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In vitro Propagation of Garlic (*Allium sativum* L) from Meristem Culture

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Abstract

The present study investigated *in vitro* multiplication and bulbification in one imported garlic accession i.e. VFG158 and eleven local garlic accessions of Mauritius viz. Beeharry, Boodnah, Bondah, Gooniah, Haulkhory, Ramdhuny, Ramjee, Rampall, Sujeebun, Sujeebun 2 and Unuth through meristem culture. The explants were subjected to fourteen shoot multiplication and three bulbification treatments. Positive results for shoot proliferation and suppression of hyperhydricity were noted on six shoot multiplication media: MS basal media with various growth regulators {G0 (0.25mg/L NAA+0.5mg/L 2iP), G1 (1.5mg/L BAP+0.5mg/L NAA), G2 (0.3mg/L NAA+3mg/L 2iP), G6 (0.5mg/L NAA+2mg/L 2iP), G14(2mg/L BAP+2mg/L NAA) and G15(1mg/L BAP+0.5mg/L NAA)}. The highest number of shoot formation was observed in G2 (0.3mg/L NAA+3mg/L 2iP) and the lowest number of shoot formation was observed in G14 (2mg/L BAP+2mg/L NAA). Genotypic difference in shoot multiplication and hyperhydricity on different media formulation was observed. The highest shoot proliferation was observed in the garlic accession Ramdhuny, while the lowest shoot proliferation was recorded in groups of similar accessions namely, Ramjee, Sujeebun and VFG 158. Bulblet formation was earlier on bulbification medium B2 (MS enriched with 12% sucrose). Largest and heavier bulblets were obtained on medium B5 (MS supplemented with 2mg/L BAP+1mg/L GA3 and enriched with 90% sucrose). A reliable protocol for rapid shoot regeneration and multiplication from meristem-tip culture and bulblet formation from multiple shoot clumps was optimised.

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Bulblet Formation;
Garlic; Growth Hormones;
Hyperhydricity;
Tissue Culture.

Introduction

Garlic (*Allium sativum* L) belongs to the genus *Allium* of the family Alliaceae. It is cultivated globally as a vegetable condiment for culinary and medicinal use. The increasing popularity of garlic is mainly attributed to its beneficial effects on human health due to the presence of allicin and other organo-sulphur compounds.[1] Garlic imports to Mauritius

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amounted to USD 3M in year 2021[2] and is expected to increase continuously in the coming years. The COVID-19 pandemic and the conflict between Russia and Ukraine have exacerbated the existing food insecurity issues in the country. With elevated food inflation, the purchasing power of Mauritian citizens has significantly declined. Consequently, an increase in domestic production of garlic would not only reduce the country's reliance on imported garlic but also yield positive economic impacts for the nation.

The local production of garlic is irregular, low and restricted to the eastern part of the island. Over the last three decades, the average annual production has decreased from around 200 t during the 1980s' to mere 31 t in 2021 This is principally attributed to the lack of improved garlic varieties and limited supply of clean planting materials. Small farmers are the main growers of garlic in Mauritius. They produce their own planting material. However, these cloves are likely the carriers of viral contamination in field and main cause of reduction in yield.

Since most garlic varieties have low flowering ability and are sterile, it is vegetatively propagated through cloves or aerial bulbils.[3,4] Consequently, it is easily infected by different types of viruses such as aphid transmitted *Potyviruses* and *Carlaviruses*, mites transmitted *Allexiviruses*.[5] Also, viruses are accumulated after successive planting cycles in garlic bulbs and increased spread of viral diseases to different regions happens through infected planting material. Considerable yield losses and decrease in quality of bulbs are caused by different viral diseases, which have severe impact on the production of garlic.[6]

Previous studies revealed that cultivation of virus-free planting material considerably increased the productivity.[7] Other studies reported that expected decrease in yield of virus-free bulbs in subsequent years due to continual viral exposure were negligible. In fact, yields obtained from cultivated virus-free bulbs after five consecutive planting cycles in the field were higher than the yields of contaminated bulbs cultivated successively for the same period. Therefore, renewal of planting material periodically is required to reduce productivity loss in garlic.[7]

The production of virus-free planting material through traditional methods is difficult. Furthermore, garlic improvement through breeding program is challenging due to low flowering ability of garlic plants.[8] Therefore, other propagation methods such as tissue culture techniques can be used to produce virus-free garlic bulbs. Previous studies reported that virus-free propagules with higher yield and improved quality could be produced by meristem culture.[9] These techniques consist of cleaning infected bulbs and mass propagation of virus-free planting material in a short period of time.[8] Indeed, application of tissue culture techniques for the mass propagation of shoots and subsequent formation of multiple bulblets has high potential in garlic improvement. Other methods used for virus elimination in garlic are meristem-tip culture,[10] meristem culture in combination with heat treatment and meristem culture combined with chemotherapy using ribavirin.[11]

To date, no research work on regeneration of Mauritian virus-free garlic clones have been conducted. Therefore, the main objective of this study was to establish and optimize a reliable protocol for the production of virus-free garlic propagules that have high yield with improved quality for cultivation by Mauritian farmers.

Materials and Methods

The research was carried out between the year 2019 and 2022 at the Tissue Culture Laboratory of Food and Agricultural Research and Extension Institute (FAREI), Mauritius.

Plant Material

One imported garlic accession namely VFG158 introduced from the World Vegetable Centre (WVC) and eleven local garlic accessions viz. Beeharry, Boodnah, Bondah, Gooniah, Haulkhory, Ramdhuny, Ramjee, Rampall, Sujeebun, Sujeebun 2 and Unuth received from Agronomy Division of FAREI. The garlic accession VFG158 was selected for this study as it is adapted to the local climatic conditions. All these accessions are grown by local growers but are not registered as improved varieties. The selected garlic accessions for this study have high demand in the local market due to their high pungency.

