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Development of Effective Protocol for four Varieties of large Cardamom

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Abstract

Large cardamom is one of the most important spices that can significantly contribute to the economical farming in the country of Nepal. It is grown in Nepal and north-eastern states of India which provide suitable agroclimatic growing conditions of high humidity, ambient temperature and high rainfall. Meeting the demand for high quality plants and yield of cardamom is challenging with traditional methods of propagation. The present study has used the plant tissue culture technique to produce high quality plants. In this regard, MS media with three different hormonal combinations were used for the development protocol for 8 weeks. Shoot length, root length, shoot number and root number were assessed at intervals. The best protocol for growth was MS media with 1 mg/L BAP + 0.5 mg/L IBA for the Ramsey variety, with no significant difference for Golsai, Dambarsai, or Sikkimae varieties. Similarly, the acclimatization and field transfer study was done. The use of any substrate composition in ratio of coco peat: soil 1:2; moss: coco peat 1:2 and sawdust: coco peat 1:2 enables transfer of healthy plants to the field. The results indicate that the varieties respond differently to the micropropagation process and to hormone concentrations indicated by differing root and shoot production. The protocol of 1mg/L BAP and 0.5mg/L IBA could be used for the Ramsai while optimal shoot production for Golsai and Sikkimae should be at 0.5mg/L and 5mg/L for shoot production. All varieties showed optimal root production at 0mg/L BAP and 0.5mg/L IBA. This study sheds light on the different responsiveness of varieties to tissue culture and hormone concentrations for both root and shoot development in micropropagation.

Keywords: Cardamom, 6-benzylaminopurine, micropropagation, *Amomum subulatum*, variety, tissue culture

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Introduction

Large cardamom, *Amomum subulatum* Roxb. belongs to the family Zingiberaceae and its cultivation is confined to the sub-Himalayan range of eastern Nepal, northern India (Sikkim and West Bengal) and Bhutan [1]. Nepal is the world's largest producer of large cardamom supplying close to 50% of the world's market demand with 14200 ha cultivated [1], these are concentrated in Taplejung, Sankhuwasabha, Panchthar, and Ilam comprosing 80% of Nepal's production [2]. The annual production is 6026 mt from productive area of 11665 hectares and supports the livelihood of more than 70,000 families directly and indirectly [3]. It has been regarded as one of the important spices upon which the livelihood of most farmers depends.

However, the quality and quantity of the cardamom have declined over the years to disease associated with it [4]. Common cardamom diseases include foorkey (nanovirus) [5], and chirke diseases (macluravirus) [6], and phoma leaf spot (*Phoma hedericola*) [7]. These have directly or indirectly affected the stakeholders and the economy of the country. The demand and the price have been declining over the years because of low quality and for this reason many farmers have stopped cultivating large cardamom. Large cardamom has a high price volatility which poses a risk to long term farming, and reduced production is caused by aging orchards, disease, poor management, and lack of new planting material [8, 9]. These problems pose potential risks to the large cardamom industry in Nepal.

A plant tissue culture technique (shoot tip culture) provides an alternate way to meet the quality and quantity of the cardamom, producing vigorous disease-free plantlets in large numbers. The techniques will help to restore the high quality of plantlets and fulfill the demand of disease-free plants in the cardamom pocket zone of Nepal [10]. This will in turn help to reestablish the ability to supply the regional and international cardamom markets.

In this regard, present investigation has developed an effective protocol for producing highly demanded large

cardamom varieties such as Ramsai, Dambarsai, Golsey, and Sikkimme.

Material and methods

Explants were collected from the nursery of NARC Sub-Station Pakhribas. The freshly collected sucker (explants) were washed under running tap water for at least 30 minutes to remove soil and other external particles attached on their surface. Then, the explants were dipped in water containing Tween 20 (0.1%) for 15-20 minutes, shaken well and again washed in running tap water until all the detergents washed off clearly and rinsed with distilled water. Thereafter, dipped into 1% sodium hypochlorite solution for 15 minutes and followed subsequently by with 70% ethyl alcohol for 2 minutes [11]. Finally, explants were rinsed thoroughly with sterile water for 5 times and ready for cut after drying in filter paper. Multiplications were made from sterile mother stock which had been established through several rounds of subculturing with the aim to minimize the need for rhizome collection and allow year-round multiplication. Plants were assessed daily to monitor growth and contamination, and were sub-cultured every 7-21 days.

