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Micropropagation and *in Vitro* Flowering of *Luffa acutangula* (L.) Roxb.- an Important Vegetable Crop

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Abstract

An efficient protocol has been established for micropropagation and *in vitro* flowering of *Luffa acutangula* (L.) Roxb. in the present study. Maximum numbers of shoots (8.2±0.29/ nodal explants) were obtained in the MS medium supplemented with 4.44 µM of 6-Benzylaminopurine (BA). Doubling of 40 mM of $FeSO_4$ and Na_2EDTA resulted in chlorosis prevention of *in vitro* leaves. Nearly 100% of *in vitro* plants induced roots in presence of ½ MS medium containing 4.90 µM of IBA within 12.4±0.40 days of culture. Cytological analysis of the donor and regenerated plantlets revealed chromosome stability with 2n=26 chromosomes. About 100 % of the *in vitro* grown plants induced both male and female flower buds in MS medium (with 3% sucrose). Nearly 90.5% of the pollen grains formed in the *in vitro* male flowers were viable. The survival rate of acclimatized plants reached 94.5% when transferred to field conditions. Therefore, the present study can be considered as a promising system for efficient micropropagation and *in vitro* flowering of *L. acutangula*, which can also be a source of viable aseptic anthers, and thus can be directly used for haploid culture.

1. Introduction

Luffa acutangula (L.) Roxb. (Family: Cucurbitaceae), generally known as ridge gourd, angled loofah or ribbed gourd, is one of the most popular vegetables which holds a desirable position in the Asian market. Cultivars of *L. acutangula* are monoecious, *i.e,* the male and female flowers are borne on the same plant. The male flowers are large, bright yellow in colour and are found in clusters; whereas the female flowers are few in number and stand alone. The ratio of male and female flowers is 40:1. This plant is nutritionally rich in vitamin A, C and Fe (Yawalkar, 1985) and has many medicinal importances. It has antitumor, antioxidant and immunomodulatory properties (Ng et al., 1992). Young green fruits of this plant are generally used as vegetable.

L. acutangula is typically propagated through seeds. But this conventional breeding method is not desirable due to poor germination and genetic instability of the seeds. Therefore, this species needs genetically stable clonal propagules derived from elite individual for regular cultivation. *In vitro* micropropagation producing multiple elite clones within a short period of time is an alternative approach in this respect. On the other hand, *in vitro* flowering can be considered as the initial and simple step for anther culture from which homozygous plants can be obtained within very short period of time. Anther culture can be of great value in crop improvement and has become a popular tool for the rapid production of haploid and isogenic lines for obtaining hybrid cultivars (Sopory and Munshi, 1996). Anther or haploid culture has reduced the time required for breeding new cultivars by at least 3 to 5 years (Tai, 2003). In Cucurbitaceae, earlier attempts to grow plants from cultured anthers have been successful for muskmelon (Dryanovska, 1985). *In vitro* flowering in vegetables is also found to be very important for selective hybridization with pollens from high yielding cultivars or rare accessions (Kielkowska and Havey, 2012). Nevertheless, *in vitro* flowering has been reported as an exceptional process by which genetic purity is highly maintained (Stephen and Jayabalan, 1998). It also provides an ideal experimental system for studying phase

Abbreviations

PGRs: Plant Growth Regulators; MS: Murashige and Skoog basal medium (Murashige and Skoog, 1962); B5: Gamborg basal medium (Gamborg et al., 1968); SH: Schenk and Hildebrandt basal medium (Schenk and Hildebrandt, 1972); BA: 6-Benzylaminopurine; Kn: Kinetin; IBA: Indole-3-butyric acid; IAA: Indole-3-acetic acid; NAA: α-Naphthalene acetic acid; *p*DB: para-dichlorobenzene.

transition and floral development (Huang et al., 2009). There is also a possibility of using *in vitro* flowering in genetic study. Moreover, it is a modern area of research and has great potential in plant breeding programs (Sivanesan and Jeong, 2007a).

There are few reports on *in vitro* micropropagation protocol for some important cucurbit vegetables like *Citrullus vulgaris* (Dong and Jia, 1991), *Momordica dioica* (Rai et al., 2012). *In vitro* flowering has been reported in *Momordica charantia* L. (Wang et al., 2001) and *Cucumis sativus* L. (Rajasekaran et al., 1983; Kielkowska and Havey, 2012). As there is no report on *in vitro* propagation of *L. acutangula* till date, the present study was undertaken to develop a standardized protocol for generating a large number of cytogenetically and morphologically uniform plants through high frequency axillary shoot multiplication and also to establish an easy protocol for *in vitro* flower induction in *L. acutangula.*

2. Materials and Methods

2.1. Plant material

Nodal portions were collected from young, healthy plants of *Luffa acutangula* (L.) Roxb., which were maintained in the experimental garden of Ramakrishna Mission Vivekananda Centenary College, Rahara.

