INTRODUCTION
Grape (Vitis vinifera L.) is a refreshing, nourishing and delicious fruit of the world. It is one of the most important fruit crops grown in the world today in terms of both total acreage and dollar value [1]. At present, its acreage of production is around 10 million hectares [2]. The world area of grape is 8.37 million hectares producing 74.30 million tons per annum [3]. It belongs to the family Vitaceae which is made up of 12 genera consisting of about 600 species and 800 varieties widely distributed all over the world [4]. Among fruits, grape occupies the first position in the world in terms of area and production [5]. As fruits, grapes are a rich source of vitamins A, C, B6, as well as essential minerals, such as potassium, calcium, iron, phosphorus, magnesium and selenium, which are necessary for human health [6]. In Bangladesh grape is a rare food item but throughout the world grape is a popular food. Preliminary observation trail conducted at BARI concluded that grape can be grown in Bangladesh as a fruit crop [7]. Recently farmers also want to make vineyard at commercial scale. But the problem is that non availability of virus free planting materials and good varieties of grape [8]. Some plants produce seeds remain viable only for a limited duration and some do not produce seeds and only require vegetative propagation. The vegetative propagation also faces challenge due to high rate of mortality and low rate of rooting in soil [9]. All grape varieties are propagated through grafting, layering and stem cutting in conventional method. Sometimes this method is hampered by seed and cutting dormancy, seedling heterozygosity, time consideration and space, and limited yield. The improvement in production and quality of grapes can be achieved by practicing genetic and sanitary clonal selection through incorporation of unconventional propagation method like tissue culture which is adopted an established method for the commercial propagation of herbaceous and woody plant species [10]. Propagation by axillary shooting has proved to be the most applicable and reliable method of in vitro propagation of Vitis vinifera L [11].

The main objectives of this study were to establish a reproducible cost effective protocol, to establish standardized HgCl2 treatment for explants...
sterilization process, to select suitable growth regulators for proper multiple shoots regeneration, elongation and root induction to produce a large number of plantlets within a short time for large scale production of *Vitis vinifera* L. in Bangladesh.

**MATERIALS AND METHOD**

**Collection of Plant Materials and Explant Preparation through HgCl₂ Treatment**

Terminal shoot segments with immature leaves from field grown plants were collected. The excess unnecessary parts like tendrils and leaves were removed from the collected materials and the remaining part of shoot segments were cut into five positions with convenient size (5-6 cm in length). The cutting positions of explant were numbered as P₁, P₂, P₃, P₄ and P₅. Shoot tips were the position number one (P₁) and nodal segments were position number P₂, P₃, P₄, P₅ respectively. Then they were collected in separated conical flask. Both the materials were washed thoroughly under running tap water for several times to reduce the dust and surface contaminants and then were taken in conical flask containing distilled water with a few drops of savlon and washed for 4-5 minutes with constant shaking. Second time washing was performed through dipping in 70% (v/v) ethanol for 30 seconds gradual change of distilled water until all traces of above chemicals were removed. The procedure of surface sterilization was carried out in side of laminar airflow cabinet. The above materials were taken into sterile flask and suspended in 0.1% HgCl₂ solution for different period to ensure contaminant free culture. The sterilized materials were washed 7-8 times with sterile distilled water immediately to remove all the traces of HgCl₂. The surface sterilized explants were sized into 2.0-4.0 cm in length.

**Media Preparation with Different Hormonal Combination and Inoculation of Explants**

Full strength MS [12] medium with different concentrations of BAP and NAA was used for shoot induction and for root induction MS medium with different combinations of IBA and NAA were used. For carbon source 3% sugar was used and the medium was solidified with 0.7% agar. In all tests the pH of the medium was adjusted to 5.7 ±1 before addition of agar. Finally the culture vessels containing the medium were autoclaved at 15 id/inch² pressures and at the temperature of 121°C for 20 min to insure sterilization. Then the vessels with the medium were allowed to cool as vertically.

Prepared explants were carefully inoculated in culture vessels (test tube) containing agar gelled nutrient medium supplemented with different concentration of hormones. The cotton plugs of the culture vessels were removed inside laminar airflow cabinet in presence of spirit lamp flame. Then the inoculation procedure was applied. Inoculation of explants was made singly per culture vessels. During inoculation, special care was taken so that the explants could touch on the medium equally and did not dip into the medium. After inoculation the mouth of culture vessels were tightly plugged and marked by glass marker with inoculation date. Then the vessels were ready for incubation. In incubation all cultures were grown in the growth chamber illuminated by 40 watts white fluorescent light intensity varied from 2000-3000 lux. The photoperiod was maintained generally 16 hours and 8 hours dark. The culture vessels were checked daily to note the response.