Plant growth assay

The protocorms produce from the explants were used for the plant growth assay. MS (Murashige & Skoog) media was prepared [12]. The different hormonal compositions (MS + 0mg/L BAP + 0.5mg/L IBA; MS+0.5 mg/L BAP + 0.5 mg/L IBA; MS + 1mg/L BAP + 0.5 mg/L IBA; MS + 5mg/L BAP + 0.5mg/L IBA) were used for the plant growth assay for all the varieties Ramsey, Golsey, Dambarsai, and Sikkimme. The plantlets were grown in aseptic conditions under a 16 h photoperiod, at 25± 2°C. Growth pattern of the plantlets were recorded after 60±5 days. Shoots were then excised and placed on fresh media to grow until field transfer.

Acclimatization and field transfer

The in-vitro plantlets were acclimatized using different substrate compositions. The substrates used were sterilized cocopeat, moss, sawdust and soil. The ratio of substrate cocopeat:soil 1:2; moss: cocopeat 1:2 and sawdust:cocopeat 1:2. The trial was kept under observation for 4 weeks in a greenhouse. The plant height was measured at weekly intervals.

Statistical analysis & growth ranking

Plant growth assays were performed independently for a minimum of eight replicates to measure the shoot and root length and number. Both were tested by ANOVA with the alpha error level set at $p \geq 0.05$ (GraphPad Prism). Normal distribution was tested on each data set (D'Agostino & Pearson), with 22% of data sets being considered normally distributed. Non-Gaussian distribution was therefore assumed for ANOVA. The control was considered to be 0mg/L BAP media. A growth ranking system was implemented to identify optimal hormone concentrations within the study due to high variability and low sample sizes. Growth rankings were determined by firstly normalizing each parameter to the highest average value. Shoot or root growth ranking was then a multiplicative product (unitless) of the two parameters (length and number).

Results

Plant growth assay

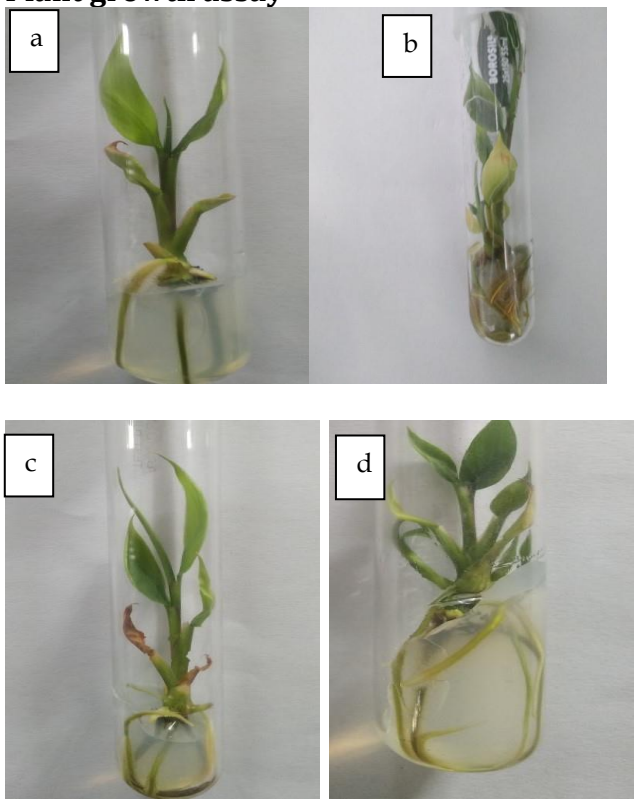

Figure 1. The plant growth assay perfomerd for 6o days *in-vitro* condition for all four varieties of cardamom Dambarsai (a), Golsai (b), Sikkimae (c), and Ramsai (d) with MS+1mg/L BAP +0.5mg/L IBA