2.2. Explant disinfection and implantation

Nodal explants were washed thoroughly under running tap water for 10 min and then surface sterilized with 2% w/v solution of Bavistin® (systemic fungicide) for 15 min followed by 5% v/v solution of Tween 20 (liquid detergent) for 5 min. Then the explants were thoroughly washed with fresh water to remove the detergent. Finally, the explants were surface sterilized with freshly prepared 0.1% w/v solution of mercuric chloride ($HgCl_2$) for 8-10 min, rinsed in autoclaved distilled water for 3-4 times to remove traces of $HgCl_2$ under the sterile condition. Then the nodal explants (0.5-1.0 cm) were placed vertically onto sterilized MS media.

2.3. Culture media and culture conditions

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) with 3% sucrose was used for breaking the dormancy of shoot buds as well as for proliferation. The pH of the medium was adjusted to 5.7 using 0.1 N KOH or 0.1 N HCl as and when required and 0.7% (w/v) agar (Merck) before autoclaving. Approximately 20 ml of molten medium was dispensed into each culture tube (25×150 mm) and plugged with non-absorbent cotton. The tubes were then wrapped in one layer of cheese-cloth and steam sterilized at 15 lb $inch^{-2}$ for 15 min. All the cultures were grown at 24±2 °C temperature and 55±5% relative humidity under 16 h photoperiod at 55 µmol $m^{-2}s^{-1}$ irradiance, supplied by Philips (Trulite 5 star 36 w/82 2700˚K G84, made in India) fluorescent tubes.

2.4. In vitro shoot multiplication

For *in vitro* shoot multiplication, nodal explants were cultured on MS medium alone as control and MS medium with different concentrations and combinations of cytokinins (BA, Kn) either individually or with auxins (IAA, NAA). The effects of different plant growth regulators were tested on multiple shoot induction, shoot elongation, and were recorded periodically. Being a member of the family Cucurbitaceae, the nodal explants or the stem portions of *L. acutangula* were supposed to produce tendrils. The *in vitro* development of tendrils from the nodal explants was also observed and photographed.

2.5. In vitro root induction

For rooting, *in vitro* grown micro shoots (3-4 cm) with more than four leaves were excised and implanted on both MS and ½ MS basal media. The cultures were examined every week and were recorded on the basis of visual observations. After this screening individual micro shoots were treated with different concentrations of IBA (ranging from 2.46 to 9.80 µM) in ½ MS medium along with 3% sucrose.

2.6. In vitro flowering and pollen viability testing

In vitro developed complete plantlets and micro shoots (3-4 cm) were used for *in vitro* flowering experiments and cultured on different basal media (MS, ½ MS, B5 and SH) along with different concentrations of sucrose (1.5%, 3% and 6%) or without sucrose. The effects of different concentrations and combinations of cytokinins (BA, Kn) and auxins (IAA, NAA) on *in vitro* flowering were also tested. All cultures were maintained in the previously mentioned culture conditions. Percentage of plantlets showing flower induction, the numbers of male and female flower buds appearing per plantlet were recorded after 30 days. The morphology of *in vitro* induced flowers was compared with that of the field grown plants. Assessment of pollen viability and fertility is a preliminary and essential condition for genetic crop breeding (Tuinstra and Wedel, 2000). In our study, pollen viability of *in vitro* grown male flower was determined in two ways (Lyra et al., 2011). Firstly, pollen grains were dissected out and stained with aceto-carmine (2%) and observed under microscope and photographed. Secondly, pollens were collected from fully opened, mature *in vitro* male flowers and cultured on the sterile sucrose solution (15% w/v) for 2 days at 24±2 °C temperature for germination of pollen tube.

2.7. Hardening of plants

For hardening, at first the tissue culture derived healthy rooted plantlets were placed at room temperature for 1 week. Then the plantlets were removed from the agar medium, washed thoroughly under running tap water and transferred to earthen pots containing sterilized soilrite (Keltech Energies Ltd, Bangalore, India). To preserve moisture, the potted plantlets

were covered with transparent polythene bag and the pots were placed on a plastic tray containing water under diffused light (16 h, photoperiod) in our poly-house for 25-30 days. After 25 days, these plants were transferred to the earthen tub (8 inch diameter) containing the mixer of garden soil, sand and vermicompost (2:1:1) and placed in the green house. After one month, surviving plants bearing both male and female flowers were transferred to the field and were hand-pollinated. Numbers of plants producing healthy fruits were observed and the viability of the seeds was also tested.

