In vitro Regeneration protocol for Mulberry (Morus alba L.) through Tissue Culture Techniques

Md. Sohel Rana[1*], Muhammad Nurul Amin[2] and Mustafa Abul Kalam Azad[2]

[1]*Regional Bamboo Research and Training Centre, Bangladesh Forest Research Institute, Domar, Nilphamari-5340, Bangladesh*
[2]*Professor, Department of Botany, University of Rajshahi-6205, Bangladesh*
**Corresponding author*

Abstract: **This research work was taken for enormous production of white mulberry (*Morus alba* L.) through micropropagation techniques. Surface sterilization of the explants collected from the field grown mature plants was important and it was done with 0.1% $HgCl_2$ (Mercuric chloride) solution at different durations. Result showed that duration of the treatment was the best at 12 minutes for both shoot tips and nodal segments. Axillary shoot proliferation of nodal explants was better than that of (terminal) shoot tips. Data analysis showed that cytokinin BAP (6-Benzyl aminopurine) was more effective than Kn (Kinetin) for axillary shoot proliferation. The MS medium supplemented with 6.0 µM was the best to grow 100% shoot proliferation of the explants where the average length was 5.57cm ± 0.05. On the other hand, different concentration and type of auxin affected greatly to induce number and percentage of roots per shoot. In this case, half MS medium supplemented with 6.0 µM IBA (Indole-3-butyric acid) was showed the highest percentage (100%) of root regeneration where the average length was 4.21cm ± 0.07.**

***Key words*: Axillary shoot, Sterilization, Micropropagation, Explants, Proliferation**

I. INTRODUCTION

Mulberry are fast growing perennial plants which are small to medium sized shrubs. The highest tall of mulberry plant is about up to 24m (80ft) wide spreading and round-topped. The role of mulberry leaves as a protein source in food formulations is satisfactory and its neuroprotective functions are also effective against neurodegenerative disorders such as Alzheimer and Parkinsonism(Butt et al., 2008). A silkworm produces luxuriant silk thread to form cocoon from mulberry leaves during larval stage(Rahmathulla, 2012).The flowers are small size, greenish-yellow colour, 4 sepals, 4 stamens and pistils bearing two styles. An oval aggregate fruit turns into individual drupelets. The fruit containing numerous brown seeds is normally white, pink or purple-black in colour. Although, over 150 mulberry species is present in the world, botanists recognize only 10–16 species widely (Datwyler et al., 2004). Generally, all mulberries grow very fast for 40–50 years and it reach upto 60–80 cm in diameter at breast height (dbh), after which their growth rates are slow. The living period of mulberry tree is about 200–300 years (Lochynska et al., 2011).

Mulberry is generally distributed in tropical, subtropical, temperate and sub-arctic areas (Machii et al., 2002).China started mulberry cultivation and silk production about 3000 years ago. From China, it was spread to Persia, India, Japan and to the East. According to western historians, the initial cultivation of mulberry had also started to India by about 140 BC. Thus, mulberry seems to be a native species either of India or China

Herb, shrubs and trees are commonly found as forest plants or weeds in Bangladesh. The plant kingdom gives food, shelter, medicinal elements for saving life and raw materials for industrial purpose in different forms. Economically and ethnobotanically mulberry seems to be an important species in traditional Chinese medicine, the fruit is used to do grey hair, to boost the blood and to treat also against constipation and diabetes(http://en.wikipedia.org).The bark is used in curing cough, wheezing, edema, fever, headache and sore eyes(http://en.wikipedia.org). Mulberry fruits also used to produce seductive drink known as mulberry wine. Being nutritious, mulberry leaves are eaten as a vegetable. The cattle are also eaten to improve the milk production.

Breeding programs and genetic manipulation play an important role to ensure high productivity in agricultural sector. In this case, tissue culture techniques are followed to propagate of plants aseptically which are also known as *in vitro* propagation or micropropagation. Now a days, micropropagation is the best used biotechnological tools for applied purpose on plant development. (Loyola-Vargas and Ochoa-Alejo, 2018). One selected elite genotype assures the best quality production of clonal plants, isolation of useful variants with better disease resistance and stress tolerance of using micropropagation techniques within a very short time and limited space(Hussain et al., 2012). Plant tissue culture also involves in industrial sector for increasing crop production, hybridization, somaclonal variation, synthetic seed formation, genetic transformation, pathogen eradication, haploid culture and preserving germplasm(Gulzar et al., 2020).In recent years, rapid multiplication of valuable endangered and threatened plants species continue sto produce elite clones through *in vitro* culture techniques (Anjum et al., 2016; Anis and Faisal, 2005; Cano-castillo et al., 2009; Karuppusamy et al., 2006; Manjkhola et al., 2005). Thus, the present investigation was an effort to develop atissue culture technique through rapid regeneration for commercial production of a variety of mulberry.