The *in-vitro* plant growth assay performed for four varieties of cardamom to investigate the optimum concentration of hormone required of the plant growth **(Figure 1**). Length and numbers of shoots and roots are indicated in **Table 1**. Shoot number was significantly higher in the Sikkimae variety on 5mg/L BAP producing 8.4±0.9 shoots per cutting. Root number was significantly lower in the Ramsai variety at 5mg/L BAP producing 1.5±0.4 roots per cutting.

Table 1: Growth patterns of Dambarsai, Golsai, Sikkimae, and Ramsai cardamom varieties in response to differing cytokinin concentration in micropropagation.

	0mg/L BAP + 0.5mg/L IBA	0.5mg/L BAP + 0.5mg/L IBA	1mg/L BAP + 0.5mg/L IBA	5mg/L BAP + 0.5 mg/L IBA
Dambarsai				
Shoot number	2.5 ± 0.5	2.9 ± 0.5	4.3 ± 0.8	3.4 ± 0.7
Shoot length (mm)	36.3 ± 15.7	41.3 ± 13.6	42.7 ± 10.3	37.9 ± 10.0
Root number	3.0 ± 1.0	2.6 ± 1.2	3.7 ± 1.0	0.9 ± 0.7
Root length (mm)	100.0 ± 52.9	68.8 ± 44.8	71.7 ± 23.1	12.9 ± 12.0
Golsai				
Shoot number	3.8 ± 1.1	7.8 ± 1.0*	4.8 ± 1.0	6.0 ± 1.3
Shoot length (mm)	53.0 ± 17.2	38.5 ± 6.4	33.3 ± 5.8	32.1 ± 7.6
Root number	3.1 ± 1.1	0.7 ± 0.4	0.9 ± 0.5	0.1 ± 0.1
Root length (mm)	51.0 ± 20.9	6.0 ± 4.1	19.6 ± 14.6	0.5 ± 0.5
Sikkimae				
Shoot number	4.4 ± 0.6	3.2 ± 1.5	3.7 ± 1.1	8.4 ± 0.9*
Shoot length (mm)	48.1 ± 20.8	35.0 ± 19.7	42.2 ± 7.1	82.3 ± 13.1
Root number	4.9 ± 1.8	5.4 ± 2.0	3.3 ± 1.0	2.3 ± 0.8
Root length (mm)	101.3 ± 47.0	63.8 ± 40.4	93.3 ± 32.6	20.5 ± 10.5
Ramsai				
Shoot number	2.2 ± 0.5	2.3 ± 0.4	2.6 ± 0.4	1.5 ± 0.2
Shoot length (mm)	61.7 ± 16.0	47.1 ± 12.2	57.5 ± 10.8	35.0 ± 3.6
Root number	5.2 ± 1.1	2.3 ± 0.8	2.7 ± 0.7	1.5 ± 0.4*
Root length (mm)	112.8 ± 29.3	96.3 ± 31.9	111.5 ± 38.4	27.5 ± 6.8*

Table 2: Growth ranking for optimal shooting and rooting of Dambarsai, Golsai, Sikkimae, and Ramsai cardamom varieties in response to differing cytokinin concentration in micropropagation. Optimal raking for the trail for shoot and root responses are shown in bold

	0mg/L BAP + 0.5mg/L IBA	0.5mg/L BAP + 0.5mg/L IBA	1mg/L BAP + 0.5mg/L IBA	5mg/L BAP + 0.5 mg/L IBA
Dambarsai				
Shoot	0.49	0.64	**1.00**	0.70
Root	**0.82**	0.49	0.72	0.03
Golsai				
Shoot	0.48	**0.73**	0.39	0.47
Root	**1.00**	0.03	0.11	0.00
Sikkimae				
Shoot	0.31	0.16	0.22	**1.00**
Root	**0.90**	0.63	0.57	0.09
Ramsai				
Shoot	0.86	0.68	**0.93**	0.32
Root	**1.00**	0.37	0.51	0.07