2.8. Somatic chromosome analysis

Chromosome analysis was done for the evaluation of cytogenetic uniformity among donor and *in vitro* derived plants. For this experiment, root tips from both the *in vitro* and field grown plants were pretreated with saturated solution of para-dichlorobenzene (*p*DB) for 4½ h at 10°C and fixed overnight in aceto-ethanol (1:3) at 16°C. The root tips were stained with 2% aceto-orcein for 3 h, then squashed and observed under microscope and photographed (Sharma and Sharma, 1980).

2.9. Statistical analysis

All the experiments were conducted under controlled conditions with three replications. Means and standard errors were carried out for each experiment and data was analyzed using one way Analysis of Variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Tukey's multiple range test at a 5% probability level. Data analysis was performed using SPSS v 16.0 software.

3. Results and Discussion

3.1. Role of plant growth regulators on in vitro shoot induction and multiplication

It was observed that for shoot induction, nodal explants responded to both MS basal medium without PGRs and MS medium supplemented with different concentrations and combinations of cytokinins and auxins. The earliest visible sign of green shoot bud (2-3) induction from nodal segments was noticeable after 6 days of culture on MS medium supplemented with BA (8.87 µM). After 4 weeks, number of shoots increased up to 8.2±0.29 per node without sub culturing (Figure 1A), whereas nearly 2.3±0.15 shoots were induced in case of medium containing Kn (9.29 µM). The effects of 2.32 µM and 4.65 µM Kn on shoot proliferation were found to be insignificant. The explants showed slight swelling at the base prior to the sprouting of shoot buds in both the cases. But, surprisingly, combined effects of both the cytokinins *i.e*., BA (4.44 µM) and Kn (2.23 µM) reduced the shoot induction and multiplication rates by 50% (Table 1).

The superior activity of BA compared to other cytokinins is reported in many plants like *Dalbergia sissoo* (Pradhan et al., 1998), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Holostemma annulare* (Sudha et al., 2000), *Sesbania rostrata*

Table 1: Effect of different cytokinins and auxins on *in vitro* shoot multiplication of *L. acutangula*

Medium (3% sucrose)	Concentrations (µM)		% of nodal explants showing shoot multiplication	No. of multiplied shoots nodal explant^{-1}	Length of micro shoots (cm)	No. of leaves micro shoot^{-1}
	Cytokinins	Auxins				
MS	0	0	31.66	1.8±0.20^{a}	10.5±0.40^{h}	9.6±0.70^{a,b}
MS	BA (2.22)	0	62.50	3.8±0.20^{c,d}	6.0±0.25^{f,g}	10.8±0.72^{a,b,c}
MS	BA (4.44)	0	95.00	8.2±0.29^{g}	6.7±0.22^{g}	17.7±0.60^{a,h}
MS	BA (6.66)	0	87.50	6.1±0.43^{f}	5.5±0.25^{e,f}	17.0±0.70^{g,h}
MS	BA (8.87)	0	72.50	5.5±0.34^{e,f}	4.6±0.25^{d,e}	14.8±0.75^{e,f,g}
MS	BA (13.31)	0	62.50	5.0±0.25^{d,e,f}	4.0±0.15^{a,b,c,d}	13.0±0.45^{b,c,d,e}
MS	BA (17.75)	0	57.50	4.7±0.30^{d,e}	3.4±0.21^{a,b}	11.2±0.50^{a,b,c}
MS	BA (22.19)	0	47.50	4.2±0.32^{c,d,e}	3.1±0.08^{a}	8.9±0.40^{a}
MS	Kn (2.32)	0	32.50	3.0±0.21^{a,b,c}	4.2±0.10^{b,c,d}	13.3±0.36^{c,d,e}
MS	Kn (4.65)	0	50.00	2.6±0.22^{a,b}	4.3±0.09^{b,c,d}	14.1±0.43^{d,e,f}
MS	Kn (9.29)	0	47.50	2.3±0.15^{a}	4.1±0.09^{a,b,c,d}	12.1±0.38^{b,c,d}
MS	BA (4.44)	Kn (2.32)	47.50	2.8±0.20^{a,b}	3.8±0.13^{a,b,c,d}	10.1±0.31^{a,b}
MS	BA (4.44)	IAA (0.57)	62.50	3.8±0.35^{c,d}	4.4±0.10^{c,d}	11.9±0.38^{b,c,d}
MS	BA (4.44)	IAA (1.14)	57.50	3.1±0.23^{a,b,c}	3.8±0.13^{a,b,c,d}	16.0±0.45^{f,g,h}
MS	BA (4.44)	NAA (0.54)	47.50	2.3±0.15^{a}	3.5±0.17^{a,b,c}	11.0±0.33^{a,b,c}
MS	BA (4.44)	NAA (1.08)	32.50	2.2±0.13^{a}	3.1±0.20^{a}	9.6±0.54^{a,b}

