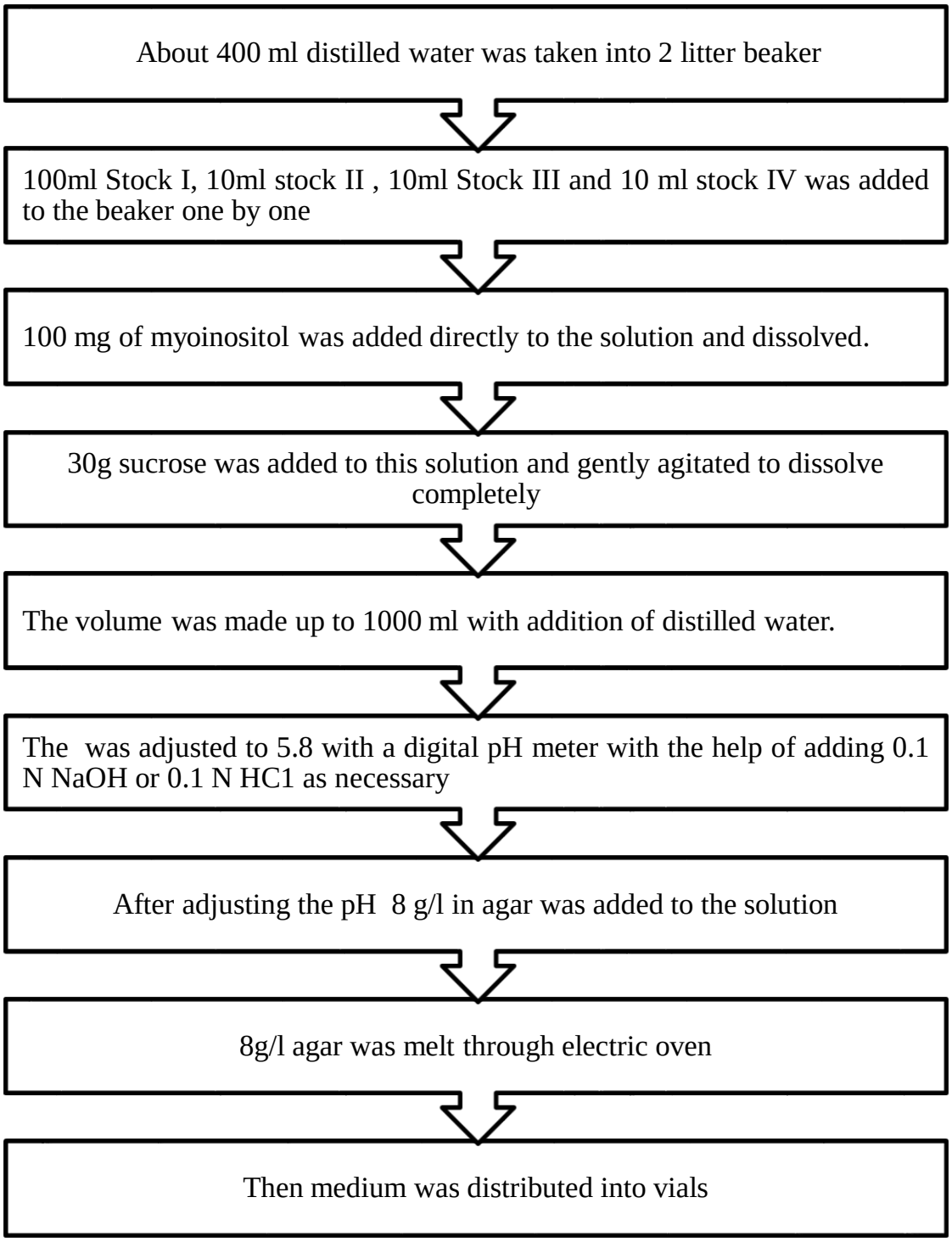


Fig 1. Flow diagram of MS media preparation

3.13 Low cost media

We were used different low cost media (LCM) for regeneration of *Gynura procumbens*.

3.13.1 Composition of coconut water

Tan *et al.,* (2014) reported that coconut water was the liquid endosperm found in the cavity of the nut. By the third month of fruit development, there are small quantities of coconut water. This amount increases and reaches the maximum when the nut is 7-9 months old. This is also when the coconut water tastes the sweetest, and is classified as young coconut water.

The coconut's composition is dependent on several factors such as age, varieties, growing seasons (monthly or yearly variability's), geographical locations and environmental conditions, including rainfall and temperature.

Coconut water harvested from nuts between 10-13 months old is classified as mature coconut water. After the nuts ripen, the amount of coconut water declines. This is because during maturation, coconut water is used to form coconut flesh inside the fruit, a phenomenon across all of the coconut's varieties.

Coconut water comprises of 95% water, with trace amounts of carbohydrates, proteins, oils, vitamins and minerals.

Physicochemical properties of coconut water

1 Titratable acidity as malic acid percentage

2 Total phenolics content, expressed as mg GAE/l

Table 2. Physicochemical properties coconut maturity stage

Physicochemical properties	Coconut maturity stage (month)		
	5-6	8-9	>12
Volume of water (ml)	684	518	332
Total soluble solids (°Brix)	5.60	6.15	4.85
pH	4.78	5.34	5.71
Titratable acidity1 (%)	0.089	0.076	0.061
Turbidity	0.031	0.337	4.051
Sugar content			
Fructose (mg/ml)	39.04	32.52	21.48
Glucose (mg/ml)	35.43	29.96	19.06
Sucrose (mg/ml)	0.85	6.36	14.37
Mineral			
Potassium (mg/100ml)	220.94	274.32	351.10
Sodium (mg/100ml)	7.61	5.60	36.51
Magnesium (mg/100ml)	22.03	20.87	31.65
Calcium (mg/100ml)	8.75	15.19	23.98
Iron (mg/l)	0.294	0.308	0.322
Protein (mg/ml)	0.041	0.042	0.217
Total phenolic compound(mg/l)	54.00	24.59	25.70
Potassium (mg/100mL)	220.94	274.32	351.10

Source: Tan *et al.*, 2014

Coconut water contains a small amount of proteins. The total protein content of coconut water increases as the coconut matures (Table 2). The amino acid composition of coconut water can be found in Table 3.

Table 3. Amino acid composition of coconut water

Amino acid	% of total protein
Alanine	2.41
Arginine	10.75
Aspartic acid	3.6
Cysteine	0.97-1.17
Glutamic acid	9.76-14.5
Histidine	1.95-2.05
Leucine	1.95-4.18
Lysine	1.95-4.57
Proline	1.21-4.12
Alanine	2.41
Arginine	10.75
Phenylalanine	1.23
Serine	0.59-0.91
Tyrosine	2.83-3.00

Source: Rethinam P., 2006.