**Sub culture for multiple shoot and root induction**

When the regenerated shoots were 2-3 cm in length, they were removed aseptically from the culture vessels and placed on a sterilized petri dish and remove the old media by washing through double sterilized distill water and again transferred into test tubes containing the same of different hormones supplemented media.

The regenerated shoots were removed aseptically from the culture vessels and placed on a sterilized petri dish and they were cut 2-4 cm in length from the basal end of the shoots. Then each of the shoot was inoculated on freshly prepared medium containing full strength of MS medium with different combination and concentration of hormonal supplements for root induction.

**Acclimatization and Transplantation of Plantlets**

The rooted shoots (neo-formed plantlets) were kept in their media for 14, 24, 34 days without subculture before acclimatization to enhance the efficiency of roots. Neo-formed plantlets were washed thoroughly to remove any medium residual. The neo-formed plantlets were treated with Benelet (antifungal) 1 g/l and potted into a mixture of peat moss 1:1 sand (v/v), then covered with white plastic bags, to maintain high humidity the plantlets and maintained in a growth chamber at 27+2°C under 16h illumination (45 μmole lux. The photoperiod was maintained generally 16 hours and 8 hours dark. The culture vessels were checked daily to note the response.

**Statistical Analysis**

Variance analysis of data was done using ANOVA program for statistical analysis. Data were analyzed using SPSS software (Ver. 11.5)

**RESULTS & DISCUSSION**

Effects of 0.1% HgCl₂ with different duration on surface sterilization and different position of explants

To establish explants from field grown plants under aseptic condition surface standardization is essential. Standardization for surface sterilization was carried out by trial and error experiments. Surface sterilization was carried out by 0.1% HgCl₂ solution at different time duration. When the explants were treated
with 0.1% HgCl₂ solution for 0.5 minute, contamination was occurred as the treatments failed to kill the microorganisms attached to the explants. 30%, 65% and 85% of explants were found contamination free with healthy tissue when they were treated for 1, 2 and 3 minutes respectively. No contamination was found but partial and complete tissue killing was observed when explants were treated for 4-5 minutes and more than five minutes (Table 1).

### Table 1: Effects of 0.1% HgCl₂ with different duration of explants surface sterilization

| HgCl₂ Treating period (Minutes) | No. of different explants | Contamination rate after days | % of survival explants |
|---------------------------------|---------------------------|------------------------------|------------------------|
| 0.5                             | 20                        | 0                            | 5                      |
| 1.0                             | 20                        | 4                            | 35                     |
| 2.0                             | 20                        | 7                            | 45                     |
| 3.0                             | 20                        | 10                           | 50                     |
| 4.0                             | 20                        | 12                           | 60                     |
| >5.0                            | 20                        | 15                           | 65                     |

Note: * = No contamination, ** = Partial tissue killing, *** = Moderate tissue killing and **** = Complete tissue killing.

When lower concentrations of HgCl₂ were used in short duration they fail to kill the microorganisms attached to the surface of the explants. When higher concentrations of HgCl₂ were used in short duration, it showed some efficiency but when those higher concentrations were applied in long duration tissue killing occurred.

In case of positional treatment of explants with 0.1% HgCl₂ solution at different time duration were survive 35% of explants on P₁, 45% of explants on P₂ and 50% of explants on P₃, 60% of explants on P₄ and 65% of explants on P₅ (Table 2).

### Table 2: Effects of 0.1% HgCl₂ with different position of explants surface sterilization

| Different position of explants | No. of different explants | Treating period in Minutes HgCl₂ | % of survival explants |
|--------------------------------|---------------------------|---------------------------------|------------------------|
| P₁                             | 20                        | 1.0, 2.0, 3.0, 4.0, 5.0         | 35                     |
| P₂                             | 20                        | 2.0, 3.0, 4.0, 5.0              | 45                     |
| P₃                             | 20                        | 3.0, 4.0, 5.0                   | 50                     |
| P₄                             | 20                        | 2.0, 3.0, 4.0, 5.0              | 60                     |
| P₅                             | 20                        | 2.0, 3.0, 4.0, 5.0              | 65                     |