Each value represents the mean±SE of 10 replicates and each experiments was conducted thrice. In each column, mean±SE followed by the same letter were not significantly different (p<0.05) according to Tukey's multiple range test

(Jha et al., 2004), and *Pterocarpus marsupium* (Husain et al., 2008). Among the concentrations of BA alone ranging from 2.22 µM to 22.19 µM, it has been observed that the higher concentrations (13.31 µM to 22.19 µM) of BA formed large amounts of white-greenish callus at the base of the nodal explants which results in stunted shoots with deformed rosette leaves. The thick layer of the basal callus might also have prevented uptake of nutrients from the medium resulting in abnormal growth of the shoots. On the other hand, lower concentrations (2.22 µM to 8.87 µM) of BA induced multiple healthy shoots with normal leaves. Moreover, among the lower concentrations of BA tested, 4.44 µM was found to be the best in terms of yielding a high number of healthy shoots with maximum number of leaves (17.7±0.6). In order to assess any further increase in multiplication rate, different concentrations of auxins (IAA and NAA) in combination with the optimal concentration of BA were also tested. But these combinations could not play any significant role on induction of shoot buds even up to 30 days (Table 1). The higher concentrations of both the IAA (1.14 µM) and NAA (1.08 µM) with BA (4.44 µM) resulted in tissue swelling and produced large amount of callus at the base of shoots suppressing shoot multiplication and elongation. These results are in agreement with some earlier reports like in *Pterocarpus marsupium* (Husain et al., 2008) and *Momordica charantia* L. (Agarwal and Kamal, 2004), where the addition of higher concentrations of auxins with cytokinin resulted in callus formation at the base of the shoots. The inhibitory effect of higher concentrations of BA on shoot induction was also reported in *Tinospora cordifolia* (Raghu et al., 2006), *Sida cordifolia* (Sinanesan and Jeong, 2007b) and *Cucurbita maxima* (Mahzabin et al., 2008). On the other hand the longest shoots (10.5±0.40 cm) with fully expanded leaves were observed only in MS basal medium, while the medium with BA (4.44 µM) produced shoots with average length of 6.7±0.22 cm. Although the MS medium alone (without PGR) exhibited longest shoot formation, it had very poor shoot multiplication rate (1.8±0.20).

Therefore, MS medium supplemented with 4.44 µM of BA was selected as optimal shoot multiplication medium and used for further study. And by repeated subculturing of nodal segments from newly formed axenic shoots, a high-frequency rapid shoot culture protocol was established.

In the present study, we also observed numerous tendrils formed from the nodes of *in vitro* multiplied shoots (Figure 1B) when cultured either on the MS basal medium or on the media with different combinations of above mentioned cytokinins and auxins. Presence of tendrils throughout the culture period was also remarkable. Our observations regarding *in vitro* tendril formation were contradictory with the observation of Kielkowska and Havey (Kielkowska and Havey, 2012) in *Cucumis sativus*. They reported that the formation of tendrils in cucumber, a member of Cucurbitaceae, is closely associated with flower bud formation. But in the present study, tendrils were formed both in *in vitro* multiplied shoots without flower buds as well as *in vitro* grown complete plants with roots and flower buds.

3.2. Role of Fe-EDTA on relieving the chlorosis of in vitro grown plants

In the present investigation, another important observation was the occurrence of chlorosis in the leaves of *in vitro* multiplied plants of *L. acutangula* when maintained for long time (about 45 days) without sub culturing in the MS medium containing 20 mM of Fe-EDTA. Therefore to prevent chlorosis, planlets were cultured on MS medium containing higher concentrations (30 mM, 40 mM and 50 mM) of Fe-EDTA. It was observed that, doubling of 40 mM of FeSO4 and Na_2EDTA resulted in chlorosis prevention of *in vitro* leaves.