The two major enzymes found (Table 3) in coconut water are polyphenol oxidase (PPO) and peroxidase (POD). Both contributes to the colouration of coconut water to pink or brown when the reaction between polyphenols and oxygen is catalyzed.

Table 4. Enzyme activity of coconut at different maturity stages before thermal treatments.

Enzyme activity (U $ml^{-1}Brix^{-1}$ min^{-1})	Coconut maturity stage (month)		
	5-6	8-9	>12
Peroxidase (POD)	0.052	0.117	0..129
Polyphenol oxidase (PPO)	0.543	0.160	0.056

Source: Tan *et al.*, 2014.

Vitamins are organic substances occurring in very small concentrations. It consists of complex chemical compositions, and is essential to normal life processes. However, vitamins cannot be synthesized by the body.

Table 5. Coconut water vitamin content

Vitamins	Amount
Vitamin B_1 (Thiamin)	0.030 mg
Vitamin B_2 (Riboflavin)	0.057 mg
Vitamin B_3 (Niacin)	0.080 mg
Vitamin B_5 (Pantothenic acid)	0.043 mg
Vitamin B_6 (Pyridoxine)	0.032 mg
Vitamin B_9 (Folates)	3 µg
Vitamin C (Ascorbic acid)	2.4 mg

Source: USDA National Nutrient database, (2018).

Table 6. Water quality parameters

Attributes	**Amount**
Iron, Fe	Max 0.2 mg/l
Manganese, Mn	Max 0.03-0.1 mg/l
Nitrate, NO_3	Max 30 mg/l
Nitrite, NO_2	Max 0.02 mg/l
Sulphate, SO_4	Max 100 mg/l
Chloride, Cl_2	Max 50 mg/l
Aggressive carbon acid, CO_2	Max 2 mg/l
Total count of bacteria	Max 100 CFU/ml
Ammonium, NH_4	Traces
Ammonia, NH_3	Max 0.5 mg/l
Phosphate, PO_4	Max 0.2 mg/l
Magnesium, Mg	Max 50 mg/l
Calcium, Ca	Max 100 mg/l
Sodium, Na	Max 200 mg/l
Chemical oxygen demand (COD), $KMnO_4$	Max 20 mg/l
Copper, Cu	Max 0.05 mg/l
Zinc, Zn	Max 1.0 mg/l
Ammonium, NH_4	Traces
Ammonia, NH_3	Max 0.5 mg/l
Ammonia, NH_3	Max 0.5 mg/l
Phosphate, PO_4	Max 0.2 mg/l
Magnesium, Mg	Max 50 mg/l
Calcium, Ca	Max 100 mg/l

Sources : Coconut Hand Book, 2014.

3.13.2 Coconut water extraction

The coconut water was extracted in sterile chamber from green fruits, filtered using a 0.45µm sterile-mesh and stored without autoclaving to avoid degradation of the organic compounds by heat.

3.13.3 Nutrive value of banana Pulp

Bananas are unique fruit with unique properties. It is fantastic fruit with sweet taste that most people enjoy. Bananas are your best sources of potassium, an essential mineral for maintaining normal blood pressure and heart function. A medium-sized banana provides 350 mg of potassium

Table 6. Nutrive content of Banana Pulp for 1 litre

Attributes	Amount
Water	80.94%
Ash	.079g
Charbohardate	16.72g
Protein	1.48g
Fat	0.07g
Total sugar	12.12g
Calcium, Ca	5.00 mg
Copper, Cu	0.078 mg
Fluoride, F	2.2 mcg
Iron, Fe	0.26 mg
Magnesium, Mg	27.00 mg
Manganese, Mn	0.270 mg
Phosphorus, P	22.00 mg
Vitamin B12	0.00 mcg
Vitamin B6	0.367 mg
Vitamin C	8.7 mg
Vitamin D	0.00 IU
Vitamin E	0.10 mg
Vitamin B12	0.00 mcg
Vitamin B6	0.367 mg

Source : Lia Hapsari and Dewi Ayu Lestari (2016).

3.13.4 Banana Pulp Extraction

The ripe banana was peeled and cut into 1.0 cm in size and 500 g of the sliced fresh material was boiled for 30 minutes with 500 ml of distilled water and the hot supernatant was filtered through steel mesh and adjusted up to 500 ml by adding distilled water (Plate 2).

3.13.5 Low Cost Media Preparation

To prepare 1000 ml Low cost media the following steps were followed:

a) 500 ml of sterile distilled water was poured into 2Litre beaker.
b) Different Concentration of Banana Pupl (100ml,150ml, 200ml) or Coconut water (10%, 15%, 20%) was added to the beakber.
c) 30g of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.
d) Different concentrations of hormonal supplements were added to the solution and mixed well.
e) The volume was made up to 1000 ml with addition of sterile distilled water.
f) The pH was adjusted at 5.8.
g) Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.
h) After melting the medium was distributed into vials.

3.14 Sterilization

In vitro Micro propagation, aseptic condition is prerequisite. So all instrument, glass ware and media were sterilized.

3.14.1 Sterilization of Media (Autoclaving)

For sterilization the culture vial containing medium was autoclaved at a temperature of 121 o C for 30 minutes at 1.06 kg/cm^2 (15psi) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explant.

3.14.2 Sterilization of glass ware and instrument

Glassware and other instruments like culture vessels, pipettes, beakers, petridishes, glassware, plastic caps, forceps, spatulas, needles, scalpels scissors were sterilized in an autoclave at a temperature of 121 ^{0}C for 30minutes at 15 psi pressure. Prior to sterilization, metal instruments were needed to be wrapped using aluminum foil.

3.14.3 Sterilization of Growth chamber and transfer area

The culture room was initially cleaned by gently washing of floors and walls with detergent along with savlon. This was followed by careful wiping those with 70% ethyl alcohol. Glass cleaner was also used for cleaning purpose. The sterilization was repeated at regular intervals. The growth room was sterilized twice a month by 70% ethyl alcohol. The transfer area was also sterilized twice a month by 70% ethyl alcohol, and UV light was on for 30 minutes before starting the transfer work. An air purifier was used for aseptic condition, air purifier was used 6h/day during research.

3.15 Culture Establishment

3.15.1 Surface Sterilization of explant

- Explant was washed thoroughly with running tap water for 30 minutes.
- The explants were then surface sterilized with 5-10 ml of Savlon
- 2-3 drops of Tween-20 was used for 8-10 minutes with gently shaking.

- The explants were then washed with 4 to 5 times using distilled water for removal of sterilizers
- Taken under laminar air flow cabinet. Under laminar air flow cabinet, the explants were than rinsed with 1% $HgCl_2$ solution for several times.
- At last the explants were than washed with double distilled water with gently shaking for the removal of $HgCl_2$ solution.