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In Ramsai this concentration also produced a lower root number and length than all other treatments with a length of 27.5±6.8 mm length. Shoot number was highest in the 0.5mg/L BAP media for Golsai at 7.8±1.0 shoots per cutting. No other measurements were significantly different (**Table 1**). The number of shoots and roots produced differed widely between varieties. The maximum number of shoots produced in a media protocol per variety from 2.6 for Ramsey to 8.4 for the Sikkimae variety. Root number varied from 3.1 (Golasi) to 5.4 (Sikkimae).

Table 2 indicates growth ranking for best shoot and root development. Ranking based on growth of shoots and roots indicated that shoot and root optimization differed within each variety. The best ranking media to produce root for all varieties was the 0.5mg/L IBA media with no exogenous BAP (**Table 2**). Highest ranking media for shoot production was 1mg/L BAP for Dambarsai, 0.5mg/L BAP for Golsai, 5mg/L BAP for Sikkimae and 1mg/L BAP for Ramsai (**Table 2**).

Values are indicated as mean ± SEM. * indicates significant difference at the end of the trial period from the control BAP concentration of 0mg/L.

Acclimatization and field transfer

The plants that were grown for 65 days were taken for acclimatization and field transfer (Figure 2). The plant height in all three different substrate compositions (cocopeat: soil 1:2; Moss: cocopeat 1:2 and sawdust: cocopeat 1:2) was recorded at 15 and 30 days. The total growth accumulated from day 0 (transfer date) is shown in **Figure 2**. The growth of plants in each substrate composition did not differ between substrates. Survival and health of plants did not differ in the 30 day trial (**Figure 2 a-c**).

Discussion

The previous study has shown the use of 0.5mg BAP + 1.0mg IBA in one liter MS media as a standard protocol for the single Ramsey variety [12]. Similarly, use of MS+ sucrose 40 g + BAP 3mg/L + 0.5 NAA + 2mg/L IBA was

demonstrated [13]. Revisiting the investigation, we have demonstrated the use of 1mg/L BAP + 0.5mg/L IBA is far better for the overall *in-vitro* plant growth in the Ramsai variety. We also show that the optimal concentration of BAP with 0.5mg/L IBA, based on this study, for shoot number production in the Golsai and Sikkimae varieties are 0.5mg/L and 5mg/L respectively. While it is useful to have high shoot numbers, the shoots themselves must also show sufficient development in size. Therefore, we implemented a ranking system to look at both number and length of shoots and roots. The optimal media for shoot growth differed by variety, however for root growth the optimal media was that which contained no exogenous BAP.

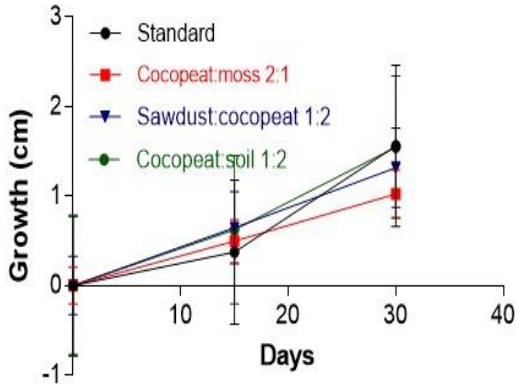

Figure 2. The plants after 65 days of growth were transfer to three different sterile substrate composition; (Sawdust: coco peat 1:2 Fig. (a); Coco peat: Soil 1:2 fig (b); Moss: coco peat 1:2 fig(c)) for 30 days. Growth measured intern of plant height for 4 weeks showing no significant difference in height during the trail at level of $p>0.05$.