Disinfection and Dissection of Garlic Cloves

Healthy garlic bulbs were separated into cloves and the outer bulb scales were removed. The cloves were washed with running tap water and domestic dish detergent (5ml/L). The cloves were then soaked in Benomyl 500WP solution (1%) containing two drops of surfactant (Tween 20) per 100 ml for 45 min with frequent agitation and washed under running tap water for 10 min. Thereafter, the cloves were subjected to two types of treatments; either no hot water treatment (P1) or hot water treatment, 37 °C for 10 min (P2). Thereafter cloves were surface-sterilized with 70% ethanol for 1 min and were shaken for 15 min in a 1% solution of sodium hypochlorite containing two drops of surfactant (Tween 20) per 100 ml. The cloves were then washed three times for 15 min with sterile distilled water. Garlic cloves were dissected and meristem-tips, about 1 mm in size, were exercised under laminar air-flow, using a microscope. The dome-shaped structure consisting of the shoot meristem and one or two leaf primordia were placed individually in an upright position in the appropriate initiation media for culture establishment. Inoculated explants were incubated under controlled temperature (25±2 ºC) and a photoperiod of 16 h with a light intensity of 2000 – 5000 lux from white inflorescent light.

Culture Conditions

The media were supplemented with 3% (w/v) sucrose and 0.24% (w/v) Sigma phytagel. The pH of the medium was adjusted to 5.86 before autoclaving for 15 min at 121 °C. Cultures were placed in growth room at 25 ±1.0 °C with a 16 h photoperiod and light intensity of 2000 – 5000 lux, which was illuminated by 24 W LED T8 fluorescent glass tubes. The cultures were transferred to fresh medium every 5-6 weeks.

Plantlet Establishment and Multiplication

After 15 days, the explants developed into rooted plantlets which were cultured on MS basal medium with different combinations of plant growth regulators, including 6-benzyladenine (BAP), gibberellic acid (GA3), Indole-3-butyric acid (IBA), isopentenyl adenine (2ip), kinetin and naphthaleneacetic acid (NAA), LS basal medium supplemented with plant growth regulators (BAP and NAA) and Gamborg B5 medium supplemented with plant growth regulators (BAP, NAA and Indole-3-acetic acid {IAA}). Fourteen media compositions for shoot multiplication were tested in three replicates for this study (Table 1). Each replicate consisted of five explants cultured in 5 × 11 cm bottle jar.

Bulblet Formation

Shoot cultures were subcultured on three media compositions for bulblets formation (Table 1) and were placed in the growth room at 25 ± 1.0°C with a photoperiod of 16 h with a light intensity of 2000 – 5000 lux. Five shoots' clumps were placed in each culture jar, and five culture jars (replicates) were used for each treatment.

Data Analysis

Data were analyzed using ANOVA and the Tukey's multiple range tests. Statistical analysis of the data was carried out using the JASP programme package.

Table 1: Different media compositions used for plantlet establishment, multiplication and bulbification

Medium code	Salts	Kinetin (mg/L)	BAP (mg/L)	NAA (mg/L)	GA3 (mg/L)	2iP (mg/L)	IAA (mg/L)	IBA (mg/L)	Sucrose (g/L)
				Establishment					
MS hormone -free	MS	-	-	-	-	-			30
				Multiplication					
G0	MS	-	-	0.25	-	0.5			30
G1	MS	-	1.5	0.5	-	-			30

G2	MS	-	-	0.3	-	3		30
G3 (G3A + G3B)	The cultures were placed in G3A media then transferred to G3B after 4 weeks							
G3A	MS		0.5	0.5				30
G3B	MS		2	2				30
G4	MS		2				0.5	30
G5 (G5A+ G5B)	The cultures were placed in G5A media then transferred to G5B after 4 weeks							
G5A	LS		0.25			0.23		30
G5B	LS		2.3	0.93				30
G6	MS			0.5		2		30
G7	MS		2	0.1				30
G8	MS							30
G9	Gamborg B5		0.5	0.1				30
G10 (G10A+G10B)	The cultures were placed in G10A media then transferred to G10B after 4 weeks							
G10A	MS			0.25		0.5		30
G10B	MS		1.5	0.5				30
G11	MS		0.5			1		30
G14	MS	-	2	2	-	-		30
G15	MS	-	1	0.5	-	-		30
Bulbification								
B2	MS	-	-	-	-	-		120
B4	MS	2	-	-	1	-		30
B5	MS	-	2	-	1	-		90

Gamborg B5: Gamborg B5 medium (Gamborg *et al*., 1968)[12]
LS: Linsmaier and Skoog medium (Linsmaier & Skoog, 1965)[13]
MS: Murashige Skoog medium (Murashige & Skoog, 1962)[14]
BAP: 6-Benzylaminopurine, 2iP: 6-(γ,γ-Dimethylallylamino) purine, GA3: Gibberellic acid, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, Kin: Kinetin, NAA: α-Naphthalene acetic acid

Result and Discussion

In tissue culture technique, contaminated explants do not develop and are thus discarded. Therefore, an effective surface sterilisation protocol should be established to obtain contamination-free explants. Two washing protocols, P1 (control) and P2 (an additional step with hot water treatment 37 °C for 10 min included) were tested for surface-sterilization of healthy garlic cloves. Protocol P2 was found to be more effective as the rate of contamination among the explants was lower than protocol P1. The contamination rate for protocol P2 varied from 5% to 10% compared to 20% to 35% for protocol P1.

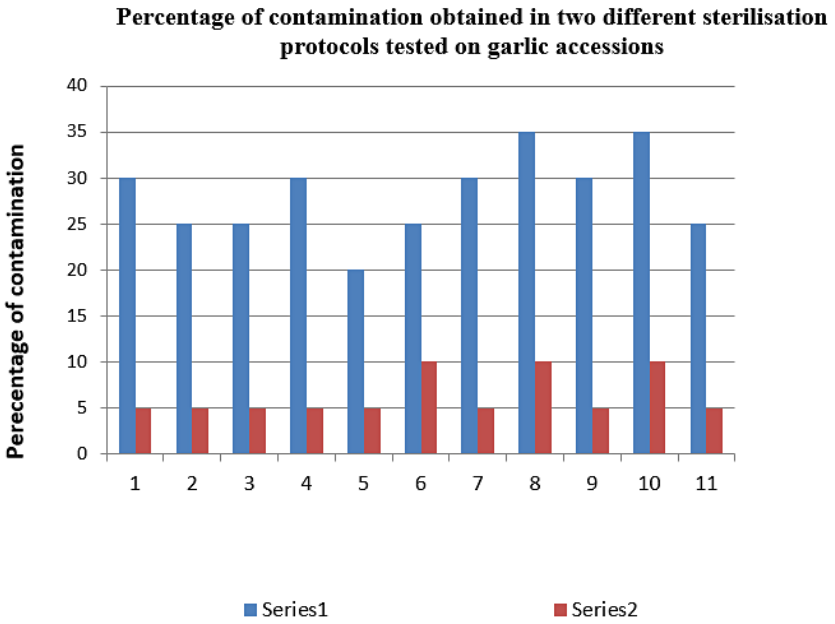

Fig. 1: Percentage of contamination recorded with the two different sterilization protocols tested on local garlic accessions