II. MATERIALS AND METHODS

Proliferating cultures of axillary shoots were established on a suitable medium from the nodal explants of field-grown mature plants of mulberry in Rajshahi University Campus. The plant materials were washed thoroughly under running tap water in the laboratory to reduce the dust and surface contaminants. The material was then separated into shoot segments and nodal segments and placed in separate flasks for surface sterilization. Surface sterilization including constant shaking was done for the shoot and nodal segments separately with 1% savlon for 10 minutes. Then the materials (shoot and nodal segments) were washed 3-4 times with distilled water and then under running laminar airflow cabinet and transferred to 250 ml sterilized conical flask. They were immersed in 0.1% $Hgcl_2$ for different duration of time. To remove trace of the sterile the material was then washed with sterile distilled water at least 4-5 changes of water. The segments containing nodes or shoot tips (1.0-1.5 cm) were prepared from the surface-sterilized material and were used as explants.

Both the surface-sterilized explants were cultured on MS medium supplemented with BA and Knin different concentrations for regenerating the axillary shoots. Data were recorded after 4 weeks of culture initiation from different treatments. The cultures were allowed to grow further on the initial medium to increase budding frequency. After 2 months, the elongated shoots (usable shoots) were excised from the proliferated cultures and transferred individually to the rooting media. Some of the shoots after removing leaves were cut into pieces having axillary buds and recultured to freshly prepared medium for multiplication of auxiliary shoots. These cultures were again produced usable axillary shoots within two weeks of subculture. The process had been repeated several times to ensure continuous production of axillary shoots.

The pH of the medium was adjusted to 5.7-5.8 using a digital pH meter with the help of 0.1N NaOH or 0.1N HCl whichever was needed. All cultures were maintained in a growth room under 16hour photoperiod with a light intensity of 2000-3000 lux provided by the cool-white fluorescent tubular lamp. The temperature of the growth room was maintained at 25±1ºC but the humidity was not controlled.

III. RESULTS AND DISCUSSION

Surface sterilization was essential before initiation of *in vitro* culture for preventing the overgrowth of microbial contaminants because microbial contamination deteriorates the growth of the excised tissue (explants). For this, surface sterilization with 0.1% $HgCl_2$ solution was applied for different durations for enhancing the viability of the usable *in vitro* cultured explants. Most of the cultured explants were affected by bacterial and fungal contamination within 3 to 10 days of inoculation without surface sterilization. Comparatively bacterial infection was more in number than both bacterial and fungal infections. There were no contamination-free shoot tips (explants) for 4 minutes surface sterilization with 0.1% $HgCl_2$ solution after 10 days whereas the maximum percentage (90%) of explants were alive and contamination-free for 12 minutes with the same concentration of $HgCl_2$. Similarly, none of the nodal segments were contamination-free for 4 minutes surface sterilization after 10 days. On the contrary, hundred percentage (100%) culturable contamination-free nodal segments were showed by treating the explants for 12 minutes surface sterilization with 0.1% $HgCl_2$ after the same period. It was observed that the killing rate of explants was increasing with the expanding duration of treatment with 0.1% $HgCl_2$ solution (Table 01). $HgCl_2$ is generally used for surface sterilization of explants in plant tissue culture to mitigate microbial and fungal contamination (Mekonnen et al., 2013). Moreover, it causes different unwanted health effect such as respiratory, dermatological, neurological, reproductive and developmental problems (Risher and Amlar, 2005; WHO, 2005; Sharma et al., 2007; Durak et al., 2010). The use information of $HgCl_2$ is present in the different research studies for surface sterilization of *ex vitro* explants.