Note: # = Contamination, *** = Complete tissue killing

### Effect of Hormones for Establishment of Grapevine culture from Shoot Tip and Nodal Segment

In vitro establishment of grapevine (Vitis vinifera L.) shoot tips and stem segments were cultured on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA). Results of this study have been presented in Table 3. In shoot tip culture the highest percentage of shoot induction (90%) was noticed in MS+ 0.5mg/l BAP and 0.1mg/l NAA. The highest mean number of shoots formed per explants was obtained 1.35 in MS+ 1.0mg/l BAP and 0.1mg/l NAA within 10-18 days. The highest length of shoot was recorded 2.84 cm in MS+ 2.0 mg/l BAP and 0.1mg/l NAA. Lowest percentage of shoot multiplication was 75% and length of shoot was 2.67 cm MS+ 0.5mg/l BAP+ 0.1mg/l NAA. However, this result could be ascribed to the mode of action of BAP as cytokinins at 1.00 mg/l that stimulate both cell division and promote growth of axillary shoots in plant tissue culture as reported by Trigiano and Gray [13] and George et al. [14]. These results are compatible with the previous studies which disclosed that BA is the most effective among other cytokinins for inducing shoot development in Vitis [15, 16]. Further, BAP at 1.00 mg/l concentration was the best for shoot proliferation in both ‘Soltanin’ and ‘Sahebi’ cultivars that was reported by Aazami [17].

In stem segment culture the highest percentage of shoot induction (95%) and the highest mean number of shoots formed per explants was obtained 1.30 in MS+0.5mg/l BAP and 0.1 mg/l within 8-15 days. The highest length of shoot was recorded 2.15 cm was obtained in MS+ 1.0 mg/l BAP+ 0.1 mg/l NAA. Lowest percentage of shoot induction was 75% and length of shoot was 0.56 cm was obtained in MS+ 1.0 mg/l BAP+ 0.1 mg/l NAA and MS+ 2.0 mg/l BAP+ 0.1 mg/l NAA respectively. This result could be ascribed to the mode of action of BAP a scytokinin that stimulate both cell division and enhance growth of axillary shoots in plant tissue culture as reported previously by Trigiano and Gray [13] and George et al. [14]. The shoot proliferation confided upon the balance of cytokinins.
and auxins and using lowest concentration of NAA affected well the initiation of grapevine in vitro reported by Tapia and Read [18]. Torrey and Reinert [19] reported that auxin increased the activating enzymes that break down starch and had the ability to increased proliferation of organogenesis.

Table-3: In vitro establishment of grapevine (Vitis vinifera L.) shoot tips and stem segment cultured on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA).

Data were recorded after four weeks

| Explants            | Hormones supplement used in MS medium mg/l | No. of Explants inoculated | % of explants responded | Days to shoot formation | Mean number of shoots formed / explants | Length of Shoots in cm (M±S.E.) |
|---------------------|-------------------------------------------|---------------------------|-------------------------|-------------------------|----------------------------------------|-------------------------------|
| Shoot tip culture   | BAP + NAA                                  |                           |                         |                         |                                        |                               |
| 0.5 + 0.1           | 20                                         | 90                        | 12-20                   | 1.35                    | 2.67±0.084                             |                               |
| 1.0 + 0.1           | 20                                         | 85                        | 10-18                   | 1.35                    | 2.71±0.14                              |                               |
| 2.0 + 0.1           | 20                                         | 75                        | 15-20                   | 1.00                    | 2.84±0.42                              |                               |
| Stem segment culture| BAP + NAA                                  |                           |                         |                         |                                        |                               |
| 0.5 + 0.1           | 20                                         | 95                        | 8-15                    | 1.30                    | 1.69±0.089                             |                               |
| 1.0 + 0.1           | 20                                         | 75                        | 6-13                    | 0.95                    | 2.15±0.26                              |                               |
| 2.0 + 0.1           | 20                                         | 80                        | 10-18                   | 0.80                    | 0.56±0.17                              |                               |

Note: M= mean and S.E. = standard error

Hormonal Effects Observation during Shoot multiplication and Root Induction

Shoot multiplication of grapevine on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA) have been presented in Table 4. The highest mean number of shoots formed per explants was obtained 3.1 and the highest length of shoot was recorded 4.2 cm in MS+ 3.0 mg/l BAP and 0.2 mg/l NAA. Lowest mean number of shoots formed per explants was obtained 2.3 and length of shoot was 2.5 cm in MS+ 3.0 mg/l BAP+ 0.1 mg/l NAA and MS+ 3.0 mg/l BAP+ 0.3 mg/l NAA respectively. For multiplication rate, cytokinins were effective in this respect when used in combination with an auxin. Butiuc-keul et al. [20] reported that supplementations of culture media with cytokinins improved the multiplication rate of grapevine for instance number of shoots/explants increased with the increase of BAP concentration until 2.0 mg/l with all NAA treatments.