Effect of Different Media formulation on Shoot Regeneration

The meristem tip cultures inoculated on growth regulator-free MS medium regenerated successfully into plantlets within two weeks of culture. However, no shoot multiplication was observed for all twelve garlic accessions after five weeks of culture in hormone-free MS medium. This result is in agreement with the findings of previous studies, which reported the non-efficacy of hormone-free MS medium to promote shoot multiplication in garlic explants.[15,16] The frequency of multiple shoot formation was low on multiplication media (G0 to G11) for all twelve garlic accessions within the first three months. Two additional shoot multiplication media (G14 and G15) were tested. Plantlets develop into clumps having multiple shoots within the first five weeks of culture.

A total of fourteen media was tested and positive results were noted on only six shoot multiplication media: MS basal media with various growth regulators {G0 (0.25 mg/L NAA + 0.5 mg/L 2iP), G1 (1.5 mg/L BAP + 0.5 mg/L NAA), G2 (0.3 mg/L NAA + 3 mg/L 2iP), G6 (0.5 mg/L NAA + 2 mg/L 2iP), G14 (2 mg/L BAP + 2 mg/L NAA) and G15 (1 mg/L BAP + 0.5 mg/L NAA) (Table 1). Shoot proliferation was significantly better in shoot multiplication media G0, G1, G2, G6, G14, G15 compared to G3, G4, G5, G7, G8, G9, G10 and G11 (Table 1).

Highly significant difference ($P< 0.05$) was noted among the garlic accessions regarding the number of multiple shoot formation. Multiple shoots formation was recorded in different shoot multiplication media after 6 weeks of culture as from fourth subculture for nine garlic accessions namely, Beeharry, Boodnah. Bondah, Haulkhory, Gooniah, Ramdhuny, Rampall, Sujeebun 2 and Unuth (Fig. 3A). The other three garlic accessions: Ramjee, Sujeebun and VFG158 did not show any proliferation even after being maintained for more than 8 weeks (Fig. 3A). The highest number of shoot formation was observed in G2 (0.3 mg/L NAA+ 3 mg/L 2iP) and the lowest number of shoot formation was observed in G14 (2 mg/L BAP + 2 mg/L NAA) among the selected six shoot multiplication media Fig. 4 (B and C). It was noted that 20 to 25 % increase in shoot number was recorded in first generation followed by a multiplicative factor of 2 over generations as shown in Fig. 4 (C).

The garlic accessions could be categorised into four groups on the basic of the visual observations made during the trials and garlic accessions in the same group showed similar responses to culture media

(Fig. 3 B). Furthermore, other studies suggested that shoot proliferation was induced on MS medium containing high concentrations of BA and low concentrations of NAA, IBA and IAA17. Actually, explants cultured on the six media (G0, G1, G2, G6, G14, G15 (Table 1) containing comparatively high concentrations of cytokinins (BA and 2ip) in combination with lower concentrations of auxins (NAA, IBA and IAA) resulted in good proliferation rate.

Researchers have also reported that explant with 1-2 primodial leaves in meristematic tissue have the ability to intake auxin and cytokinins that promote growth and development of the explant.[18] Previous studies suggested that the cytokinins 2iP and BA in the culture media have interchangeable responses in the induction of shoots.[17] According to researchers it is more advantageous to use 2iP in media as the shoot produced are healthier and appeared to inhibit callus formation on the basal plate of the explants. The present results are in agreement with previous studies as callus formation was observed at the basal plate of the explant in media supplemented with NAA and BA (G1, G14 and G15), while no callus was recorded with media supplemented with 2ip (G0, G2 and G6). Furthermore, researchers suggested that the absence of callus at the base of the explant is beneficial for shoot regeneration and genetic stability as it is the preferred pathway to produce true-to-type clones. In contrast to previous reports,[17,19] shoot regeneration was not achieved with LS and Gamborg (B5) medium. Moreover, the present result was not in agreement with other studies which reported better shoot regeneration in media supplemented with IBA or IAA in combination with BA. Actually, root regeneration was observed instead shoot of regeneration, when the combination IBA or IAA and BA were used.

