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Research Article

In vitro Seeds Germination and Seedling Growth of *Begonia Malabarica Lam.* (Begoniaceae) a Source for Anthocyanin

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Abstract

*Begonia malabarica La*m. is an ornamental species. It occupies a prominent place in indoor landscaping programms. These plants are pharmacologically useful due to their anti inflammatory, anti hepatotoxic, antioxidant and antiseptic activities, which have been attributed by anthocyanin. Generally, B. *malabarica* is propagated through seeds. The propagation of this species from seeds is particularly challenging because of dormancy of seeds and also lose their viability within a short span of time. In addition, propagation from seeds often leads in a high variability among the progeny. In this juncture, the aim of the present study was *in vitr*o seed germination by breaking dormancy and determination of the optimal parameters for germination to propagate this plant on a large scale. Seeds were standardized with different surface sterilization protocols and inoculated on different basal media without growth regulators. Various culture media, its strengths, the type and concentration of carbon source were investigated. Responses to germination varied with the culture conditions also. Seeds in half strength MS medium germinated quickly and expressed remarkable germination rate. Similarly, the *in vitro* growth performances of plantlets varied with the basal medium composition. The optimal growth performances of plants were displayed on half strength MS basal medium. Further, 3% sucrose in the culture medium was more optimal for maximum growth of plantlets. B. *malabarica* is a source of anthocyanin. So, future studies are planned to induce callus, cell line culture from these *in vitr*o germinated seedlings to extract, purify and fractionate the pigments.

Keywords: *Begonia malabarica*; *In vitro*; Germination media

Introduction

Indoor landscaping plants are produced worldwide for their aesthetic importance. Creation of novel variation in terms of qualitative characters like leaf nature, color of the flower, its fragrance, longevity, shape and architectural feature are the major economic objectives for the horticulture industries. Further, production of superior quality saplings through genetic modifications requires basic information regarding *in vitro* seed culture protocols [1]. In some ornamental genotypes shows non flowering or low production of seeds or often associated with irregular germination. Diseases also often accumulate in the saplings resulting in infected plants and low yields. Challenges with the vegetative multiplications include high perishability, handling and transportation labour costs and inconvenient weight and bulk of the samples [2].

Further, immature embryo, difficulty to obtain samples in all seasons of the year, poor germination rate, failure in seedling emergence and growth were the other hurdles associated with traditional cultivation techniques. Seed size has a special role in crop production. Small seed size indicates a low protein synthesizing ability, which is normally attributed to less availability of substrate, energy, active enzymes and machinery for protein synthesis [3].

Mass multiplication of ornamental species is labour oriented, and for that reason, *in vitro* multiplication of plants is an efficient system and used by many commercial nurseries and institutes globally for rapid plant propagation, germ plasm conservation, pathogen elimination, genetic manipulations, and for phytochemical production [4].

Begonia of *Begoniaceae* comprises approximately 1000 species and many of these are ideal indoor ornamentals and medicinal herbals. Begonia is herbaceous species with attractive foliage and can be utilized for horticultural purposes. Many begonias respond best to warm, moist conditions. Further, they prefer well drained

soil rich in compost or organic matter and shade [5]. Generally the seeds are very small with poor germination rate and time. The B. *malabarica* is commonly used by the locals for curing many skin borne disorders and also as an antioxidant herbal tonic. Ramesh et al. [6] reported the claim of the usefulness of the plant in respiratory tract infections and also suggests its use in diarrhoea and skin diseases caused by pathogenic bacteria. The plants are reported for its rich anthocyanin content. In this scenerio, the objectives of the present study were to design a protocol for mass multiplication of the plants via the *in vitro* seeds culture. This includes initiation of aseptic cultures, surface sterilization, inoculation, effect of different media, MS medium strength, effect of carbohydrate source and germination % and growth parameters.

Materials and Methods

Plant Material and Initiation of Aseptic Cultures

The fresh healthy plants of *Begonia malabarica* were collected from the plant grown on wild habitat from Wayanad hills and Idukki district, Kerala and the voucher specimen was deposited in the herbarium of University College, Trivandrum (UCB 1207). Generally, due to the succulent and pubescent nature of *Begonia*, micropropagation seems to be laborious due to high rate of contamination. Thus, direct *in vitro* seed germination is an alternative and B. *malabrica* produces hundredths of dormant seeds in the capsule. So an attempt was made during the initial phase of the study using excised leaves, petioles, rhizomes and capsules (with seeds) as explants for *in vitro* micropropagation. These explants were thoroughly washed in running tap water for 1 h and treated with 5% teepol (v/v) for 20 min with continuous agitation. Subsequently, the teepol content from the explants were removed by repeated washing with double distilled sterile water.