3.15.2 Inoculation

During inoculation, special care was taken that the explants must touch the medium equally and do not dip into the medium. For inoculation, the nodal segments were cut into 1-2 cm long pieces and placed on their proximal end on the medium surface. Explants were cut out carefully with the help of scapled by holding with the forceps.The excised explants were then inoculated in the culture bottles containing medium using forceps for shooting.

3.15.3 Incubation

The culture vials was transferred to growth chamber in controlled environment. The temperature of the growth chamber room was maintained within $25 \pm 2\ ^0C$ by an air conditioner and 16hour photoperiod was maintained along with light intensity of proper growth and development of culture**.**

3.15.4 Subculture

Micro Shoot formed in the vials were taken out after after four weeks of inoculation. The shoots were dissecting them in the sterile environment of laminar air flow cabinet with sterile dissecting needle and forceps. They were again placed in the vails containg fresh media.Subcultures were carried out regularly at an interval of 15 days.

3.15.5 Root formation on regenerated shoot

Newly formed shoot with adequate length were excised individually from the culture vial and transferred to rooting media. The growth regulator IBA was used in (0.5mg/l) for rooting. The observations on development pattern of root were made throughout the entire culture period.

3.15.6 Hardening

Young rooted plant were taken out of the test tube, washed with distilled water and planted in poly bag containing acclimatization media. Plants were watered once in two days initially in a day after eight to ten days. Then they were transferred to greenhouse after two weeks for further acclimatization.

3.15.6.1 Hardening Media

3.15.6.1.1 Soil

The garden soil was used as control for standardization harden media of *Gynura procumbens*.

3.15.6.1.2 Coco peat

Coco peat is coir fibre pith that having coconut husk as its base. It is a soil conditioner and growing medium. Its uniquenss is that it can hold 8 times of water of its weight. It relase nurteints in solution over long intervals . It was best for commercial and home gardening application . it provides breathing space , *i.e* letting out of air for roots which help better growth . Added to above mentioned characters, it encourage favorable micro organisms around the root zone. Since it is having slow degradation level it was universally accepted. It will re-wet easily without the use of chemical wetting as it was hydrophilic by nature. It can easily be mixed with growing media.

3.15.6.1.3 Vermicompost

Vermicompost was the product or process of composting using various worms, usually red wigglers, white worms and others earthworms to create a heterogeneous mixture of decompositing vegetable or food waste, bedding materials and vermicast. Vermicompost , aslo called worm castings, worm humus or worm manure , is the end product of the breakdown of organic matter by an earthworm. Containing water solubale nutrients, vermicompsot is an excellent , nutrient rich in many nutrients than compost produced by other composting methods. It has also outperformed a commercial plant medium with nutrients added. It was rich in microbial life which converts nutrients already present in the soil into plant avialbale forms . Unlike other compost, worm casting also contain worm mucus which helps prevent nutrients from washing away with the first watering and holds moisture better plains soil.

3.16 Details of experiment

3.16.1 Experiment 1: Effect of different Media on shoot multiplication

Design: Completely Randomized Design (CRD)

Number of factor: Single Factor

Treatment: 7

Replication: 3

3.16.1.1 Treatment

1. **Conventional MS media** (control)
2. **LCM-1 :** 100ml/ l Coconut water
3. **LCM-2 :** 150ml/ l Coconut water

4. **LCM-3 :** 200ml/ l Coconut water
5. **LCM-4:** 100ml/ l Banana Pulp
6. **LCM-5 :** 150ml/ l Banana Pulp
7. **LCM-6:** 200ml/ l Banana Pulp

All low cost media was supplanted with 30g table sugar + 8g/l agar and 2mg/l BA + 0.5mg/l NAA for shooting.

3.16.1.2 Data Collection on shooting

3.16.1.2.1 Days required for shoot initiation

In case of shoot initiation the data were collected by proper visual observation of cultured media. The observation of cultured media was started from 7^{th} day after inoculation and the data were recorded on a weekly basis up to 28^{th} days. The culture was keenly observed for shoot initiation. The mean values of the data provided the days required for shoot initiation.

3.16.1.2.2 Shoot initiation percentage

The percentage of shoot initiation was noted after 8 weeks days of cultured explants .

3.16.1.2.3 Number of shoots per plantlet

The total number of shoots per plantlet was counted after eight weeks of inoculation in the experiment on culture establishment and expressed as mean.

3.16.1.2.4 Shoot length

The shoot length was measured from base to the top of the shoot with scale .

3.16.1.2.5 Number of leaves per plantlet

The number of leaves produced per shoot was counted after 8 weeks of inoculation and expressed as means **.**

3.16.2 Experiment 2: Effect of different media on rooting

Design: Completely Randomized Design

Number of factor: Single Factor

Treatment Combination: 7

Replication: 3

3.16.2.1 Treatment

1. **Conventional MS media** (control)
2. **LCM-1 :** 100ml/ l Coconut water
3. **LCM-2 :** 150ml/ l Coconut water
4. **LCM-3 :** 200ml/ l Coconut water
5. **LCM-4:** 100ml/ l Banana Pulp
6. **LCM-5 :** 150ml/ l Banana Pulp
7. **LCM-6:** 200ml/ l Banana Pulp

All media was supplanted with 30g table sugar + 8g/l agar and 0.5mg/l IBA for rooting.

3.16.2.2 Collection of data on rooting

3.16.2.2.1 Number of days taken for shoot initiation

The number of days taken for root initiation was counted and expressed as mean number of days .

3.16.2.2.2 Number of root

The numbe of roots produced per shoot was counted after four weeks of inoculation and expressed as mean.

3.16.2.2.3 Root Length

Root Length of the longest root from the collar region to the root tip was measured with a scale and expressed in centrimeter.

3.16.3 Experiment 3: Standardization of hardening medium for regenerated plantlet

Design: CRD

Number of factor: Single Factor

Treatment Combination: 3

Replication: 3

3.16.3.1 Treatment combinations:

1. **T_0 :** Garden Soil
2. **T_1 :** Garden Soil+ Cocopeat (1:1,v/v)
3. **T_2 :** Gargen Soil+ Vermicompost (1:1,v/v)
4. **T_3 :** Garden Soil+Vermicompost+Cocopeat (1:1:1,v/v)

3.16.3.2 Data collection on hardening

3.16.3.2.1 Survival percentage of plantlets

The number of plantlets survived out of total platlets subjected to hardening was counted and expressed as percentage.

3.16.3.2.2 Plant height

The plant height was measured from the base to top of the plantlet using a scale after four weeks of hardening and average was expressed in centrimeter **.**

3.16.3.2.3 Number of leaves per plantlet

The number of leaves produced from single plantlet was counted after four weeks of hardening and expessed as mean.