Different concentrations of 2.0; 4.0; 6.0; and 8.0 µM BAP on MS medium were used to induce axillary shoot proliferation on two different explants shoot tips and nodal segments from field-grown mature plants of mulberry. They were grown to produce shoots *in vitro* condition aseptically. These types of shoot segment were used as explants to compare with field-grown explants for proliferating axillary shoots. In these case, the highest 60% shoot tips with average length number of 3.40± 0.07 cmof field- grown explants produced axillary shoot proliferation on MS medium supplemented with 6.0 µM BA while nodal segments showed 70% shoots regarding average length number of 3.92 ± 0.08 cmon the same medium (Table 02). Similarly, the highest 100% nodal segments were average length number of 5.57 ± 0.05 cmand 80% shoot tips followed by average length number of 5.12 ± 0.08cm grown axillary shoot in *in vitro* condition on 6.0 µM BAP medium respectively (Table 03). It was seen that nodal segments of the *in vitro* and mature plants are better to produce axillary shoots than the shoot tips of explants. Moreover, nodal segments derived from*in vitro* plants were the best to proliferate axillary shoots rapidly. (Nalawade et al., 2003) also proved that nodal explants were better to regenerate plantlet than shoot tip explants in *in vitro* plantlet regeneration recast.

Table 1. Surface sterilization of shoot tip and nodal segments

Treatment duration(min)	Type of explants	Rate of contamination (after days of treatment)					Contamination-free explants after 10 days (%)
		2	4	6	8	10	
	Shoot tip						
4	,,	-	4	6	10	10	0
6	,,	-	-	6	8	10	0
8	,,	-	-	7	8	8	20
10	,,	-	-	-	4	5	50
12	,,	-	-	-	4	1	90
14	,,	-	-	-	-	-	100*

16	,,	-	-	-	-	-	100**
18	,,	-	-	-	-	-	100***
Nodal segment							
4	,,	-	4	7	8	10	0
6	,,	-	3	5	8	10	0
8	,,	-	-	2	4	5	50
10	,,	-	-	-	1	2	80
12	,,	-	-	-	-	-	100
14	,,	-	-	-	-	-	100*
16	,,	-	-	-	-	-	100**
18	,,	-	-	-	-	-	100***

"-" indicates no contamination

"*" indicates culture death due to tissue killing (* = 5-25%, **= 26-50%, ***= 50-100%)

Table 2. Effect of different concentrations of BAP on (field-grown) nodal and shoot tip explants for axillary shoot proliferation

Growth regulator (BA µM)	Type of explants	Explants showed proliferation (%)	Total No. of shoot/culture	Length of shoot/ culture (cm)
2.0	Shoot tip	40	2.22 ± 0.08	2.61 ± 0.06
4.0		50	2.40 ± 0.06	2.94 ± 0.05
6.0		60	2.63 ± 0.04	3.40 ± 0.07
8.0		50	2.51 ± 0.07	3.08 ± 0.09
2.0	Nodal segment	40	2.32 ± 0.09	3.25 ± 0.05
4.0		60	2.74 ± 0.07	3.64 ± 0.06
6.0		70	2.92 ± 0.06	3.92 ± 0.08
8.0		50	2.45 ± 0.08	3.40 ± 0.06

Table 3. Effect of different concentrations of BAP on nodal and shoot tip explants from *in vitro* proliferated shoots for axillary shoot proliferation

Growth regulator (BAP µM)	Type of explants	Explants showed proliferation (%)	Total No. of shoots/culture	Length of shoot/culture (cm)
2.0	Shoot tip	50	2.40 ± 0.08	3.68 ± 0.07
4.0		70	2.71 ± 0.06	4.72 ± 0.05
6.0		80	2.96 ± 0.05	5.12 ± 0.08
8.0		60	2.62 ± 0.07	4.08 ± 0.06
2.0	Nodal segment	70	2.71 ± 0.07	4.54 ± 0.05
4.0		90	2.90 ± 0.06	5.28 ± 0.06
6.0		100	3.25 ± 0.04	5.57 ± 0.05
8.0		80	2.88 ± 0.07	4.94 ± 0.08

Shoot tips and nodal segments collected from the field grown plants were cultured to get the maximum numbers of shoot proliferation and for choosing the best cytokinin concentration on MS medium supplement with BAP and Kn alone at 2.0, 4.0, 6.0 and 8.0 µM concentration. When explants cultured at concentration of 6.0µMKn, the effect of Kn was the most to regenerate shoots among the other concentrations of Kn where the average length of shoots was 4.52 ± 0.05 cmand culture formed usable shoots were 80%. On the other hand, the average lengths of shoots was5.48 ± 0.02cmand culturable shoots were 100% at concentration of 6.0 µM BAP which was the best result among the other concentrations of BAP(Table 04). Therefore, the results ensured that the activity of BAP is more effective to form axillary shoots than Kn. There were other plants like like *Ocimumsanctum* (Jamal et al.,2016), *Rosmarinus officinals* (Leelavathi et al., 2013), *Arachishypogaea* (Al-Joboury., 2012) and*Atropa belladonna* (Ahmed et al., 2019) whereas the superior activity of BAP to other cytokinin was proven to grow *in vitro* shoots reproduction.