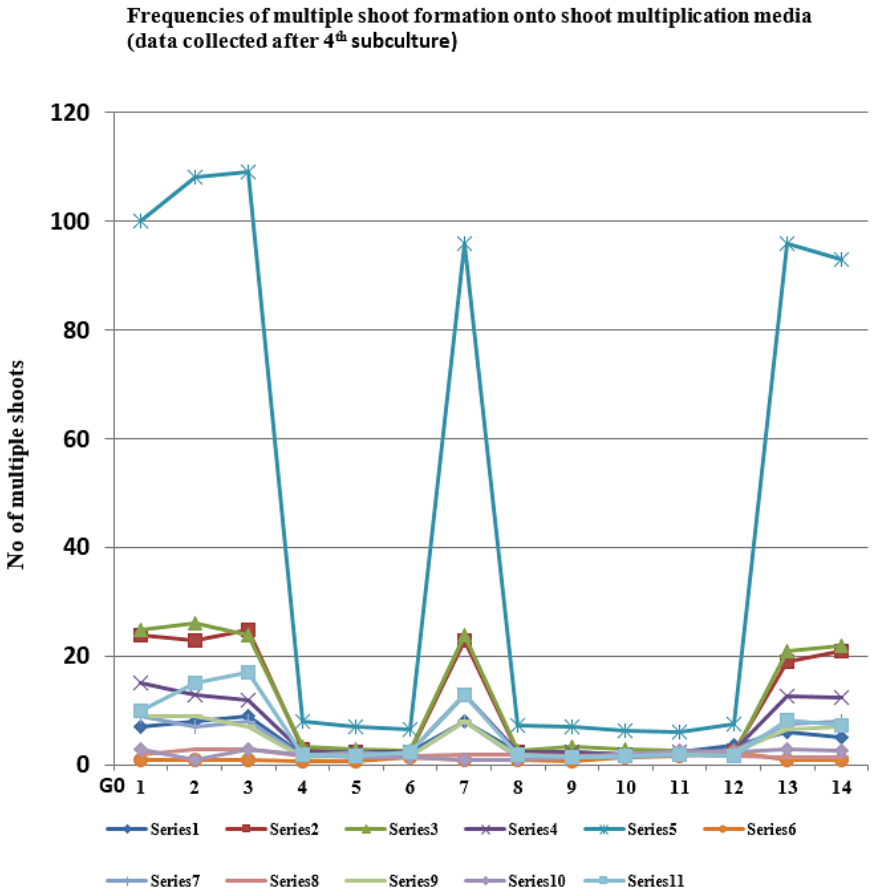

Fig. 2: Frequencies of multiple shoot formation recorded in garlic cultures on different shoot multiplication media.

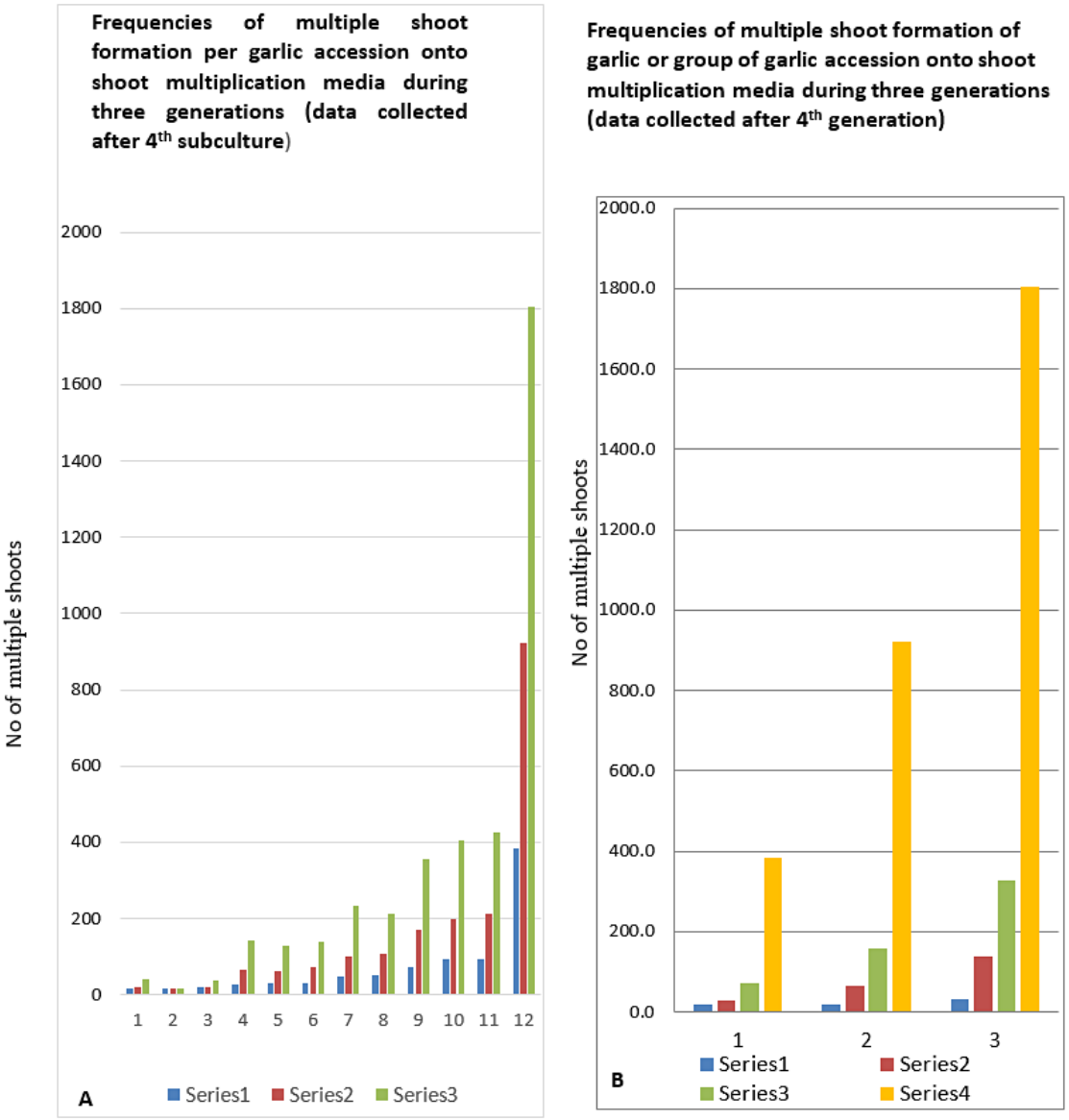

Fig. 3: Frequencies of multiple shoot formation recorded in garlic cultures. (A) Frequencies of multiple shoot formation on different shoot multiplication media during three generations. (B) Frequencies multiple shoot formation per of different garlic or group of garlic accessions on different shoot multiplication media during three generations.