3.16.3.2.4 Number of shoot per plantlet

Number of shoot per plantlet produced from single plantlet was counted after four weeks of hardening and expessed as mean

3.17 Cost of Analysis:

The current market price of the conventional and the alternative source of MS media. Based on the quantities used per liter of the medium the cost of each treatment was calculated as follows.

$$\frac{\text{Amount used in Culture medium (g/l)} \times \text{price of amount bought (Tk)}}{\text{Amount Bought}}$$

Difference in cost between the conventional culture media and alternative nutrient media were then determined and their percentage evaluated.

3.18 Data Analysis:

The collected data on different parameters under this investigation were statistically analyzed by using Statistical Package for the Social Sciences (SPSS). Recorded data of all parameters were analyzed statistically using analysis of variance technique (ANOVA) and means were compared by Duncan's Multiple Range Test (Steel *et al.*, 1997).

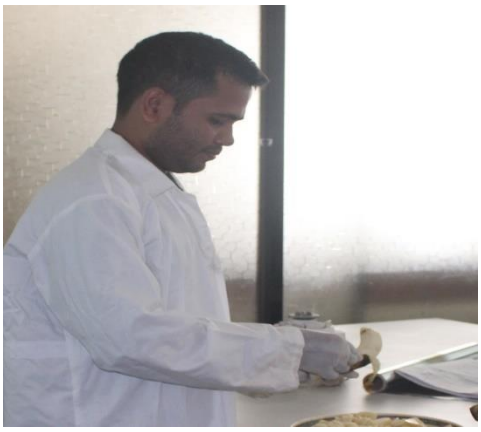
(a) *Gynura procumbens* (Mother plant)

(b) Nodal segment (Explant)

(c) Tender coconut

(d) Tender coconut water extract

Plate 1. (a) *Gynura procumbens* (Mother plant), (b) Nodal segment (c) Tender coconut, (d) Tender coconut water extract.



(a) Peeling of banana

(b) Sliceing banana pulp

(c) Weighing banana pulp

(d) Boiling banana pulp

(e) Seving banana extract

(d) Banana pulp extract

Plate 2. (a) Peeling banana, (b) Slicenig banana pulp, (c) Weighing of banana pulp, (d) Boiling banana pulp, (e) Seving banana extract , (f) Banana Pulp extract.

(a) Digital pH meter

(b) Distilled water plant

(c) Growth chamber

(d) Air Purifier

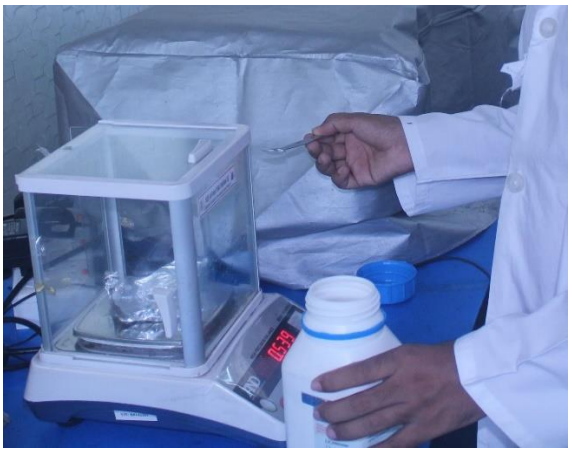
(e) Laminar air flow

(f) Dry oven

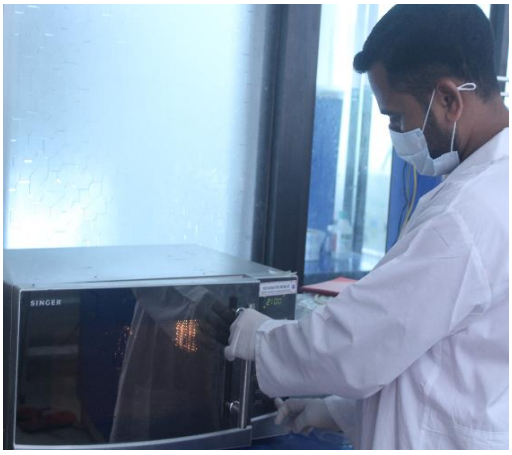
Plate 3. (a)Digital pH meter, (b) Distilled water plant, (c) Growth chamber, (d) Air purifier, (e) Laminar air flow, (f) Dry oven.

(a) Hot plate stirrer

(b) Magnet



(c) Digital weigh machine



(d) Oven

(e) Autoclave machine

(f) Media distribution into vials

Plate 4. (a) Hot plate stirrer, (b) Magnet, (c) Digital weigh Machine, (d) Oven (e) Autoclave machine, (f) Media distribution into vials

CHAPTER IV
RESULTS AND DISCUSIIONS

CHAPTER IV

RESULTS AND DISCUSSIONS

The result obtained in the present investigation entitled "Low cost media for *in vitro* Regeneration of *Gynura procumbens* (Lour.) Merr." are presented under the following heading in this chapter**.**

4.1 Experiment 1: Effect of different media on *in vitro* micro shoot regeneration of *Gynura procumbens*

4.1.1 Days required for shoot initiation

Significant difference were observed in different media for the days required for shoot initiation (Table 8). In case of control the growth of explant was healthy and started shoot induction within 7.33±0.33 days. Among different low cost media explant growth in LCM-2 (150ml coconut water + 30g table sugar + 8g agar + 2mg/l BA+0.5mg/l NAA) also found healthy but start of shoot induction within 11.60±.33 days . In other hand the LCM-4 (100ml banana + 30g table sugar + 8g agar + 2mg/l BA+0.5mg/l NAA) took the highest number of days (19.00±.57) for shooting . Alizah *et al.,* (2014) found that initially one shoot bud per explant emerged after 5-8 days of inoculation in MS media supplemented with 3mg/l BAP and after 6week 100% of the explant produced shoots on induction medium. Chan *et al.,* (2009) reported that 2mg/l BA + 0.5mg/l NAA produced 2-3 leaves from nodal segment could be notified after 10days.

4.1.2 Shoot initiation percentage

The percentage of shoot initiation was influenced by the different composition of culture medium the full strength of MS medium supplemented with 2mg/l BA+0.5mg/l NAA (control) produced the highest percentage (98.43±.63%) of

shoots. Among different low cost media LCM-2 produced 93.10±1.36% which was healthy as Control and Similar to LCM-1, in contrast the LCM-4 showed lowest percentage (59.47±1.00) of shoots. All the other treatments showed an intermediate result compared to the highest and the lowest Shoot initiation percentage (Table 8).