Table 4.Effect of different concentrations of cytokinin alone in MS medium in direct regeneration of shoots from explants of mulberry

PGR (µM)		% of culture forming usable shoots	Average length of shoots (cm)	Total no. of nodes per culture	Days taken for shoot initiation
Control		-	-	-	-
BAP	2.0	70	4.30 ± 0.05	3.60 ± 0.37	7.0
	4.0	80	4.40 ± 0.04	4.00 ± 0.31	6.0
	6.0	100	5.48 ± 0.02	6.42 ± 0.51	6.0
	8.0	90	4.95 ± 0.04	5.12 ± 0.56	5.0
Kn	2.0	50	3.80 ± 0.06	3.20 ± 0.51	8.0
	4.0	60	4.07 ± 0.05	3.62 ± 0.51	7.0
	6.0	80	4.52 ± 0.05	5.02 ± 0.58	6.0
	8.0	50	4.15 ± 0.06	4.02 ± 0.59	6.0

Micro-shoots (2.0-3.0cm long) collected from the *in vitro* culturable shoots cultured on ½ MS medium. In this case, different concentration with 2.0 µM, 4.0 µM, 6.0 µM and 8.0 µM of either IBA or NAA (α-Naphthalene acetic acid)were taken for root initiation. After 4 weeks of culture, the percentage of root formation, number of roots per shoot and length of the longest roots were counted to compare with these two auxins. From the observation, it was seen that the concentration of 6.0 µM IBA was the best at the highest 100% root formation regarding4.21cm± 0.07 average length of roots among the other concentrations of IBA. On the other hand, 4.0 µM concentration of NAA was the best at the highest 70% root formation followed by 2.36cm±0.05average length of roots among the other concentrations of NAA (Table 05). Therefore, it was cleared that IBA was better to produce roots than NAA. There were other plants such as *Daturametel*(Amiri et al., 2010),*Elaeocarpus robustus* (Rahman et al.,2009)and *Punica granatum* (Prabhuling et al., 2018) whereas the same observation was seen to produce roots *in vitro* condition.

Table 5. Effects of different concentrations of auxins for root formation on half strength of MS medium *in vitro*

PGR (µM)		Root formation (%)	No. of roots/cutting	Average length of roots (cm)	Days to root formation
Control		-	-	-	-
IBA	2.0	80	3.80 ± 0.28	1.78 ± 0.04	15.0
	4.0	80	4.70 ± 0.44	2.08 ± 0.05	14.0
	6.0	100	8.50 ± 0.84	4.21 ± 0.07	14.0
	8.0	90	6.40 ± 0.45	3.16 ± 0.06	15.0
NAA	2.0	60	3.60 ± 0.32	2.02 ± 0.07	14.0
	4.0	70	4.40 ± 0.22	2.36 ± 0.05	14.0
	6.0	60	5.90 ± 0.18	1.96 ± 0.06	15.0
	8.0	50	3.20 ± 0.16	1.72 ± 0.05	15.0