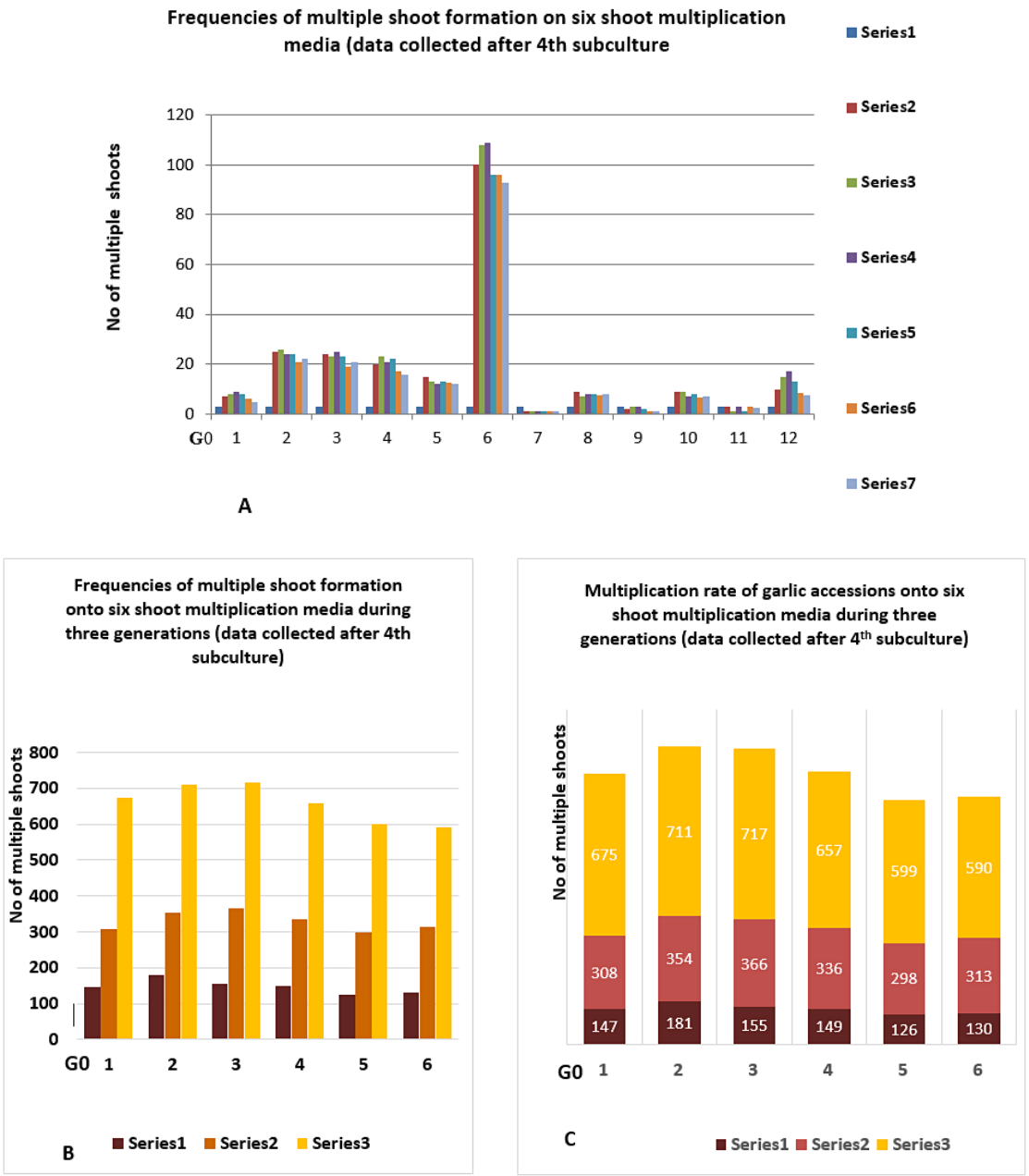

Fig. 4: Frequencies of multiple shoot formation recorded in garlic cultures on six selected media. (A) Frequencies of multiple shoot formation per garlic accession on six selected shoot multiplication media. (B) Frequencies of multiple shoot formation of garlic accessions on six selected shoot multiplication media during three generations. (C) Multiplication rate of garlic accessions on six selected shoot multiplication media during three.

Table 2: Effect of shoot multiplication media on shoot proliferation of different garlic accessions

Media	Sum	Average	Variance
G0	1130	376.67	73232.33
G1	1246	415.33	73046.33
G2	1238	412.67	80594.33
G6	1142	380.67	66012.33
G14	1023	341	57319
G15	1033	344.33	53636.33

Values represent sum, mean and variance of 15 explants per treatment in three repeated experiments

Table 3: Effect of shoot multiplication media on shoot proliferation during three generations

Media	Sum	Average	Variance
Gen 1	888	148	389.6
Gen 2	1975	329.17	740.17
Gen 3	3949	658.17	2936.97

Values represent sum, mean and variance of 15 explants per treatment in three repeated experiments

Effect of Genotypes

Several studies have reported genotypic variation in shoot multiplication in garlic.[20,21] In general, each genotype has different response to different media formulation. The results showed that there is high significant difference ($P<0.05$) in multiplication rate among garlic accessions or groups of similar accessions. The highest shoot proliferation was observed in the garlic accession Ramdhuny, while the lowest shoot proliferation was recorded in groups of similar accessions namely, Ramjee, Sujeebun and VFG 158 on different media formulations tested in the present study. The result also indicated high influence of genotype regarding multiplication of each garlic accession or groups of similar accessions on different media formulations (Fig. 3B and Fig. 4A). The result is in accordance with previous studies, which reported that garlic accessions or groups of similar accessions have different source of genetic material.[20,22] It is obvious that genotypes played an important role in shoot multiplication of different garlic accessions (Fig. 3B and 4A). Furthermore, the obtained result indicated that some garlic accessions have high regeneration capacity, while others are less responsive to the effect of hormones or combinations of hormones in different media. Therefore, these findings confirmed the hypothesis of previous reports that the efficiency of tissue culture techniques in garlic is strongly genotype-dependent.[23]

Hyperhydricity

Hyperhydricity formerly termed as vitrification is a morphological abnormality that occurs during *in vitro* culture.[24,25] Vitrified cultures have a glassy appearance with enlarged, thick and water-soaked translucent stems and leaves.[26] Several stress factors including cytokinins[27,28] and excess level of mineral elements in culture media cause this phenomenon during micropropagation. However, the factors that induce hyperhydricity in tissue culture are not fully understood. According to previous study, regeneration of normal and mature clonal shoots through micropropagation is inhibited by this physiological disorder.[29] Consequently, the multiplication factor and vigour of *in vitro* culture are affected, which a serious problem.[30] Furthermore, it also reduces the survival rate of plantlets in free living conditions. (Table 7). While there was significant difference ($P<0.05$) regarding hyperhydricity over generation. Data in Table 7 and Fig. 5 C indicated that hyperhydricity was suppressed on six media namely G0, G1, G2, G6, G14 and G15. Besides, the effect of different shoot multiplication media on hyperhydricity is shown in Fig. 5A-D. A decrease of 50% in the frequency of hyperhydricity from first to second generation and second to third generation was observed in the tested garlic accessions as shown in Fig. 2 (A). It was observed that frequency of vitrified shoots decreased and shoots proliferation increased with repeated subculture. This result is not in accordance with previous study, which reported reduction in proliferative shoots due to increase in frequency of hyperhydricity with repeated subculture.[31] Several researchers mentioned that BAP is a very effective cytokinins compared to kinetin, 2ip and zeatin (Z) in micropropagation of different plants.[32] However, previous studies reported that BAP also induced hyperhydricity and is amplified with increasing concentration of BAP.[33,34] Therefore, lower concentration of BAP or 2ip was used to avoid vitrified shoots. Data in Table 2 and Fig. 5C indicated that low concentrations of BAP ranging from 1 to 2 mg/l or 2ip with concentrations ranging from 0.5 to