Surface Sterilization and Inoculation

Various surface sterilization protocols were adapted using sterilants such as mercuric chloride 0.01%-0.1% for 3-8 min, sodium hypochlorite - 5%-15% for 5-10 min or calcium chloride 10-15% for 10 - 20 min for all the excised explants. Meanwhile, in the case of fully matured seeds that are about to burst out from the capsule were directly treated with mercuric chloride (0.1%) for 3-5 min. Invariably, Tween 20, the poly ethoxylated sorbitan ester, an emulsifying, wetting, dispersant, hydrophilic and solubilizing agent was also added with all the treatments.

The disinfected explants were rinsed with sterile distilled water for several times and were dissected into small pieces of explants like excised leaves, petioles, rhizomes and capsules (with seeds). Subsequently, the leaves and rhizome measuring approximately 1cm2 and petiole about 1cm long were cultured on different culture media such as MS medium [7], B5 [8], SH [9], MC [10], Chu N6 [11] and Whites medium with various combinations of hormones. The media were supplemented with 1-3% sucrose and gelled by 0.3%-0.7% agar. PH was adjusted to 5.8 ± 0.02 prior to autoclaving at a pressure of 1210 C for 20 min. The cultures were incubated at 25 ± 20C with 12/12. 8/16, 16/8 hours light/dark photoperiod through white fluorescent tubes (1000 lux).

Seed Sources and Surface Sterilization

Subsequently, the mature capsules of B. *malabarica* were collected from the natural habitat. The capsules were stored in air tight containers at room temperature. Under a laminar airflow cabinet, the capsules or dissected seeds from the capsules were initially surface-sterilized with 70% (v/v) ethanol (60 sec.). Subsequently, the capsules were treated with different sterilants such as 2.5, 5, 7.5, 10% (w/v) calcium hypochlorite solution (5-30 min), 0.01-0.1% mercuric chloride for 3-8 min, sodium hypochlorite - 5%-15% for 5-10 min or calcium chloride 10%-15% for 10 - 20 min and finally rinsed thoroughly with three times sterile distilled water. The treated capsules or seeds were soaked overnight in 100 mL beaker containing 50 mL of sterile distilled water (SDW). After soaking overnight, the water was discarded, the seeds were rinsed 3-4 times with SDW, and the seeds were used for in vitro germination trials.

Germination % and Growth Parameters

Germination was calculated as the appearance of 2 mm radical and was considered as physiological state of germination. Germination was monitored regularly for until no further germination was recorded and the mean germination time (mgt) was calculated using the formula given below [12]

$$mgt = \frac{\Sigma (nd)}{\Sigma n}$$

where n is the number of seeds newly germinated at each day at 25°C; d represents days from the beginning of the germination test; Σn is the total number of seeds germinated at the termination of the experiment [13]. Four weeks after sowing, seedling height, epicotyl and primary root length, and the number of leaves, branches, and secondary roots were recorded. The dry weights of aerial and root systems were obtained by drying them in an oven at 65°C for 72 h until a constant dry weight.

Effect of Different Basal Media

The capsules or seeds were cultured on six basal media such as MS [7], B5 [8], SH [9], MC [10] and Chu N6 [11] (CHU) and Whites medium. Different types of sugars were added to these basal media without growth regulators. The pH of the culture media was adjusted to 5.8 before adding 0.6% (w/v) agar. Culture medium without any fortification of nutrients was considered as control.

Effect of MS Medium Strength

To optimize germination of surface sterilized seeds and the in vitro development of plantlets, the seeds were inoculated on three strengths of MS medium, namely, full strength MS salts, half strength MS salts (½ MS), and quarter strength MS salts (¼ MS).