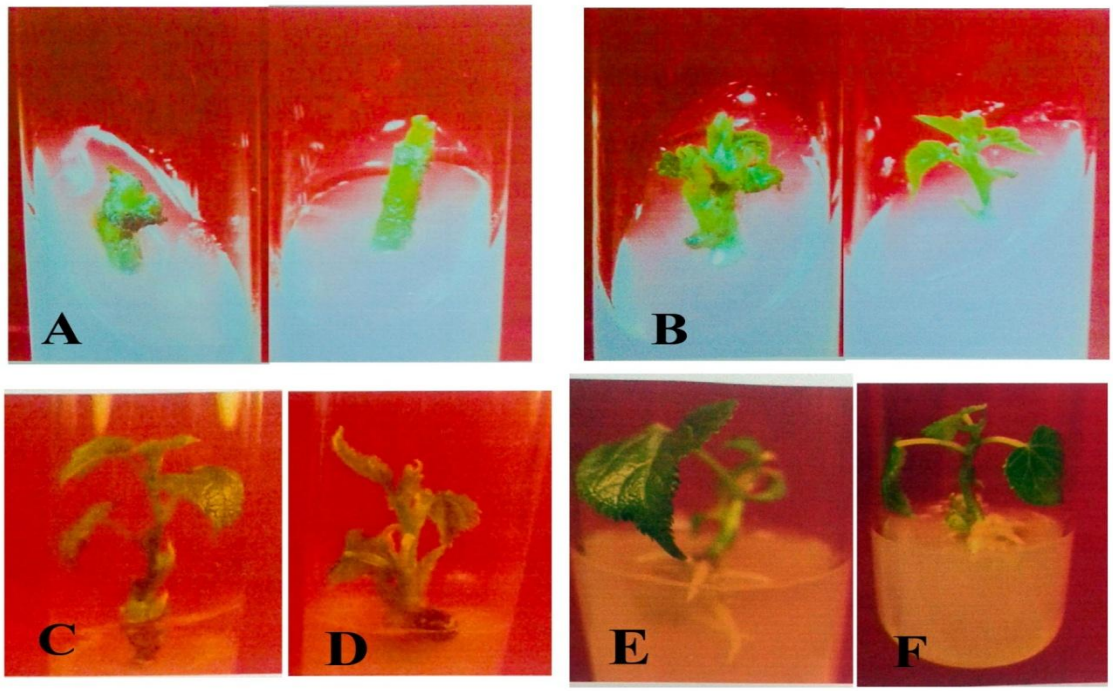

Figure A-F: ***In vitro*** **development of shoots and roots of complete planlets in white mulberry Roxb**
A: The initial culture of nodal segments on MS medium with 6.0 µM BA.
B: The initial culture of shoot tips on MS medium with 6.0 µM BA.
C: Axillary shoot proliferation from nodal segments on MS medium with 6.0 µM BA.
D: Axillary shoot proliferation from shoot tips on MS medium with 6.0 µM BA.
E: The development of roots on half strength of MS medium with 6.0 µM IBA.
F: The development of roots on half strength of MS medium with 6.0 µM NAA.

IV. CONCLUSION

A suitable research protocol was proved for large production of white mulberry using shoot tips and nodal segments through different experiments of micropropagation. Overall, based on axillary shoot proliferation, culture multiplication and root formation of the *in vitro*shoots, the nodal explants were better than the shoot tips.

ACKNOWLEDGMENTS

I am very grateful to the Plant Tissue Culture Laboratory authority of Rajshahi University for their sincere cooperation to execute research activities.

REFERENCES

[1] Ahmed, H. and Ammen, B. Z. (2019). Atropa belladonna L. By Tissue Culture and Determination of Some Alkaloids in Callus. Horticulture Science and Landscape Design (Plant Tissue Culture), University of Mosul, College of Agriculture and Forestry.

[2] Al-Joboury, Kh. R. (2012). In Vitro Propagation of Groundnut (Arachis hypogea L.). Iraq Natural History Research Center and Museum, University of Baghdad.

[3] Amiri, S., Kazemitabar, S. K., Ranjbar, G. A. and Azadbakht, M. (2010). In vitro propagation and whole plant regeneration from callus in Datura (Datura stramonium. L). African Journal of Biotechnology, 10(3), 442-448.

[4] Anis, M. and Faisal, M. (2005). In vitro regeneration and mass multiplication of Psoraleacorylifolia-an endangered medicinal plant. Indian Journal of Biotechnology, 4 (2), 261-264.

[5] Anjum, S., Haider Abbasi, B. (2016). Biomimetic synthesis of antimicrobial silver nanoparticles using in vitro-propagated plantlets of a medicinally important endangered species (Phlomisbracteosa). International Journal OfNanomedicine, 11, 1663-1675.

[6] Butt, M. S., Nazir, A., Sultan, M. T. &Schoën, K. (2008). Morus alba L. nature's functional tonic, Trends in Food Science & Technology, 19, 505–512.

[7] Cano-castillo, M., Serrano-Martinez, F., and Casas, J.L. (2009). In-vitro propagation of Astragalus nitidiflorus (Leguminosae), an endemic and endangered species from South-East of Spain. Acta Horticulturae, 812, 545-550.

[8] Datwyler, S. L. and Weiblen, G. D. (2004). On the origin of the fig: Phylogenetic relationships of Moraceae from NDHF sequences, pp. 767-777.