Chlorosis has been a problem in tissue culture in many plant species like *Carica papaya* (Castillo et al., 1997), watermelon (Thomas et al., 2000), *Scrophularia takesimensis* (Sivanesan et al., 2008) and *Hibiscus rosasinensis* (Christensen et al., 2008). The green pigment chlorophyll is required for photosynthesis in plants. $Fe^{2+}$is an important element responsible for chlorophyll biosynthesis, and chlorosis is usually caused due to lack of it (Spiller et al., 1982). Chlorophyll content of some species may even be promoted by increasing the level of iron source than that recommended by Murashige and Skoog (Murashige and Skoog, 1962; Sivanesan et al., 2008). In case of *Luffa*, the amount of Fe^{2+}present in normal MS medium could not induce optimum level of chlorophyll biosynthesis resulting in chlorosis, whereas doubling of FeSO4 and Na_2EDTA content from 20 mM to 40 mM, could increase the chlorophyll content, thereby preventing chlorosis.

3.3. Induction of roots from in vitro grown micro shoots

To develop a successful and consistent micropropagation protocol, easy and high frequency rooting from micro shoots is very important. Root induction was achieved on both the MS and ½ MS basal media, but it was noted that in ½ MS, roots were induced quickly than in MS medium (Table 2). The morphology of the induced roots was healthy and thick. Therefore, only ½ MS medium was selected for subsequent studies. To facilitate the induction of maximum number of roots within a short period of time, ½ MS medium was supplemented with different concentrations of IBA (ranging from 2.46 to 9.80 µM). The earliest (12.4±0.40 days) and highest percentage (100%) of root induction with maximum number (11.0±0.45) of roots per explants (Figure 1C) was achieved on ½ MS medium containing 4.90 µM of IBA. Similar to our observation, lower salt concentration proved to be effective

on *in vitro* root induction of *Ixora singaporensis* (Malathy and Pai, 1998). Misra and Datta (Misra and Datta, 1999) had also suggested that medium containing half concentration of salts was optimum for root induction in *Ixora*. The enhancing role of IBA among different auxins on *in vitro* root induction has been reported to be the most effective in many previous experiments (Vengadesan et al., 2002; Feyissa et al., 2005; Husain and Anis, 2006). Monteuuis and Bon (Monteuuis and Bon, 2000) also reported that exposing the microshoots of *Acacia mangium* to 4-6 µM IBA in darkness significantly increased root induction. Jha and Jha (Jha and Jha, 1989) have also reported that the half strength of MS medium containing 4.90 µM of IBA is effective for root induction in *Allium hookeri* Thw. However, the induction of roots on higher concentration (9.80 µM) of IBA containing medium was accompanied by the development of profuse callus, which inhibited further development of roots. This result was consistent with the similar findings in root induction of *Acacia chundra* (Rout et al., 2008) where, at higher concentration (7.36 µM) of IBA, the percentage of rooting was reduced and callus was formed at the basal cut end.

Table 2: Effect of IBA on *in vitro* root induction of *L. acutangula*

Nutrient medium (3% Sucrose)	Concentrations of IBA (µM)	% of micro shoots showing root induction	No. of roots induced micro shoot $^{-1}$	Days taken for root induction
MS	0	80.00	6.6±0.22^{b}	24.2±0.49^{a}
½ MS	0	95.00	9.0±0.36^{a}	17.3±0.30^{b}
½ MS	IBA (2.46)	95.00	8.5±0.34^{a}	16.8±0.25^{b}
½ MS	IBA (4.90)	100.00	11.0±0.45^{c}	12.4±0.40^{c}
½ MS	IBA (9.80)	47.50	3.1±0.23^{d}	22.2±0.36^{a}

Each value represents the mean±SE of 10 replicates and each experiments was conducted thrice. In each column, mean±SE followed by the same letter were not significantly different (p<0.05) according to Tukey's multiple range test

3.4. *In vitro flower development and viability of pollens*

Nearly 100% of the *in vitro* grown plants induced flower buds when cultured on MS medium along with 3% sucrose (Table 3). Table 3 showed different responses of ½ MS (with 1.5% sucrose), B5 (with 3% sucrose) and SH media (with 3% sucrose) on *in vitro* flower bud induction. Plants cultured on MS medium devoid of sucrose did not flower at all (Table 3). The *in vitro* grown plants were monoecious bearing racemes of male flower buds (Figure 1D) and solitary female flower buds (Figure 1E). The induction of first flower bud (male) occurred after 4 weeks of transfer of the micro shoots to the full strength MS medium. *In vitro* grown complete plantlets, with 4 to 5 elongated internodes, induced highest number of male flower buds (32.2±1.53) after 10 weeks of implantation (Table 3). Male flower buds generally clustered in racemes in nodal region. Each cluster contained 6 to 11 male flower buds. Maximum number of female flower buds (3.2±0.25) was obtained on the same medium (Table 3) but the induction was delayed by 2 weeks compared to the male flower buds. Each male bud took 3 to 4 days to open completely.