Effect of Carbohydrate Sources

The best culture medium from the previous experiment was selected for testing dif¬ferent sources of carbohydrate. Thus, in this germination medium contain different doses of sugars such as 1-3% of sucrose or glucose or fructose have been added to analyze their effectiveness in promoting the germination and subsequent growth of the seedling.

Statistical Analysis of Data

The whole experiment was completed out in a completely randomized design. 50 seeds were used for each of the following experiments. Germination days, % of germination, plant height, number of leaves, root length and biomass variables were analyzed against the media such as MS, B5, SH, MC, Chu N6, Whites and control. Subsequently, the plant height, number of leaves, root length and biomass variables were analyzed with different strength MS media such as full strength, half strength, and quarter strength MS salts. Half strength MS medium was supplemented with different carbon sources like glucose, fructose, sucrose vs plant height, number of leaves, root length and biomass variables were evaluated. Finally, the half strength MS medium against different concentrations of sucrose (1, 2 and 3%) were also analyzed in terms of plant height, number of leaves, root length and biomass.

Each experiment was repeated for six times. The data were subjected to normality test using descriptive data analysis. It was found that skewness for the above parameters were nearly zero and kurtosis was almost 3. Test also for the chi-square in terms for goodness of fit was analyzed. p - value > 0.05 was considered as probability level of significance. All these were carried using statistical software program Statistical version 7.

Results and Discussion

Initiation of Aseptic Cultures, Surface Sterilization and Inoculation

Standardization to develop aseptic *in vitro* cultures as per the pre-treatment protocols with mercuric chloride or calcium chloride or sodium hypochlorite with the selected explants resulted in high rate of contamination. This may be due to the abundance of epidermal trichomes and succulent nature. Thus, a protocol was designed to generate aseptic cultures of B.*malabarica* through *in vitro* seed germination.

Seed Sources and Surface Sterilization

Generally, *in vitro* responses in plants varied greatly based on the medium, explants, hormonal combinations and culture maintenance conditions. $HgCl_2$ sterilant yielded the optimal results with seeds when compared to other sterilants. Direct pre-treatment of capsule with 0.1% $HgCl_2$ for 5 min was effective when compared to direct treatment of seeds (i.e., it leads to dormancy, retarded growth with chlorosis with the germinated seedlings) (Figure 1).

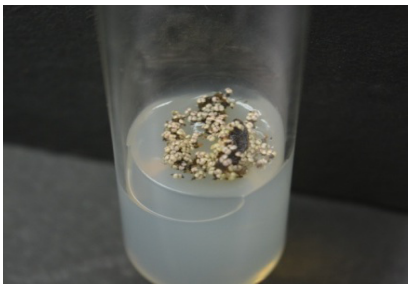

Figure 1: Directly pretreated seeds with 0.1% mercuric chloride showing nitrification and seed dormancy.

While the seeds from the pre-treated excised capsule with HgCl2 0.1% for 5 min inoculated on various medium showed optimal seed germination with negligible rate of contamination. Sen et al. [14] evaluated the effect of sterilizing substances like ethanol, mercuric chloride, flugal, nystatin, and sodium hypochlorite on germination and development of explants of Achyranthes aspera. The percentage of contamination and germination, as well as seed color, growth pattern, and shoot let development varied depending on the sterilants.

Effect of Different Basal Media

After incubation on the cul¬ture media, seeds became swollen quickly and germination occurred within the four weeks of culture (Figure 2a & b). The mean germination time (mgt) and the mean germination % of the seed on different culture media after a 6-week incubation period are tabulated. Highest germination % and mean germination days was shown by MS medium i.e., 90 and 20 respectively. Mean growth % was not significantly influenced by the different culture media tested.