4.1.3 Number of shoots per plantlet

The number of shoot produced after 8 weeks of inoculation differ significantly (Table 8). The full strength of MS medium supplemented with 2mg/l BA+0.5mg/l NAA (control) produced the highest number of shoot (17.33±.67) per plantlet. Among different low cost media the LCM-2 produced produced 15.00±.57 shoot. per plant . On the other hand LCM-4 showed lowest number of shoot (4.30±.34). All the other treatments showed an intermediate result compared to the highest and the lowest number of shoot per plantlet. Chan *et al.*, (2009) reported that MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA was found to be the most effective medium for shoot multiplication resulting in the formation of an average of 18.2 ± 0.6 shoots per explant.

4.1.4 Number of leaves per plantlet

The different low cost medium showed significant variation in case of number of leaves per plantlet (Table 8). The maximum number of leaves per plantlet (5.67±.33) was recorded in MS media supplemented with 2.0 mg/L BAP and 0.5 mg/l. In LCM-2 produced 5.33±.33 leaves. In contrast the lowest number of leaves per plantlet (2.33±.67) was recorded from the LCM-4. All the other treatments showed an intermediate result .

4.1.5 Shoot length

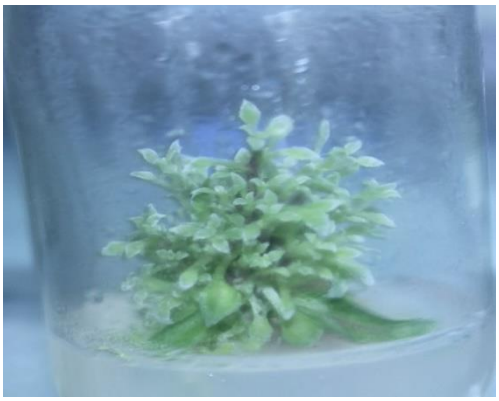
Significant variation was observed among the different media regarding the shoot length (Table 8). The shoot length was recorded after 8 weeks. Among their full strength of MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l showed the longest shoot (7.23±.21) . In LCM-2 we observed 6.93±.88 cm long shoot which was almost similar to control . On the other hand LCM-4 produced minimum shoot length 2.28±±.12cm . Farzan *et al.,* (2014) reported that 1mg/l BAP showed 8 cm longest shoot was produced with in 1mgl/l BAP.

Table 8. Effect of different Media on Shoot multiplication of *Gynura procumbens* [y]

Treatment[x]	Days required for shoot initiation	Shoot initiation %	Number of Shoot	Shoot Length (cm)	Number of leave per plantlet
Control(MS media)	7.33±0.33 d	98.43±0.63 a	17.33±.67 a	7.23±.21 a	5.67±.33 a
LCM-1	14.00±.33 b	89.90±1.36 b	7.67±.33 e	2.61±.33d	3.67±.88bc
LCM-2	11.60 ±.33 c	93.10±1.36 b	15.00±.57 b	6.93±.88a	5.33±.33ab
LCM-3	13.33 ±.57 b	75.68±1.20 c	10.00±.57 c	4.23±.50b	3.67±.33bc
LCM-4	19.00 ±.57 a	59.47±,1.00 e	4.30±.34 e	2.28±.12d	2.33±.67c
LCM-5	17.00 ±.33 a	70.65±1.12 d	4.60±.88 d	2.87±.27cd	2.66±.33c
LCM-6	17.38 ±.88 a	62.90±1.42 e	6.60±.33 d	3.97± .76bc	3.00±.57c
Sig. Level	***	***	***	***	***

[y]Mean±Standard error with in a colum followed by similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05level of probability.

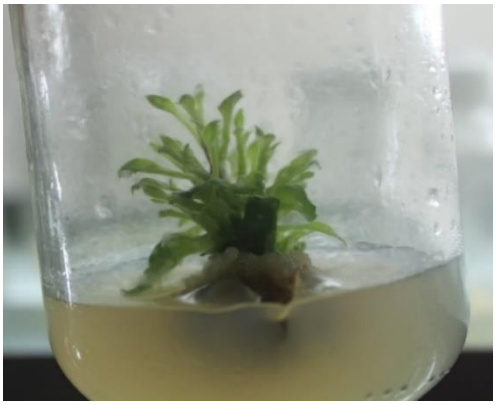
[x]Control, Conventional MS media ; LCM-1, 100ml/L Coconut water+30g table sugar + 8g agar ; LCM-2, 150ml/L Coconut water+30g table sugar + 8g agar; LCM-3, 200ml/L Coconut water+30g table sugar + 8g agar; LCM-4, 100ml/L Banana pulp+30g table sugar + 8g agar; LCM-5, 150ml/L Banana pulp +30g table sugar + 8g agar; LCM-6,Banana pulp+30g table sugar + 8g agar; All media are supplemented with 2mg/l BA+0.5mg/l NAA for shooting.



(a) Conventional MS media (Control)

(b) LCM-1

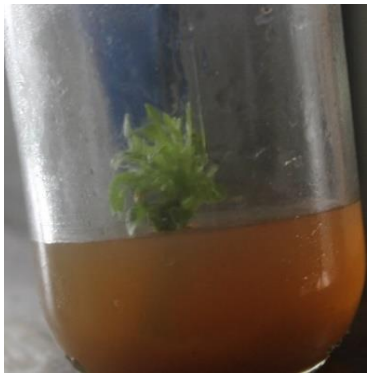
(c) LCM-2



(d) LCM-3

(c) LCM-4

(c) LCM-5



(c) LCM-6

Plate 5. (a) Conventional MS media (Control); (b) LCM-1, 100ml coconut water; (c) LCM-2, 150ml coconut water; (d) LCM-3, 200ml coconut water; (e) LCM-4, 100ml banana pulp ; (f) LCM-5, 150ml banana pulp; (g) LCM-6, 200ml banana pulp; All low cost media was supplemented with 30g table sugar and all media supplemented with 2.0mg/l BA and 0.5mg/l NAA for shooting.

4.2 Experiment 2: Effect of different media on in vitro micro root regeneration of *Gynura procumbens*

4.2.1 Days required for root initiation

Significant difference were observed in different media for the days required for root initiation (Table 9). In case of control the micro shoot stared rooting within 13.33±.33 days and amnong different low cost media the LCM-2 (150ml coconut water + 30g table sugar + 8g Agar + 0.5mg/l IBA) also found healthy rooting induction within 14.66±.33 days . In other hand the LCM-4 (200ml banana + 30g table sugar + 8g agar+ 0.5mg/l IBA) took highest number of days (22.00±.57) for rooting .

4.2.2 Number of root per plantlet

Significant differences were observed for number of roots per plantlets (Table 9). The maximum number of roots (15.33±.88) per plantlets was recorded in control, and in LCM-2 also find similar result 14.67±.57. Oppositely the lowest number of roots (5.66±.88) per plantlet was produced by the LCM-4. All the other treatments showed intermediate result compared to the highest and the lowest number of roots per plantlet .