Nearly 47.5% of the plantlets cultured on MS medium with BA (4.44 µM) produced 10.9±0.64 male flower buds and 1.2±0.20 female flower buds (Table 3); whereas 32.5% of the plantlets produced 8.50±0.63 male flower buds when cultured on Kn (2.32 µM) containing medium. MS medium containing both

Table 3: Effect of basal media and PGRs on *in vitro* flowering of *L. acutangula*

Nutrient medium	Concentrations (µM)		% of plantlets showing flower induction	No. of male flower buds plantlet^{-1}	No. of female flower buds plantlet^{-1}
	Cytokinins	auxins			
½ MS (1.5% Sucrose)	0	0	72.50	13.2±0.62^{a}	2.0±0.21^{cd}
MS (3% Sucrose)	0	0	100.00	32.2±1.53^{d}	3.2±0.25^{a}
B5 (3% Sucrose)	0	0	90.00	18.3±0.99^{e}	2.4±0.16^{bc}
SH (3% Sucrose)	0	0	86.50	15.8±0.74^{f}	1.5±0.16^{de}
MS (6% Sucrose)	0	0	97.50	23.8±0.86^{g}	2.8±0.20^{ab}
MS (3% Sucrose)	BA (2.22)	0	47.50	13.7±0.66^{a}	1.4±0.22^{de}
MS (3% Sucrose)	BA (4.44)	0	47.50	10.9±0.64^{h}	1.2±0.20^{e}
MS (3% Sucrose)	Kn (2.32)	0	32.50	8.50±0.63^{b}	0
MS (3% Sucrose)	Kn (4.65)	0	22.50	6.70±0.42^{bc}	0
MS (3% Sucrose)	BA (4.44)	Kn (2.32)	62.50	5.50±0.42^{c}	0
MS (3% Sucrose)	BA (4.44)	IAA (0.57)	0	0	0
MS (3% Sucrose)	BA(4.44)	NAA (0.54)	0	0	0
MS (0% Sucrose)	0	0	0	0	0

Each value represents the mean±SE of 10 replicates and each experiments was conducted thrice. In each column, mean±SE followed by the same letter were not significantly different (p<0.05) according to Tukey's multiple range test

BA 4.44 and Kn 2.32 µM produced only male flowers buds (5.50±0.42) and inhibited female flower induction (Table 3). There was no flower bud induction on the media when 4.44 µM of BA was added in combination with 0.57 µM of IAA or 0.54 µM of NAA. In the present study, it has been observed that the media containing 3% sucrose (w/v) has better effect than 6% sucrose in terms of percentage of flowering and number of flowers.

In the present investigation it has also been observed that *in vitro* flowering of *L. acutangula* occurred in 16 h photoperiod when cultured on MS basal medium along with sucrose as a carbon source. Flowers produced from tissue culture system presented normal morphological aspects like the field grown plants. Female flower buds had normal inferior ovary (Figure 1E), one of the significant characters of cucurbits. Normal anthesis was also observed in *in vitro* mature male flowers (Figure 1F). Two different approaches were adopted to estimate pollen viability: staining pollen with dye (aceto-carmine) and *in vitro* germination assay. Staining techniques aim to determine cell membrane integrity and nuclear viability. On the other hand *in vitro* germination assays determine the actual germination ability of pollens under suitable conditions (Tuinstra and Wedel, 2000). Positive nucleus staining with aceto-carmine and *in vitro* pollen tube formation proved that about 90.5% of the pollens from *in vitro* male flowers was viable (Figure 1G & 1H). Therefore, it can be suggested that the present study of *in vitro* flowering of *L. acutangula*, can be a source of viable aseptic anthers, and thus can be directly used for haploid culture.

It was observed that the present *in vitro* flowering protocol does not require any PGR and was differentially influenced by types of basal media along with different concentrations of sucrose. It has been reported that different concentrations of carbon sources (mono or disaccharides) in the medium have a determining influence on the formation of reproductive buds (Sivanesan and Jeong, 2007a). Although cytokinins and auxins individually or in combination had been shown to play a critical role in *in vitro* flower induction in different plant species like *Vitex negundo* (Vadawale et al., 2006), *Pentanema indicum* (Sivanesan and Jeong, 2007a), *Dioscorea zingiberensis* (Huang et al., 2009), we have not found such observation in *Luffa*. This result coincides with the earlier reports on *Pisum sativum* (Franklin et al., 2000) and *Gentiana trifolia* (Zhang and Leung, 2002). The inhibitory effect of Kn on induction of flowers in *Chamomilla recutita* L. was reported earlier by Kintzios and Michaelakis (Kintzios and Michaelakis, 1999).