3 mg/l used in shoot multiplication media (G0, G1, G2, G6, G14 and G15) promoted shoot proliferation, which is similar to findings of previous studies.[33,34] Other researchers also reported the influence of genotypes in micropropagation of garlic cultures, which is confirmed by the results obtained in this study (Fig. 5 B and D).[20,22] The difference in frequency of hyperhydricity in the different genotypes of the garlic accessions is evident in Table 2. It is noted that frequency of hyperhydricity was very low or nil, when garlic accessions Bondah and Boodnah were treated with the six shoot multiplication media (G0, G1, G2, G6, G14 and G15). This result confirmed the effect of genotypes in garlic accessions[20,22]

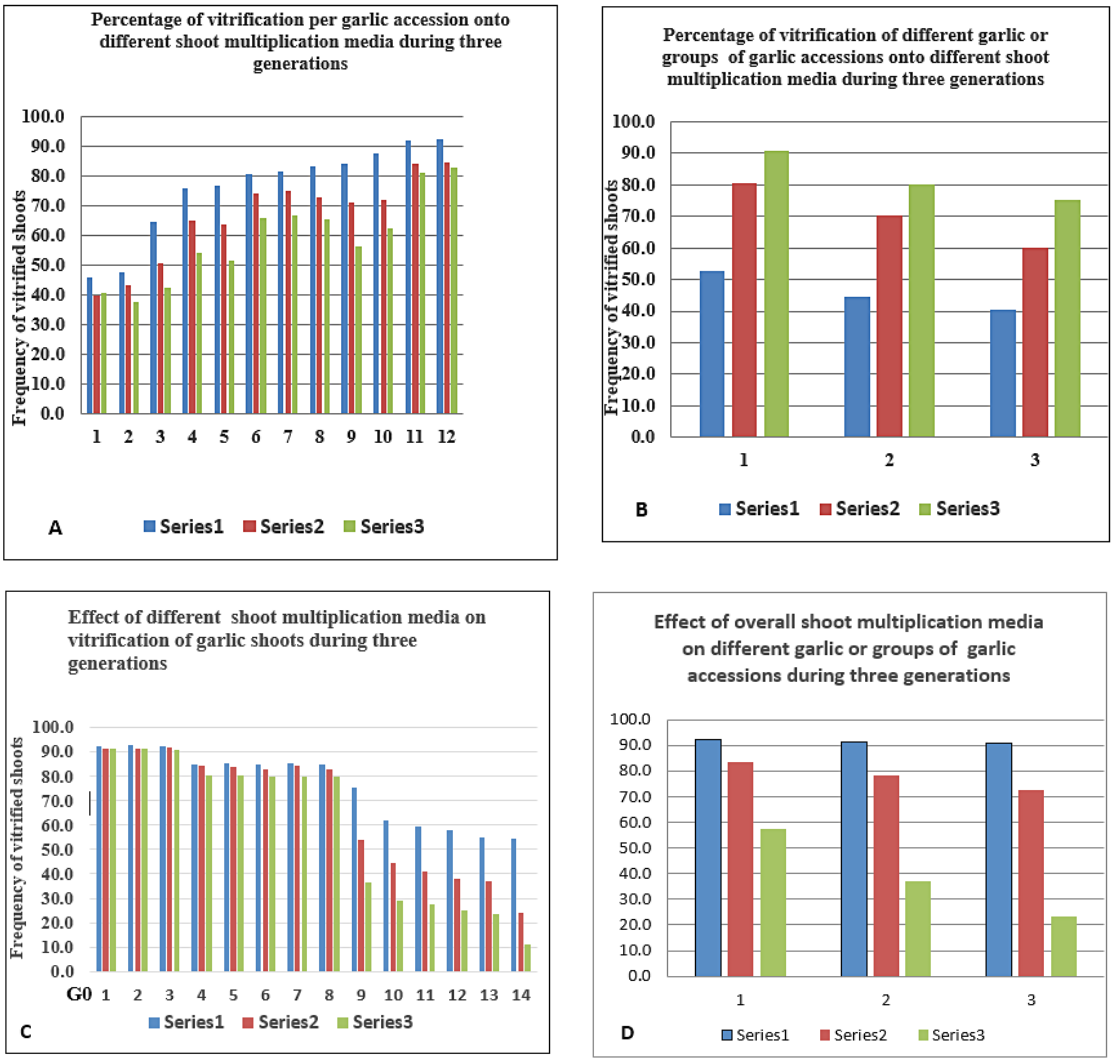

Fig. 5: Percentage of vitrification recorded in garlic cultures. (A) Percentage of vitrification for each garlic accession on different shoot multiplication media during three generation. (B) Percentage of vitrification different garlic or group of garlic accessions on different shoot multiplication media during three generation. (C) Effect of different shoot multiplication media on vitrification of garlic shoots formed during three generation. (D) Effect of overall shoot multiplication media on different garlic or group of garlic accessions during three generations.