Table-4: Effect of different concentrations and combinations of BAP, NAA for multiple shoots proliferation of grapevine (Vitis vinifera L.). Data were recorded after four weeks

| Sub culture for multiple shoots proliferation | Hormones supplement used in MS medium mg/l | Mean number of shoots formed / explants | Length of shoots in cm (M±S.E.) |
|----------------------------------------------|-------------------------------------------|----------------------------------------|-------------------------------|
| BAP + NAA                                    | 2.0+0.1                                   | 2.5                                    | 2.9±0.23                     |
|                                              | 2.0+0.2                                   | 2.4                                    | 3.3±0.12                     |
|                                              | 2.0+0.3                                   | 2.7                                    | 2.7±0.20                     |
|                                              | 3.0+0.1                                   | 2.3                                    | 3.1±0.15                     |
|                                              | 3.0+0.2                                   | 3.1                                    | 4.2±0.15                     |
|                                              | 3.0+0.3                                   | 2.6                                    | 2.5±0.18                     |

Results of root induction of regenerated shoot on MS medium supplemented with different concentration and combinations of plant growth regulators (NAA and IBA) have been presented in Table 5. The highest percentage of root induction per shoot was 85%, the highest mean number of roots formed per explants was obtained 3.3 and highest length of root was recorded 4.3 cm was obtained in MS+ 0.5 mg/l NAA + 1.0 mg/l IBA. Lowest percentage of root induction per shoot was 65% and length of root was 2.5 cm was obtained in MS+ 1.0 mg/l NAA + 1.0 mg/l IBA and MS+ 0.5 mg/l NAA + 0.5 mg/l IBA respectively. These results were close to those of Barreto and Nookaraju [21] and Butiuc-keul et al. [20] who reported that in V. vinifera cv. "Perlette" up to 95% rooting of micro-cuttings were obtained on MS medium supplemented with IBA and NAA.

Acclimatization

Acclimatization of plantlets is one of the most important steps in tissue culture to free living conditions. Data in Table 6 represented that mean survival percentage of plantlets was 58% after keeping plantlets in rooting medium for 14 days before acclimatization. The survival percentages were increased to 83% after 34 days on rooting medium before acclimatization. These results were similar to that obtained by Thomas [22] who found that large survival percent of neo-formed plantlets were successfully acclimatized and cultivated in greenhouses.
Gok et al. [23] clarified that the survival percent of neo-formed plantlets (80%) were exposed to open filed environmental conditions. It is clear that leaving the neo-formed plantlets that kept in the rooting medium for longer period of time for increased the efficiency of roots which led to the increase the survival percentages of acclimatized plantlets [24].

### Table-5: Effect of different concentrations and combinations of NAA, IBA for roots formation of grapevine (Vitis vinifera L.)

| Sub culture for root induction | Hormones supplement used in MS medium mg/l | % of rooted / shoot | Mean number of roots formed / explants | Length of roots in cm (M±S.E.) |
|-----------------------------|------------------------------------------|---------------------|----------------------------------------|-------------------------------|
| NAA+ IBA                   | 0.5+0.5                                   | 75                  | 1.9                                    | 2.5±0.36                     |
|                            | 0.5+1.0                                   | 85                  | 3.3                                    | 4.3±0.14                     |
|                            | 0.5+1.5                                   | 80                  | 3.0                                    | 3.8±0.23                     |
|                            | 1.0+0.5                                   | 75                  | 2.5                                    | 4.0±0.13                     |
|                            | 1.0+1.0                                   | 65                  | 2.7                                    | 4.2±0.17                     |
|                            | 1.0+1.5                                   | 70                  | 3.1                                    | 4.1±0.15                     |

### Table 6: According to time (14, 24, 34 days) effect of deferent concentrations of auxin (NAA & IBA) in rooting media on survival percentage of grapevine (Vitis vinifera L.) in acclimatization stage

| Days formation for roots | Survival % of plantlets in different concentration of hormones supplement used in MS medium mg/l NAA+ IBA | Mean % of Survival plantlets |
|--------------------------|-----------------------------------------------------------------------------------------------|-------------------------------|
|                          | 0.5+0.5                                                                                      | 40 60 65                        |
|                          | 0.5+1.0                                                                                      | 65 75 85                        |
|                          | 0.5+1.5                                                                                      | 75 75 80                        |
|                          | 1.0+0.5                                                                                      | 80 85 83                        |
|                          | 1.0+1.0                                                                                      | 85                               |
|                          | 1.0+1.5                                                                                      | 83                               |

### CONCLUSION

This study provides an in vitro rapid micro propagation of grapevine (Vitis vinifera L.) through observing HgCl₂ and hormonal effects. We obtain best and adequate concentration of HgCl₂ for surface sterilization as well as appropriate combination of hormones for the achievement of better-quality grapevines (Vitis vinifera L.).