It was also noted that nearly100 % of the *in vitro* grown plants induced both male and female flower buds in MS medium (with 3% sucrose). In the present study, the basal media with 3% sucrose induced more flower buds than 1.5% sucrose containing ½ MS medium and surprisingly plants cultured on carbohydrate less medium could not produce any flower bud. Such observations were also reported by Kanchanapoom et al., (Kanchanapoom et al., 2011) in *Gypsophila paniculata* where they have mentioned that MS medium without sucrose is unable to induce *in vitro* flower buds. But, on the other hand, Dickens and Van Staden reported stimulatory effect of 2% to 4% sucrose in *in vitro* flowering of *Kalanchoe blossfeldiana* (Dickens and Van Staden, 1988). Therefore, it can be concluded that sucrose at optimum concentration (3%) along with MS medium plays a key role in *in vitro* flower induction in the present study. Our result agrees with the observation of Zhang in *in vitro* flowering of *Perilla frutescens* (Zhang, 2007). He reported that among different concentrations of sucrose, only 3% sucrose was the most effective one for inducing maximum flowers. The same observations were also reported by Lee et al., (Lee et al., 1989) in ginseng, Al-Wareh et al., (Al-Wareh et al., 1989) in potato.

It has also been noted that, *in vitro* grown plants flower regularly and repeatedly throughout the year after initiation of flowering. Scorza (Scorza, 1982) reported that the induction of *in vitro* flowering in many plant species was triggered by the interaction of sucrose and photoperiod. Previous work in several plants demonstrated that the molecular mechanism of floral bud induction is accompanied by light regulated import of exogenous metabolizable sugars (mainly sucrose) through an up-regulation of sugar transporters (Maurel et al., 2004; Decourteix et al., 2008; Girault et al., 2010; Henry et al., 2011). Therefore, the observations reported here could offer novel opportunities for further studies about the molecular physiology of flowering, particularly to understand how light dependent sugar-mediated pathways regulate floral transition in *L. acutangula* under controlled *in vitro* conditions. In the present study nearly 90.5% of pollen grains formed in *in vitro* male flowers were found to be viable (Tuinstra and Wedel, 2000), which enabling them to use in aseptic anther culture for further studies.

The complete process of micropropagation includes successful transplantation of in vitro grown plantlets. For this, both the in vitro derived, healthy rooted plantlets and the plantlets with male and female flower buds were hardened in controlled conditions (Figure 1I) and then transferred to the field (Figure 1J). It was observed that 94.5% of the acclimatized plants survived and produced both the male and female flowers after 4 weeks of transplantation to the field. Being monoecious, the *in vitro* grown plantlets of *L. acutangula* need a suitable mode of *in vitro* cross-pollination for seed setting. In the present study these plants were hand-pollinated after hardening in the field. About 66.5% of the hand-pollinated plants bearing both male and female flowers produced an average of 12±2.0 healthy