Most significantly, root initiation is higher in the presence of a lower cytokinin concentration. The role of cytokinin's in root formation is complex and depends on many interacting factors, and affects both the type and amount of root growth [14-16]. In all varieties, the optimal root production as a rank was best in a media containing no exogenous cytokinin with 0.5mg/L IBA. Previous studies have shown variability across cytokinin concentrations with respect to root number formation in Zingeriberaceae. No clear trend between BAP concentration and root growth was shown in *Kaempferia parviflora* tissue culture studies with 0, 1.5 and 2.5mg/L producing the same number of roots [17]. Similarly micropropagation experiments using BAP and NAA showed no consistent correlation with cytokinin concentration and root number in *Amomum subulatum*, whereas increasing cytokinin resulted in increasing shoot number up to 4µM BAP concentration [13].

It may be beneficial to implement 2 stage *in vitro* propagation systems for cardamom whereby shoot multiplication is carried out on 0.5mg/L BAP + 0.5mg/L IBA for Golsai, 1mg/L BAP + 0.5mg/L IBA for Dambarsai and Ramsai, and 5mg/L BAP + 0.5mg/L IBA Sikkimae. For root development all should be transferred to media containing nil exogenous cytokinin.

Although not the primary aim of this study, it has become apparent that there are different responses of varieties to tissue culture. When looking at the number of shoots and roots produced per variety, these varied widely per variety. This outcome suggests that the Sikkimae variety is more responsive to the micropropagation process than others. This is not unexpected and has been observed in other species including oil palm (*Elaeis guineensis)* [18], potato (*Solanum tuberosum*) [19], gerbera (*Gerbera jamesonii*) [20], and grapes (*Vitis vinifera*) [21], among

many others. Further studies may establish this so we suggest additional research is undertaken. This outcome presents an opportunity to focus production on varieties who respond better to tissue culture, provided they also have desirable field characteristics. Varieties with lesser responses may need to be the focus of additional research to optimize and meet demands.

The acclimatization and field transfer are important steps for the mass propagation. In this regard, the all three-substrate combination showed uniform growth and survival rate for plants growth. Any of these substrate combination (cocopeat: soil 1:2; Moss: cocopeat 1:2 and sawdust: cocopeat 1:2) can be used for acclimatization based on the availability which allows for greater production flexibility. Other studies have shown optimal hardening of Zingiberaceae species cow dung, coir dust,soil:sand and cow dung mixes all with high survival rates of 90-100% [22]. Studies using peatmoss, sand, vermiculite and perlite mixtures found optimal survival of 100% on peatmoss alone [23]. Further work may be carried out to determine whether Golsai, Sikkimae, Dambarsai, and Ramsai varieties respond differently to other auxins and cytokinins to optimize the protocol further. Establishments of *in vitro* mother stock healthy plantlets is the major outcome of the present study. This will allow for ongoing propagation of clones year-round and will no longer be dependent on rhizome formation. This will also reduce the risk of disease contamination being introduced by continued field collection and allow clonal propagation every 60 days which will increase productivity to meet grower demand.

Conclusion

The present investigation revisited the protocol that was already established to optimize the hormonal concentrations for maximum plant growth. In this regard, the present study has developed the protocol with the use of a lower concentration of hormone for the four varieties of plant. The MS media composition with 1mg/L BAP + 0.5mg/L IBA can be recommended as standard protocol for cardamom at this stage. Similarly, substrate (cocopeat: soil 1:2; moss: cocopeat 1:2 and sawdust: cocopeat 1:2) depending upon the availability can used for acclimatization process. Further research may elucidate better protocols including the use of different plant growth regulators, carbon sources, and nutrient sources. Similarly, a two-stage process should be studied; where shoot production is carried out on media containing optimal BAP concentrations per variety, and transferred to cytokinin free media for root development.

Author Contributions

Author 1 and 2 designed the research experiment and performed the entire research. Author 3 established the *in-vitro* plantlet. Author 2 wrote the manuscript. Authors 1, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 revised the manuscript. Author 6 supervised the entire research work.

Competing Interests

The authors declare no competing interests.

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Data availability

Please contact the corresponding author for access to the data.

Ethical Approval

No ethical approval was required, sought or granted for this study.

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