### REFERENCES

1. Galletta GJ, Himerlic DG; Small Fruit Crop Management. Prentice Hall, New Jersey, 1989; 383.
2. Jalal MK; Grapes Information and cultivation Method (in Bengali). Notore Town Press, Natore. 1997; 34.
3. FAO; Food and Agriculture Organization of the United Nations Rome, 2002; 2(2): 111-114.
4. Olmo HP; Origin and distribution of grape. In: N.W. Simmonds (ed.) Evolution of crop plants Longman, London and New York, 1976; 294-298.
5. Mondal MF, Amin MR; Pholer Bagan. Club Building (First floor) Bangladesh Agricultural University, Mymensingsh, campus, Mymensingsh, 1999; 283.
6. Yerbolova LS, Ryabushkina NA, Oleichenko SN, Kampitova GA, Galiakparov NN; The Effect of Growth Regulators on in vitro Culture of Some Vitis vinifera L. Cultivars. World Applied Sciences Journal, 2013; 23 (1): 76-80.
7. Biswas M, Nazrul MI; Evaluation of some Grape lines. Bangladesh J Agril Res., 1997; 22: 51-56.
8. Chowdhury MMH, Ashrafulzaman M, Begum SN, Islam MM, Dhar P; Regeneration of plantlets from grape (Vitis vinifera L.) through different explants. International Journal of Sustainable Crop Production, 2012; 7(2):12-18.
9. Sajid GM, Siddique S, Ishtiaq M, Haq I, Anawer R; In-vitro conservation laboratory manual. PGRI, NARC 2003; 1.
10. Lewandowski VT; Rooting and acclimatization of micropropagated Vitis labrusca Delaware. Horticulture Science, 1991; 26: 586-589.
11. Sagawa Y, Kunisaki IT; Clonal propagation of orchid by tissue culture. In: Plant Tissue culture (A. Fujiwara, Ed.), 1982; 683-684.
12. Murashige T, Skoog F; A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant, 1962; 15: 473-497.
13. Trigiano RN, Gray DJ; Plant Tissue Culture Concepts and Laboratory Exercises, (2ndEd). CRC Press, Boca Raton London New York, Washington, D.C. 2000; 430.
14. George EF, Hall MA, Klerk GJD; Plant Propagation by Tissue Culture 3rd Edition Springer, 2008; 175-204.
15. Banilas G, Korkus E; Rapid micro-propagation of grapevine (cv. Agiorgitiko) through lateral bud development. E. J. Sci. Tech., 2007; 2: 31-38.
16. Papiya M, Nafisa H, Misra SC, Rao VS; In vitro propagation of a grape rootstock, de Grasset (Vitischampinii Planch.); Effects of medium compositions and plant growth regulators. Scientia Horticulture Sci. Hort. Amsterdam, 2010; 126(1): 13-19.
17. Aazami MA; Effect of some growth regulators on in vitro culture of two Vitis vinifera L. cultivars. Romani Bio. Let., 2010; 15(3): 5229-5232.

18. Tapia MI, Read PE; Propagation of grape hybrids by in vitro culture of Axillary. Agro. Ciencia, 1998; 14(1): 35-41.

19. Torrey JG, Reinert J; Suspension cultures of higher plant cells in synthetic media. Plant Physiology, 1961; 36: 483-491.

20. Butiuc-keul AL, Cotse A, Brcaciunas, Halmagyl A, Deliu A, Farago C, Iliescu M, Iuoras R; In vitro clonal propagation of several grapevine cultivars, Acta Horticulture (ISHS), 2009; 83: 151-156.

21. Barreto MS, Nookaraju A; Effect of auxin types on in vitro and ex vitro rooting and acclimatization of grapevine as influenced by substrates. India. Journal Horticulture, 2007; 64(1): 11-17.

22. Thomas P; Contribution of leaf lamina of grape nodal microcuttings to rooting, root vigour and plantlet growth in vitro. Journal of Plant Physiology, 1998; 153: 5-6.

23. Gok S, Ergenoglu F, Kuden ABJ, Dennis Jr; Propagation of several grape varieties and rootstocks by meristem culture. Acta Horticulture, 1997; 44(1): 245-250.

24. Abido AIA, Aly MAM, Sabah A, Hassanen, Rayan GA; In vitro Propagation of Grapevine (Vitis vinifera L.) Muscat of Alexandria cv. For Conservation of Endangerment. Middle-East Journal of Scientific Research, 2013; 13 (3): 328-337.