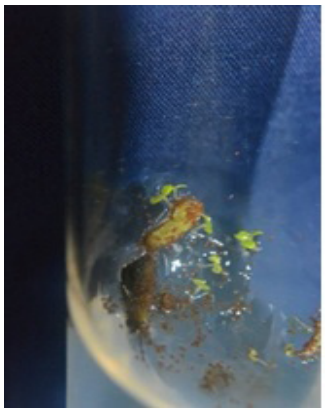

Fig: 2a

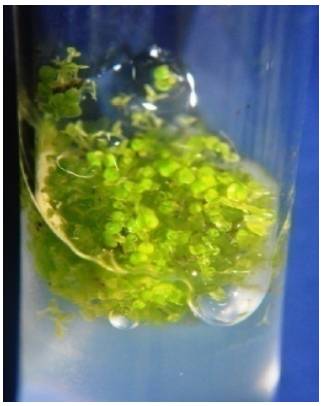

Fig: 2b

Fig: 2c

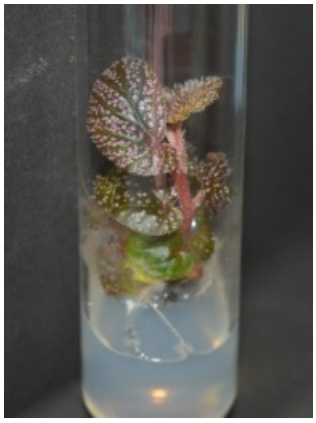

Fig: 2d

Figure 2a, b, c, d: Germinated seedlings on ½ MS medium with 0.5% agar and 3% sucrose.

The mean values of growth parameters of plantlets obtained from the explants on different basal media after culturing for four weeks were presented in Table 1. Significant differences in performance/ development was observed. Furthermore, among the six culture media tested, the highest size of the seedling (5.6 ± 0.84 cm) and root length (3.5 ± 0.43 cm) were observed with the plants developed from seeds on MS basal medium (Table 2). All tested media contain mineral salts that vary not only in their concentrations but also in their available forms.

	Control	MS	McCown	B5	SH	CHU	Whites
Mean Germination days	52	20	58	45	60	61	45
Germination (%)	36	96	55	77	60	63	60

Table1: Mean germination time and germination % of B. malabarica seeds incubation on different basal media.

Media culture	Plantlets height (cm)	Number of leaves	Root length (cm)	Biomass (g)
Control	3.76 ± 0.08	2.40 ± 0.24	2.07 ± 0.04	0.12 ± 0.001
MS	5.6 ± 0.84	8.76 ± 0.52	3.5 ± 0.43	0.51 ± 0.01
B5	4.10 ± 1.39	6.14 ± 1.57	2.65 ± 3.44	0.25 ± 0.03
McCown	3.99 ± 0.77	5.25 ± 0.98	2.98 ± 4.59	0.25 ± 0.02
SH	3.08 ± 0.60	4.82 ± 0.59	2.68 ± 3.29	0.22 ± 0.004
CHU	4.20 ± 1.48	4.43 ± 0.39	2.81 ± 3.12	0.21 ± 0.02
Whites	5.08 ± 0.60	4.55 ± 0.39	2.81 ± 3.12	0.21 ± 0.02

Table 2: Growth performance of B. *malabarica in vitro* plantlets derived from different basal media after four weeks of cultivation.

The media used in the present analysis varied from one another in their chemical composition. The remarkable feature of the MS inorganic salts is their high level of nitrate, potassium, and ammonium in comparison to other media formulations. MS medium is highly enriched with macro- and microelements and the inorganic salts in this medium were enough to support the maximum growth of the plant. The concentration and the quality of nitrogen in MS medium may be the reality of prolific growth obtained with plant derived seed types incubated on this medium. Indeed, nitrogen is supplied to medium in inorganic form as NO3- anion or the NH4+ cation.

Kone et al. [15] reported that the ammoniated form of nitrogen was more appropriate than the nitrate form yielded the fastest growth of Bambara groundnut seedlings at 50-100 mg-L-1 NH4NO3. Further, Chen and Chang [16] noticed that an optimal concentration of organic and inorganic nitrogen components can induce the growth of explants. In addition, better plant growth from embryonic axis was observed on MS medium compared to other basal media in Juglans regia [17].

Effect of MS Medium Strengths on the Growth of Seedlings Developed from the Seeds

Among the different strengths of MS basal medium employed, a remarkable difference was recorded for growth parameters when comparing the plantlets derived from seeds (Table 2). Full and ¼ strengths of MS medium, plantlet height, root length, and the biomass were similar. Significant difference was seen between the half strength MS basal medium with others in terms of leaf number, length of root and plantlets biomass derived from seeds. But a significant reduction in plantlet height was observed on 1/4 MS (Table 3). Thus, the result suggests that a low profile of macro- and micronutrients is not effective for plantlet growth. Half strength of MS produce optimal results for Begonia seedling development suggesting that an adjustment can be carried from the full composition of MS basal medium without any significant reduction in plantlet growth (Fig. 2c). Thus, half MS was selected as the culture medium for the subsequent assessment of carbohydrate sources.