4.2.3 Root length

Root length varied significantly due to the different media. The longest root (6.89±.34cm) was produced by the MS medium (control) and the shortest root (2.90±.13) was observed LCM-4 medium.In LCM-2 we showed 6.51±.15cm long root which was similar to control. All the other treatments showed an intermediate result compared to the highest and the lowest root length (Table 9). Parvin *et al.*, (1995) recorded that the average root length was 07±1.1 cm observed on MS medium supplemented with IBA 0.5 mg/l

Table 9. Effect of different media on rooting of *Gynura procumbens* [y]

Trewtment[x]	Root initiation	Number of root	Root length (cm)
Conventional MS media (Control)	13.33±0.33d	15.33±.88a	6.89±.34a
LCM-1	17.33±.33c	9.66±.88b	4.56±.53cd
LCM-2	14.66±.33d	14.67±.33a	6.51±.15a
LCM-3	16.67±.57c	11.00±.57b	5.40±.24bc
LCM-4	22.00±.57 a	5.66±.88c	2.9±.13e
LCM-5	19.33±.33b	7.33±.33c	3.7±.32de
LCM-6	17.33±.33c	9.85±.88c	6.00±.10ab
Level of sig.	***	***	***

[x]Mean±Standard error with in a colum followed by similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05level of probability.

[y]Control , MS media; LCM-1, 100ml coconut water; LCM-2, 150ml coconut water; LCM-3, 200ml coconut water; LCM-4, 100ml banana pulp; LCM-5, 150ml banana pulp; LCM-6, 200ml banana pulp; All low cost media was supplemented with 30g table sugar and all media supplemented with 0.5mg/l IBA for rooting**.**

(a) Conventional MS media

(b) LCM-1

(c)LCM-2

(d) LCM-3

(e) LCM-4

(f) LCM-5

(g) LCM6

Plate 6. (a) Conventional MS media (control), (b) LCM-1, 100ml coconut water; (c) LCM-2, 150ml coconut water; (d) LCM-3, 200ml coconut water; (e) LCM-4, 100ml banana pulp ; (f) LCM-5, 150ml banana pulp; (g) LCM-6, 200ml banana pulp; All low cost media was supplemented with 30g table sugar and all media supplemented with 0.5mg/l IBA for rooting.

4.3 Experiment 3: Standardization of hardening medium for regenerated plantlets

4.3.1 Survival percentage of plantlet

The survival percentage differed significantly among the different harden media (Table 10). The maximum survival rate (96.45±.87%) was noticed on T_3 (Garden Soil + vermicompost + coco dust). and control (T_0) showed lowest survival rate (67.63±.50%). On the other hand other media showed intermediate result between highest and lowest.

4.3.2 Number of leaves per plantlet

Number of leaves per plantlet differed significantly among the different harden media (Table 10). The maximum number of leaves (33.66±2.7) per plantlet was noticed on T_3, Garden Soil+ vermicompost + coco dust (1:1:1). And T_0 showed lowest Number of leaves per plantlet (21.33±1.7). On the other hand other media showed intermediate result between highest and lowest.

4.3.3 Number of node per plantlet

There was found significant difference among the different harden media (Table 10). The maximum number of node (10.00±.57) per plantlet was noticed on T_3, Garden Soil+ vermicompost + coco dust (1:1:1). And control (T_0) showed lowest number of node per plantlet (3.00±.57). On the other hand other media showed intermediate result between highest and lowest treatment.

4.3.4 Plant height

The plant height increased gradually with the increase in age of plants. Variation in plant height due to the effect of different treatments was found significant (Table 10). The maximum plant height (21.22±.66cm) was found on T_3 after 60

days of planting, whereas the minimum plant height (12.28±.43cm) was found from control (T_0).

Table 10. Effect of different hardening media combination on hardening of *Gynura procumbens*[Y]

Treatment[x]	Survival percentage of plantlets	Number of leave/ plantlet	Number of node per plantlet	Plant height
T_0	67.63±.50d	21.33±.1.76d	3.00±.57c	12.28±.43d
T_1	81.80±.26b	25.33±1.20c	6.66±.88b	15.37±.53c
T_2	78.53±.36c	26.00±1.52b	7.06±.87b	17.80±1.0b
T_3	96.45±.87a	33.66±.2.7a	10.00±.57a	21.22±.66a
Sig. level	***	***	***	***

[x]Mean±Standard error with in a colum followed by similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05level of probability.

[Y]**T_0,** Garden Soil**; T_1 ,** Garden Soil+ Cocopeat (1:1,v/v)**; T_2**, Gargen Soil+ Vermicompost (1:1,v/v)**; T_3,** Garden Soil+Vermicompost+Cocopeat (1:1:1,v/v).

(a) **T_0**

(b) **T_1**

(c) **T_2**

(d) T_3

Plate 7. (a) T_0, Garden Soil; (b) T_1 , Garden Soil+ Cocopeat (1:1,v/v); (c) T_2, Gargen Soil+ Vermicompost (1:1,v/v); (d) T_3, Garden Soil+Vermicompost+Cocopeat (1:1:1,v/v)

4.4 Cost analysis

The current market price of the conventional and the alternative source of MS media. Based on the quantities used per liter of the medium the cost of each treatment was calculated as follows.

$$\frac{\text{Amount used in Culture medium (g/L)} \times \text{price of amount bought (Tk)}}{\text{Amount Bought}}$$

Table 11. Cost of conventional MS media

Stock I	**Major Salts(10X)**	g/l	Taka
	KNO_3	19	9.00
	NH_4NO_3	16.5	165.00
	$MaSO_4.7H_2O$	3.7	5.18
	$CaCl_2.7H_2O$	4.40	4.40
	KH2PO4	1.70	5.10
Stock II	**Micro salts (100X)**	g/l	Tk
	KI	0.83	13.00
	H BO3	6.20	6.875
	MnSO4.4H2O	22.3	26.76
	ZnSO4.7H20	8.6	12.04
	NA2MOO4.2H2O	0.25	0.75
	CUSO4.5H2O	0.025	0.05
	CACL2	0.025	0.25
Stock III	**Iron EDTA Sol^n(100X)**	g/l	TK
	FeSO4.7H2O	2.78	3.89
	Na2EDTA.2H2O	3.73	7.46
Stock IV	**Organics (100x)**	g/l	TK
	Myo- Inositol	1.00	48
	Nicotinicacid	0.5	20
	Pyridoxin-HQ	0.5	20
	Thiamine-HCl.	0.1	6.4
	glycine	2.0	80
Carbon Source	Sucrose	30	40
Solidifying agent	Agar	8	80
Total	-	-	554

4.4.1 Cost analysis of different low cost media

The rate of Banana Pulp extract was 175Tk/kg , The rate of Coconut water was 150tk/L and table sugar was 65 Tk/ kg.