Table 4: Effect of shoot multiplication media on shoot vitrification of different garlic accessions

Garlic accessions	Sum	Average	Variance
Boodnah	126.83	42.27	11.21
Bondah	128.28	42.76	24.53
Ramdhuny	158	52.66	126.49
Unuth	194.9	64.96	118.91
Sujeebun2	192.21	64.07	159.57
Gooniah	221.07	73.69	53.94
Haulkhory	223.21	74.4	51.68
Beeharry	221.79	73.93	78.83
Rampall	211.52	70.51	191.82
Sujeebun	222.23	74.079	160.61
VFG158	257.11	85.71	32.02
Ramjee	259.76	86.58	27.14

Values represent sum, mean and variance of 15 explants per treatment in three repeated experiments

Table 5: Effect of shoot multiplication media on shoot vitrification during three generations

Generation	Sum	Average	Variance
Gen 1	912.76	76.06	242.44
Gen2	796.28	66.35	214.97
Gen3	707.91	58.99	214.82

Values represent sum, mean and variance of 15 explants per treatment in three repeated experiments

Table 6: Anova table

Source of Variation	SS	df	MS	F	P<0.05
Accession	7080.72	11	643.7	45.11	*
Generation	1759.67	2	879.83	61.65	*
Error	313.945	22	14.27		
Total	9154.34	35			

High significance difference (P<0.05) using Tukey's multiple range tests is indicated by the symbol*

Table 7: Frequency of hyperhydricity of different garlic accessions on different shoot multiplication media

Row Labels	Beeharry	Bondah	Boodnah	Gooniah	Haulkhory	Ramdhuny	Ramjee	Rampall	Sujeebun	Sujeebun2	Unuth	VFG 158
G0	46.1	0.0	0.0	49.2	43.2	45.8	58.8	53.7	47.8	35.9	45.0	58.0
G1	42.0	0.0	7.2	44.2	41.8	42.3	73.4	46.8	50.3	47.6	47.7	68.1
G10	97.4	71.4	66.7	93.9	97.7	62.8	100.0	79.4	83.0	72.3	71.8	100.0
G11	96.7	69.9	69.1	94.3	97.7	61.8	100.0	79.2	83.9	72.0	71.6	100.0
G14	41.1	0.0	0.0	43.3	40.3	41.0	58.4	52.4	48.6	36.1	40.1	59.7
G15	27.6	0.0	0.0	30.0	38.2	22.0	58.7	32.6	43.0	25.6	28.6	53.0
G2	48.4	2.8	3.3	45.0	47.1	40.9	77.0	52.8	56.9	43.7	46.4	76.8
G3	100.0	67.0	65.2	100.0	100.0	66.4	100.0	100.0	100.0	100.0	100.0	100.0
G4	100.0	67.0	65.3	100.0	100.0	66.4	100.0	100.0	100.0	100.0	100.0	100.0
G5	100.0	66.9	66.2	100.0	100.0	64.0	100.0	100.0	100.0	100.0	100.0	100.0
G6	44.8	46.6	47.6	46.1	45.3	35.9	85.9	50.6	77.9	53.4	46.7	82.2
G7	100.0	66.7	66.1	96.7	96.8	62.6	100.0	79.0	81.4	69.1	70.9	100.0
G8	96.1	70.0	66.6	94.4	96.8	62.6	100.0	80.8	81.4	69.9	70.7	100.0
G9	96.7	70.4	68.6	94.4	96.8	62.9	100.0	79.9	82.9	71.4	70.2	100.0

The gradual increase in hyperhydricity among different garlic accessions with different shoot multiplication media used.

Bulblet Formation

Many advantages are associated with *in vitro* bulblets in garlic. These structures are good propagules, easy to maintain and manage, can be stored before hardening and do not require immediate hardening. Swelling of the bases of the shoots is an indication of bulblets formation.

In the present study, bulblets induction was achieved by transferring shoots clumps on different

bulbification media: B2 (MS enriched with 12% sucrose), B4 (MS supplemented with 2mg/L Kin+1mg/L GA3 and enriched with 3%) and B5 MS supplemented with 2mg/L BAP+1mg/L GA3 and enriched with 9% sucrose). Significant differences ($P<0.05$) in bulblet formation were noted when the garlic cultures were incubated on different bulbification media under 25 ± 2°C and 16L/8D (Fig. 6). It was observed that shoots clumps transferred on bulbification media supplemented with BA or kinetin and gibberellic acid (B5 and B4) did not show swelling during the first six weeks of incubation in contrast to cultures transferred to media B2. Bulblet formation of shoots was observed only after eight weeks of culture for bulbification media B5 and 12 weeks for media B4. This result of early bulblet formation is comparable to previous studies, wherein researchers reported that bulblet formation is induced earlier in hormones- free media, which reduced the multiplication factor of the plantlets.[35,36] Furthermore, most of the swollen shoots formed on culture media B4 did not develop into bulblets compared to the culture media B2 and B5. The main reason for the failure of the swollen portion of the shoots to form bulblets in culture media B4, could be related to the low amount of carbon source in the B4 medium as it was enriched with only 3% sucrose. According to researchers, media enriched with 12% sucrose enhanced bulblet weight, while larger bulblets are obtained in hormone-free MS medium.[37] However, largest and heavier bulblets were obtained in B5 medium compared to B2 medium which is in contrast to the findings of previous study.[37]

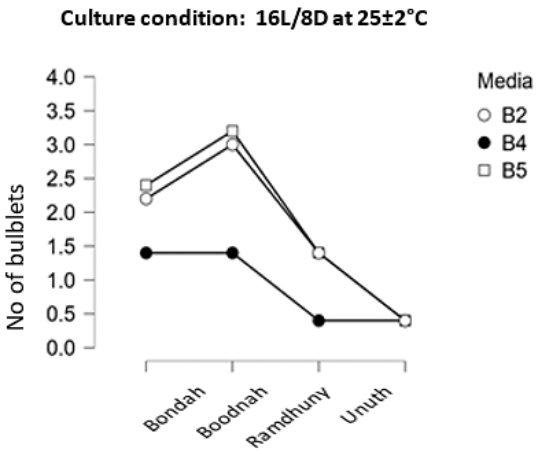

Fig. 6: Bulblet formation in different bubification media when cultured at 25±2°C and 16L/8D.