Media culture	Plantlets height (cm)	Number of leaves	Root length (cm)	Biomass (g)
Full MS	4.4 ± 0.15	5.38 ± 0.52	2.99 ± 0.54	0.51 ± 0.05
Half MS	5.6 ± 0.84	8.76 ± 0.52	3.87 ± 0.43	0.67 ± 0.01
¼ MS	2.9 ± 0.04	6.11 ± 0.44	2.16 ± 0.1	0.38 ± 0.03

Table 3: Growth performance of B. malabarica in vitro plantlets derived from different strengths of Half MS basal medium after four weeks of culture.

Effect of Carbohydrate Sources

Plants growing under *in vitro* culture conditions are semi autotrophic and leaves formed during *in vitro* growth may never attain photosynthetic competence fully [18]. Moreover, plantlets growing under *in vitro* conditions have limited accessibility to CO_2 inside the culture vessel [19]. Therefore, sugar was supplemented as carbon source to maintain an optimal usage of carbon source for *in vitro* multiplication and growth of plant cell, tissue, and organs or whole plantlets. Continuous supply of carbohydrates to plants cultured *in vitr*o is essential because the photosynthetic activity of *in vitro* grown tissues is usually low. These compounds are also act as osmolytes in the culture media. Gibson [20] revealed that sugars have potential role on the physiology, growth, and differentiation of cells. Therefore, the optimal carbon source needs to be consid¬ered. Thus, different sugars such as glucose, fructose, and sucrose at 3% (w/v) were incorporated into the 1/2 MS basal medium. After six weeks of growth, the plants were analyzed and presented in Table 4.

Media culture	Plantlets height (cm)	Number of leaves	Root length (cm)	Biomass (g)
Glucose	5.30 ± 0.21	4.90 ± 0.43	5.0 ± 0.18	0.03 ± 0.01
Sucrose	6.4 ± 0.33	10.6 ± 0.09	5.4 ± 1.49	0.025 ± 0.01
Fructose	5.94 ± 0.57	5.35 ± 0.18	4.4 ± 0.65a	0.04 ± 0.004

Table 4: Growth performance of B. *malabarica in vitro* plantlets growth on 1/2 MS containing glucose, sucrose, and fructose.

Plants grew effectively in the medium in the presence of sugar. The type of sugar (sucrose, glucose, or fructose) seems to have significant effect on the leaf number, length of the root, and the plant biomass. Eckstein et al. [21] have also been reported a dissimilar result in *Arabidopsis thaliana* (L.) Heynh. However, Smith [22] reviewed that among the three types of sugars, the significant plant height (7.94 cm) was observed on medium containing 3% sucrose. The positive effects of sucrose on growth of explants under *in vitro* condition were linked with its high solubility in water, its electrical neutrality, and its lack of inhibitory effect on the majority of biochemical processes. These positive effects of sucrose resulted in its wide application in tissue culture as carbon source. Supplementation of sucrose in growth medium meets the energy demands for growth and physiological function of the plantlets.

Effect of Different Concentrations of Sucrose

Development of *in vitro* plantlets was further investigated to study the effect of different concentrations of sucrose, 1, 2, 3, 4, 5 and 6% (w/v), on plant growth. The results obtained after six weeks of culture were recorded in Table 5. Increasing the sugar concentration from 1 to 3% has visually stronger influence on plant height and biomass production. But, above 3% concentration of sucrose, no significant difference was noticed (Figs. 2c & d). Moreover, among the different concentrations of sucrose tested, a non significant difference was recorded for the leaf number and the root length. From these results, 3% sucrose concentration in the basal medium seems to be effective for normal plant growth. Sucrose is the most widely used carbon source in most of the plant *in vitro* cultures, as it is the major sugar form translocated via the phloem.