Table 12. Cost of different Low cost media

Attribute / Name of media	Banana pulp ml/l	Cost of banana	Coconut water ml/l	Cost of coconut tk	Cost of table sugar(30g) Tk	Cost of Agar(8g) Tk	Total Cost Tk
LCM -1	-	-	100	15	1.95	80	96.95
LCM-2	-	-	150	22.5	1.95	80	104.45
LCM-3	-	-	200	30	1.95	80	111.95
LCM-4	100	17.5	-	-	1.95	80	99.45
LCM-5	150	26.25	-	-	1.95	80	108.2
LCM-6	200	35	-	-	1.95	80	116.95

Table 13. Comparisaion of cost beween Convention MS media and different Low cost media

Atrributes	Control	LCM-1	LCM-2	LCM-3	LCM-4	LCM-5	LCM-6
Cost of Media Tk/L	554	96.95	104.45	111.95	99.45	108.20	116.95
Number of Plant per litre	17.33	7.67	15.00	10.00	4.30	4.60	6.60
Cost of per plantlet	31.97	12.64	6.96	11.19	23.12	23.52	17.71
Cost Reduction%	0	60.30	78.22	64.99	26.43	27.68	44.60

Note: Where other costs was constant

The table 13 showed cost of different media. The conventional Ms media(control) showed higest cost and LCM-1 showed low cost .This table also showed cost of plantlet is highest in control (31.97 Tk) and lowest cost in LCM-2 (6.96tk) when other cost was constraint. Form this table 13, we showed the percentage of cost

reduction per plantlet . The cost reduction percentage was highest in LCM-2 (78.22%) and lowes cost reduction was showed in LCM-4(26.43%). Different low cost media can effectively reduce production cost and hence the research for such alternatives is significant. The use of coconut water as nutrient and Table sugar as carbon source source was found efficient alternative for conventional costly ingredients.

CHAPTER V

SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

5.1 Summary

Gynura procumbens is commonly known as diabetics plant in Bangladesh and belongs to a family Asteraceae. It has been used as traditional medicine for household remedy against various human ailments such as hypertension, diabetes mellitus eruptive fevers, rash, kidney disease, migraines, constipation and cancer. Its leaves extract of has anti-herpes simplex virus anti hyperglycemic, anti-oxidative antilipidemic and anti-inflammatory effects. It is usually propagated though cutting which gives low quality seedling with low multiplication rate. This method cannot meet the increasing demand of this plant used as the raw material for the preparation products .Tissue Culture technology can be used to produce high quality seedlings instead of the traditionally used cuttings. But high production cost is an impediment to tissue culture adoption which has further limited the technology. Cost effective micro propagation would facilitate commercialization of the technology. Present investigation is an innovative approaches to develop a low-cost protocol for micro propagation of *Gynura procumbens* using Banana pulp, coconut water alternate to micro and macro nutrients, using table sugar alternative to sucrose. Nodal segment was used as explant to carry out the research. For shooting we used six different low cost media(LCM-1, 100ml coconut water + 30g/l table sugar + 8g agar; LCM-2, 150ml coconut water + 30g/l table sugar + 8g agar; LCM-3, 200 ml coconut water + 30g/l table sugar + 8g agar ; LCM-4, 100ml Banana + 30g/l table sugar + 8g agar; LCM-5, 150ml Banana pulp + 30g/l table sugar + 8g agar; LCM-6, 200 ml Banana pulp + 30g/l table sugar + 8g agar and Conventional MS media (control) to compared the result of the research. All the media was supplemented

with 2mg/l BA+0.5mg/l NAA for shooting. Among different low cost media LCM-2 showed best performance due to its required low days (11.60±.33) for shoot initiation, and maximum shoot initiation percentage (93.10±1.36), maximum number of shoot (15.00±.57), maximum shoot length (6.93±.88cm) , maximum number of leaf (5.33±.33) per plantlet . Whereas the lowest performance showed in LCM-4 . In LCM-2 shoot initiation days is high and number of shoot is low comparatively Conventional MS media but other quality is more or less same. In rooting we used 0.5mg/l IBA in all the media. Among different low cost media LCM-2 showed best performance than other low cost media. Four different media, viz T_0, Soil ; T_1 , Soil+Coco dust(1:1,v/v); T_2, Soil+vermicompost (1:1,v/v); T_3, Soil+Coco dust+Vermicompost (1:1:1 ,v/v); wrer used for hardening of plantlet . The T_3 Soil+Coco dust+Vermicompost (1:1:1 ,v/v) gave maximum survival percentage (96.45±.87) with vigorous performance than other media. Whereas T_0, Soil showed lowest performance. In LCM-2 showed maximum cost reduction percentage (78.22%) and lowest cost reduction (26.43%) was observed in LCM-4 among different low cost media (when other cost was constrant).

5.2 Protocol for regeneration of *Gynura procumbens*

a. Nodal segment should be used as an explant which derived from the one year old mature plants.
b. Explant was washed thoroughly with running tap water for 30 minutes.
c. The explants were then surface sterilized with 5-10 ml of Savlon
d. 2-3 drops of Tween-20 was used for 8-10 minutes with gently shaking.
e. The explants were then washed with 4 to 5 times using distilled water for removal of sterilizers
f. Under laminar air flow cabinet, the explants were than rinsed with 1% $HgCl_2$ solution for several times.
g. At last the explants were than washed with double distilled water with gently shaking for the removal of $HgCl_2$ solution

h. After sterilization explant should be cut about 1.0 cm in length and cultured on Sterilized LCM (150ml/l coconut water +30g/l table sugar+8g/l agar) supplemented with 2mg/l BA+0.5mg/l NAA for shooting.
i. After four week the micro shoot should be subculture on same fresh media at regular interval 15 days
j. The micro shoot with 2-3 tiny leave should be transferred to LCM-2 (150ml/l coconut water +30g/l Table sugar+8g/l Agar) supplemented with 0.5g/l IBA for weeks for root initiation.
k. Then rooted plantlet should be washed gently to remove media and transferred to the hardening Soil+Coco dust+vermicompost; (1:1:1, v/v) medium for four week in green house.