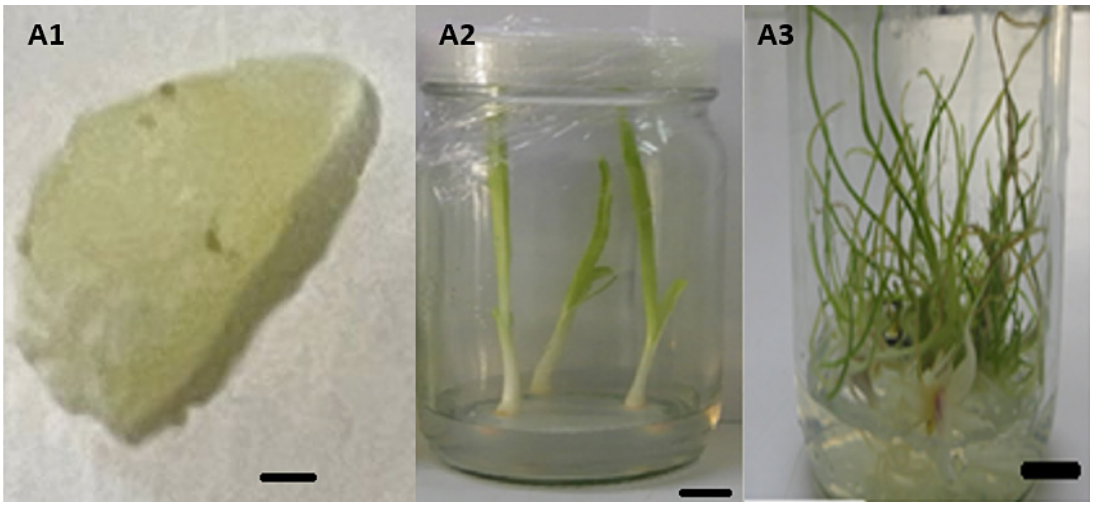

Fig. 7: Initiated meristem-tip culture and shoot development of garlic accession. (A1) Initiated meristem-tip culture {Bar=0.02 mm}. (A2) Shoots development on hormone-free medium (MS) after two weeks of culture {Bar= 1 cm}. (A3) Multiple shoots of garlic accession on G0 media (0.25mg/L NAA+0.5mg/L 2iP) after 5 weeks of culture (MS) {Bar= 1 cm}.

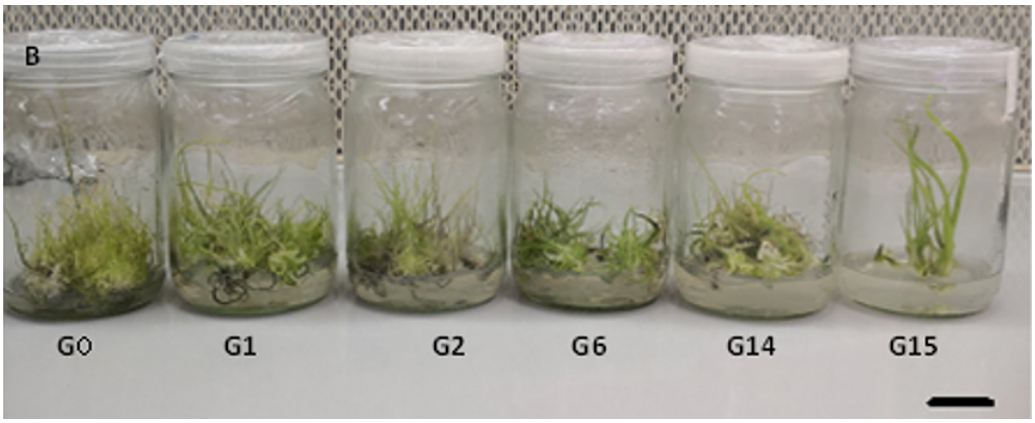

Fig. 8: Effect of different media formulations [G0 (0.25mg/L NAA+0.5mg/L 2iP), G1 (1.5mg/L BAP+0.5mg/L NAA), G2 (0.3mg/L NAA+3mg/L 2iP), G6 (0.5mg/L NAA+2mg/L 2iP), G14(2mg/L BAP+2mg/L NAA) and G15(1mg/L BAP+0.5mg/L NAA)] on multiplication rate of garlic accession (Gooniah){Bar= 1 cm}

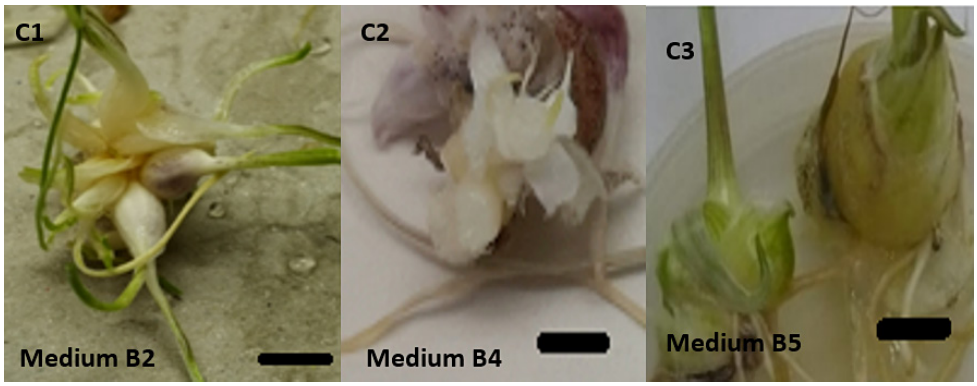

Fig. 9: *In vitro* bulblets of garlic accession (Gooniah) on different bulbification media after 12 weeks of culture. (C1) Medium B2 (MS enriched with 12% sucrose) {Bar= 1 cm}. (C2) Medium B4 (MS supplemented with 2mg/L Kin+1mg/L GA3 and enriched with 3% sucrose) {Bar= 0.5 cm} (C3) Medium B5 (MS supplemented with 2mg/L BAP+1mg/L GA3 and enriched with 9% sucrose) {Bar=1 cm}.

Fig. 10: Hardening of garlic accessions. (D) Harvested bulblets of garlic accession (Gooniah) {Bar= 1 cm}. (E) Hardened garlic plants {Bar=1 cm}.

Conclusion

A reliable protocol for rapid shoot regeneration and multiplication from meristem-tip culture was optimised in the present study. The different important steps of shoot regeneration, plantlets proliferation and bulblet formation were studied, which could be successfully used for mass propagation of garlic to produce virus-free planting material and increase the local garlic production. The use of *in vitro* techniques for garlic mass propagation will alleviate the problem of unavailability of clean planting material and continuous accumulation of viruses in the local garlic accessions.

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Conflict of Interest

There is no conflict of interest among the authors.

Authors' contributions

Boodhram Indira Kumari Devi: proof reading, manuscript proposal and manuscript revisions; Greedharry Pratima: Undertaking research activities, data collection, analysis, manuscript writing and revisions; Koyelas Chandasa: assistance provided during the initial stage of the project. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Not applicable

Ethics Statement

Not applicable

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