[9] Durak, D., Kalender, S., Gokce, F., Demyr, F. and Kalender, Y. (2010). Mercury chloride- induced oxidative stress in human erythrocytes and the effect of vitamins C and E in vitro. African Journal of Biotechnology, 9(4), 488-495.

[10] Gulzar, B., Mujib, A., Malik, M. Q. J., Rukaya, S. and Zafar, N. (2020). Transgenic Technology Based Value Addition in Plant Biotechnology, pp. 25-49.

[11] Hussain, A., Qarshi, I. A., Nazir, H. and Ikram, U. I. (2012). Plant Tissue Culture: Current Status and Opportunities, Intech Open Limited, London, United Kingdom, pp. 1-28.

[12] Jamal, M. A. H. M., Sharif, I. H., Shakil, M. M., Rahman, A. N. M. R-B., Banu, N. A., Islam, M. R. and Nazmuzzaman, M. (2016). In vitro regeneration of a common medicinal plant, Ocimum sanctum. L. for mass propagation, 15 (24), pp. 1269 -1275.

[13] Karuppusamy, S., Kiranmai, C., Aruna, V., and Pullaiah, T. (2006). Micropropagation of Vanasushavapetada- anendangered medicinal plant of South India. Plant Tissue Culture of Biotechnology, 16(2), 85-94.

[14] Leelavathi, D. and Kuppan, N. (2013). In vitro regeneration from apical bud of Rosmarinus officinalis L. An important medicinal Plant. Banat's Journal of Biotechnology, 4(8), 14-19.

[15] Lochynska, M. and Oleszak, G. (2011). Multi-use of the white mulberry (Morus alba L.). Department of Silkworms Breeding and Mulberry Cultivation, Institute of Natural Fibres and Medicinal Plants, WojskaPolskiego, Poznan, Poland. Journal of Ecological Questions, 15, 91 – 95.

[16] Loyola-Vargas, V.M. and Ochoa-Alejo, N. (2018). An Introduction to Plant Tissue Culture: Advances and Perspectives. Humana Press, New York, pp. 3-13.

[17] Machii, H., Koyama, A. and Yamanouchi, H. (2002). Mulberry Breeding, Cultivation and Utilization in Japan, National Agriculture and Food Research Organization, Japan.

[18] Manjkhola, S., Dhar, U. and Joshi, M. (2005). Organogenesis, embryogenesis and synthetic seed production in Arnebiaeuchroma- a critically endangered medicinal plant of the Himalaya. In-vitrocelluler and Developmental Biology-Plant, 41(3), 244-248.

[19] Mekonnen, T., Diro, M. and Sharma, M. (2013). An alternative safer and cost effective surface sterilization method for sugarcane (Saccharum officinarum L.) explants. African Journal of Biotechnology, 12(44), 6282-6286.

[20] Nalawade, S. M. and Sagare, A. (2003). Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Botanical Bulletin Academia Sinica Taipei, 44 (2), 79-98.

[21] Prabhuling, G. and Huchesh, H. (2018). In Vitro Regeneration in Pomegranate (Punica granatum L.) cv. Bhagwa using Double Nodal Segments. Centre for Horticulture Biotechnology, Directorate of Research, University of Horticulture Sciences, Bagalkot, Karnataka, India. Research Journal of Biotechnology, 13 (8), 1-8.

[22] Rahman, M. M., Amin, M. N., Ishiguri, F., Yokota, S., Sultana, R. S., Takashima, Y., Lizuka, K. and Yoshizawa, N. (2009). In vitro plantlet regeneration of "dwarf" Indian olive (Elaeocarpus robustus Roxb.): A fruit plant of Bangladesh. Plant Biotechnology reports, 3 (3), 259-266.

[23] Rahmathulla, V.K. (2012). Management of Climatic Factors for Successful Silkworm (Bombax mori L.) Crop and Higher Silk Production. A Review, Psyche: A Journal of Entomology, 1-12. https://doi.org/10.1155/2012/121234

[24] Risher, J.F. and Amler, S.N. (2005). Mercury exposure: evalution and intervention, the inappropriate use of chelating agents in diagnosis and treatment of putative mercury poisoning. Neuro Toxicology, 26(4), 691-699. .

[25] Sharma, M.K., Sharma, A., Kumar, A. and Kumar, M. (2007). Spirulina fusiformis provides protection against mercuric choride induced oxidatives stress in Swiss albino mice. Food chemical Toxicology, 45, 2412-2419.

[26] WHO, (2005). Mercury in drinking water.