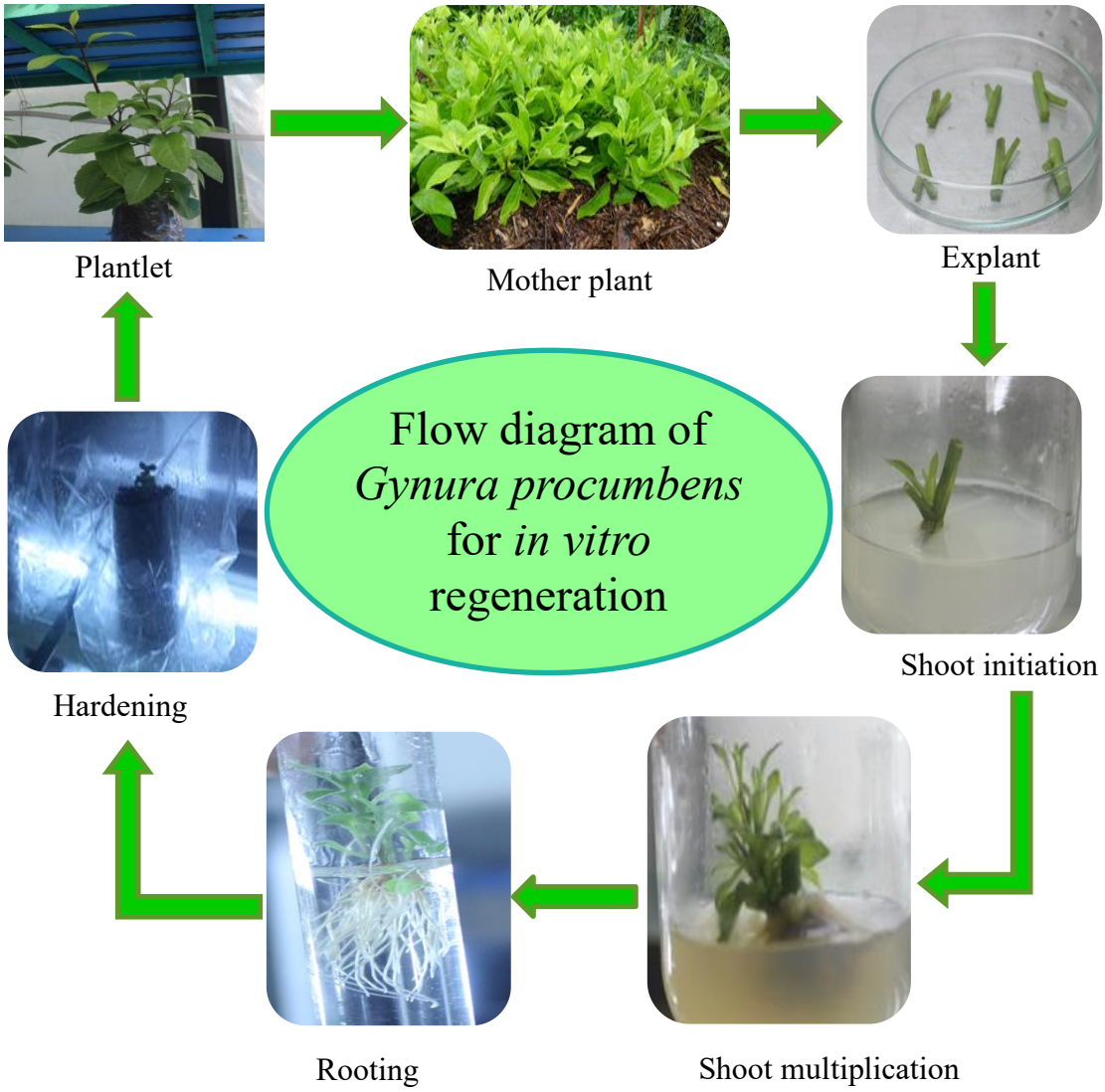


Plate 8. Flow diagram of different steps of *Gynura procumbens* for *in vitro* regeneration

5.3 Conclusion

Following conclusion can made from the present study:

I. A low cost *in vitro* regeneration protocol has been developed for *Gynura procumbens* by using low cost ingredients .
II. Based on the results LCM-2 (150ml coconut water + 30g Table sugar + 8g agar) is the best for *Gynura* regeneration
III. This protocol is simple and easiy and allows a low cost strategy to obtain high quality in vitro microporpagules with mass multiplication.
IV. This study showed that substitution of conventional ingredients with 150ml coconut water and table sugar reduced the cost of tissue culture about 78.22% of *Gynura procumbens*.
V. Low cost media effectively reduce production cost , hence this protocol is significant,
VI. Garden soil+ Vermicompost+ Coco dust, (1:1:1, v/v) was the best media for hardening .

5.4 Recommendation

Based on the finding of this research recommendations are:

1. This protocol is useful for invitro plantlets production commercially.
2. Further research is needed to optimatize the media and ensure consistent production of higher number of plantlets with low production cost.

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CHAPTER VII
APPENDICES

APPENDICES

Appendix I. Analysis of variance of the data on Shoot initiation

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	303.619	6	50.603	62.510	.000
Error	11.33	14	.810		
Replication	314.952	20			

Appendix II. Analysis of variance of the data on shoot initiation percentage

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	4250.480	6	708.413	128.714	.000
Error	77.053	14	5.504		
Replication	4327.533	20			

Appendix III. Analysis of variance of the data on Shoot number after 8 weeks

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	459.519	6	76.603	80.433	.000
Error	13.33	14	0.952		
Replication	472.952	20			

Appendix IV. Analysis of variance of the data on Shoot length after 8 weeks

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	73.901	6	12.317	26.106	.000
Error	6.605	14	.472		
Replication	80.506	20			

Appendix V. Analysis of variance of the data on number of leave per plantlet after 8 weeks

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	29.810	6	4.968	5.716	.003
Error	12.000	14	.857		
Replication	41.810	20			

Appendix VI. Analysis of variance of the data on root initiation

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	140.286	6	23.381	54.566	.000
Error	6.000	14	.429		
Replication	146.286	20			

Appendix VII. Analysis of variance of the data on root number 4 weeks

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	296.571	6	49.429	31.455	.000
Error	22.000	14	1.571		
Replication	318.571	20			

Appendix VIII. Analysis of variance of the data on root length 4 weeks

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	38.522	6	6.420	23.957	.000
Error	3.752	14	.268		
Replication	42.274	20			

Appendix IX. Analysis of variance of the data on Survival percentage of harden plantlet.

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	1272.191	3	424.064	275.502	.000
Error	12.305	8	1.538		
Replication	1284.496	11			

Appendix X. Analysis of variance of the data on plant height

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	128.759	3	42.920	29.971	.000
Error	11.457	8	1.432		
Replication	140.216	11			

Appendix XI. Analysis of variance of the data on number of leave on harden plantlet.

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	238.917	3	79.639	9.653	.005
Error	66.000	8	8.259		
Replication	304.917	11			

Appendix XII. Analysis of variance of the data on number of node per harden plantlet

Source of variance	Sum of square	df	Mean square	F	Sig.
Treatment	46.917	3	15.639	9.383	.005
Error	13.333	8	1.667		
Replication	60.250	11			