Sucrose (%)	Plantlets height (cm)	Number of leaves	Root length (cm)	Biomass (g)
1	3.08 ± 0.45	3.63 ± 0.18	4.76 ± 0.86	0.013 ± 0.0
2	4.03 ± 0.34	6.14 ± 0.09	4.97 ± 1.21	0.023 ± 0.001
3	7.99 ± 0.18	11.47 ± 0.35	5.9 ± 0.50	0.061 ± 0.002
4	7.89 ± 0.28	10.56 ± 0.19	4.7 ± 0.24	0.056 ± 0.004
5	6.66 ± 0.30	7.56 ± 0.17	4.8 ± 0.34	0.066 ± 0.003
6	6.0 ± 0.27	6.76 ± 0.20	4.5 ± 0.27	0.056 ± 0.008

Table 5: Mean values of growth parameters of B. *malabarica* plantlets on 1/2 MS containing different concentrations of sucrose.

As a carbon source, sucrose supports growth of plant cells in culture. A sucrose concentration of 1-5% is generally used for *in vitro* tissue culture, since it is also synthesized naturally by the tissue. For tissue culture, researchers commonly use 3% sucrose in the medium as per recommendation. Besides serving as energy source, it also provides the carbon precursors for synthesis of structural and functional components.

Hoque et al. [23] analyzed seed dormancy status evaluated using different media with or without sugar (3%) in rice cultivars. Jhora cultivar showed strong seed dormancy than the wild and cultivated rice genotypes.

Fruit plants were widely analyzed for their economic importance and are cultivated mainly for fruit juice. To obtain a continuous source of material for screening of secondary metabolites, zygotic embryo culture was attempted among 62 *Passiflora* species, starting from seeds mainly collected in the wild. 29 species produced calli, which had different growth rates. Plants were successfully regenerated from calli of 13 different species [24]. Similarly, Hossain et al. [25] designed an efficient protocols for in vitro seed germination, neo-formation of secondary protocorms from primary protocorms and multiple shoot buds and protocorm-like body induction from pseudo-stem segments of in vitro-raised seedlings of *Cymbidium giganteum* using four nutrient media, such as MS, Phytamax, Mitra, and Knudson 'C'. Hassanein and Azooz [26] reported seed drying decreased both the percentage of seed germination and the number of seedling per seed. Germination of seeds was better on MS medium supplemented with 0.5 mg benzyl amino purine than in soil. Bae and Yoon [27] reported that soaking of seeds in GA3 solution remarkably promoted germination up to 60%, but the control (0 mg/L) was not effective (> 5%). Toma and Rsheed [28] analyzed *in vitro* propagation through seed

culture and regeneration of *Asparagus densiflorus* L. through callus cultures derived from hypocotyls.

Hardening of in vitro culture raised plants

Seed germinated and regenerated plantlets that are well developed were transplanted on sand medium and showed remarkable acclimatization to the environment (95% survivability) (Figure 3). The acclimatized plantlets were successfully transferred in the field with 93% survivability. The field transferred plants showed absence of variability with respect to the *in vivo* generated plants.

Figure 3: Hardening of tissue culture raised plants.

Conclusion

The overall objective of this investigation was to define the optimal conditions for *in vitro* seed germination and plant growth of *Begonia malabarica*. The major results showed that the composition of the germination medium influence the germination capacity of seeds used in B. *malabarica*. The best seedling growth was observed with the seeds on half MS medium containing 3% sucrose. This established protocol would provide sufficient materials as source of explants for initiating different types of in vitro callus and cell culturing in the species.