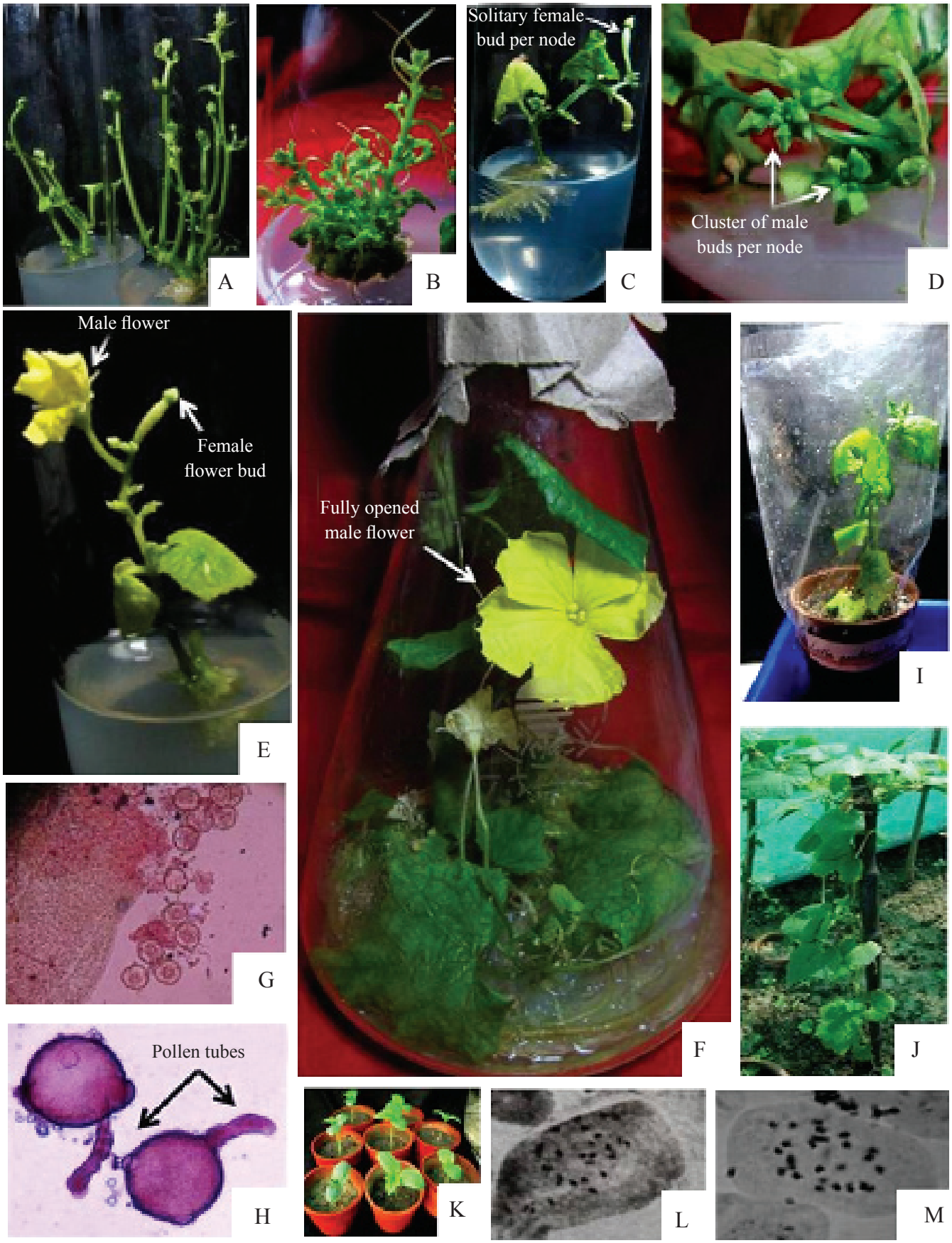
A
B
Solitary female bud per node
C
Cluster of male buds per node
D
Male flower
Female flower bud
E
Fully opened male flower
F
I
G
J
Pollen tubes
H
K
L
M

Figure 1: (A-M) Micropropagation and *in vitro* flowering of *Luffa acutangula* (L.) Roxb. A: Maximum number of *in vitro* multiplied shoots; B: Development of numerous tendrils from each node of the *in vitro* multiplied shoots after 30 days of culture; C: Shoot bearing female flower buds with highest number of induced roots; D: Induced 10 to 11 male flower buds clustered in racemose infloresence per node; E: Induction of both male flower and female flower bud from micro shoot; F: *In vitro* grown complete plantlet bearing fully opened male flowers; G: & H: Microscopic view of aceto-carmine stanined viable pollen grains and *in vitro* germinated pollens with viable pollen tubes, respectively; I: Acclimatized plantlet; J: Tisssue culture derived plant of *L. acutangula* growing in the green house; K: Germinated seedlings of *L. acutangula*; L &M: Somatic metaphase plates showing 2n=26 chromosomes of the mother plant and one of the tissue culture raised plants, respectively.

fruits with normal seeds after 8 weeks of transplantation. Nearly 70.0% of the seeds were germinated in earthen pots containing the mix of garden soil and sand (1:1) and formed young seedlings within 2 weeks (Figure 1K). All of these seedlings were apparently uniform and did not show any morphological variations and are being maintained in the experimental garden of the R.K.M.V.C. College, Rahara.

Chromosomal study was also employed with thirty in vitro grown plants for the evaluation of cytogenetic uniformity. The study revealed the presence of stable diploid chromosome number (2n=26) as that of the mother plants (Figure 1L & 1M).

4. Conclusion

Therefore it can be concluded that, the present approach for efficient and rapid micropropagation through shoot multiplication offer a potential system for a large scale propagation of multiple elite clones of *L. acutangula*. On the other hand, easy flowering of *L. acutangula* under *in vitro* conditions can be used for plant breeding and anther culture studies for the production of homozygous plants which can also accelerate breeding programs. Consequently, *in vitro* flowering of *L. acutangula* can also be a prospective way to save time in future genetic improvement programs using intra-or inter-specific crosses and can help to reveal novel properties and characteristics pertaining to fruit quality.

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