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References

1. Simona L, Cerasela P, Giancarla V, Maria B (2012) *In vitro* culture initiation and phytohormonal influence on ornamental plants. Journal of Horticulture, Forestry and Biotechnology 16: 203-205.
2. Ogero K, Mburugu OGN, Mwangi M, Ombori O, Ngugi M (2012) *In vitro* micropropagation of Cassava through low cost tissue culture. Asian Journal of Agricultural Sciences 4: 205-209.
3. Yang G, Shen X, Jackson R, Lu Z (2013) Factors affecting *in vitro* seed germination and shoot proliferation of galax [Galax urceolata (Poir.) Brummitt]. Australian Journal of Crop Science 7: 1766-1771.
4. Akinyosoye ST, Adetumbi JA, Amusa, OD, Olowolafe, MO, Olasoj JO (2014) Effect of seed size on *in vitro* seed germination, seedling growth, embryogenic callus induction and plantlet regeneration from embryo of maize (Zea mays L.) seed. Nigerian J Genetics 1-7.
5. Kumaria S, Kehie M, Das Bhowmik SS, Singh M, Tandon P (2012) *In vitro* regeneration of *Begonia rubrovenia* var. *meisneri* C.B. Clarke— A rare and endemic ornamental plant of Meghalaya, India. Indian Journal of Biotechnology 11: 300-303.
6. Ramesh N, Viswanathan MB, Saraswathy A, Balakrishna K, Brindha P, et al. (2002) Phytochemical and antimicrobial studies of *Begonia malabarica*. Journal of Ethnopharmacology 79: 129-132.
7. Murashige T and Skoog F (2002) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.
8. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50: 151-158.
9. Schenk RU and Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany 50: 199-204.
10. Lloyd G, MCcown B (1980) Commercialy feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings International Plant Propagators'Society 30: 421-427.
11. Chu CC (1978) The N6 medium and its applications to anther culture of cereal crops, in Proceedings of the Symposium on Plant Tissue Culture. (Science Press, Beijing, China) : 43-50.
12. Ellis RH and Roberts EH (1981) The quantification of ageing and survival in orthodox seeds. Seed Science and Technology 9: 373-409.
13. Trivedi DR, Joshi AG (2014) Studies on seed germination of *Stereospermum suaveolens* with respect to different parameters. Environmental and Experimental Biology 12: 33-37.
14. Sen K, Mostofa Jamal MAH, Nasrin S (2013) Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured *in vitro*. Environmental and Experimental Biology 11: 119-123.
15. Kone M, Kone T, Silue N, Soumahoro AB, Kouakou TH (2015) *In vitro* seeds germination and seedling growth of Bambara Groundnut (*Vigna subterranea* (L.) Verdc. (Fabaceae)). Scientific World Journal: 1-9.
16. Chen JT and Chang WC (2002) Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in Oncidium 'Gower Ramsey. Plant Cell, Tissue and Organ Culture 69: 41-44.
17. Sanchez-Zamora MA, Cos-Terrer JD, Frutos-Tom´as, Garcıa-Lopez R (2006) Embryo germination and proliferation *in vitro* of Juglans regia L. Scientia Horticulturae 108: 317-321.
18. Hazarika BN (2003) Acclimatization of tissue-cultured plants. Current Science 85: 1704-1712.
19. Van Huylenbroeck JM and Debergh PC (1996) Impact of sugar concentration *in vitro* on photosynthesis and carbon metabolism during ex vitro acclimatization of *Spathiphyllum* plantlets. Physiologia Plantarum 96: 298-304.

20. Gibson SI (2000) Plant sugar-response pathways, Part of a complex regulatory web. Plant Physiology 1532-1539.

21. Eckstein A, Zieba P, Gabry´s H (2000) Sugar and light effects on the condition of the photosynthetic apparatus of Arabidopsis thaliana cultured in vitro. Journal of Plant Growth Regulation 31: 90-101.

22. Lea PJ, Leegood RC, Eds Smith C (1995) Carbohydrate chemistry, in Plant Biochemistry and Molecular Biology. (JohnWiley & Sons, Chichester, UK): 73-111.

23. Hoque MN, Rahman L, Hassan L (2007) Effect of culture media on seed dormancy and callus induction ability of some wild and cultivated rice Genotypes. Biotechnology 6: 61-63.

24. Guzzo F, Ceoldo S, Andreetta F, Levi M (2004) *In vitro* culture from mature seeds of *Passiflora* species. Scientia Agricola (Piracicaba, Brazil) 61: 108-113.

25. Hossain MM, Sharma M, Jaime A, Silva TD, Pathak P (2010) Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. Scientia Horticulturae 123: 479-487.

26. Hassanein AM and Azooz MM (2003) Propagation of *Citrus reticulata via in vitro* seed germination and shoot cuttings. Biologia Plantarum 47: 173-177.

27. Bae KH, Yoon ES (2015) Seed Germination and *in vitr*o plant regeneration through callus culture of two *Lychnis* Species. Plant Tissue Culture & Biotechnology 25: 1-12.

28. Toma RS and Rsheed KA (2012) *In vitro* propagation through seed culture and regeneration of Asparagus densiflorus through callus cultures derived from hypocotyls. International Journal of Pure and Applied Sciences and Technology 9: 94-102.