



Article

Alterations in Microrhizome Induction, Shoot Multiplication and Rooting of Ginger (*Zingiber officinale* Roscoe) var. Bentong with Regards to Sucrose and Plant Growth Regulators Application

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Abstract: Ginger (*Zingiber officinale* Roscoe) var. Bentong is a monocotyledon plant that belongs to the Zingiberaceae family. Bentong ginger is the most popular cultivar of ginger in Malaysia, which is conventionally propagated by its rhizome. As its rhizomes are the economic part of the plant, the allocation of a large amount of rhizomes as planting materials increases agricultural input cost. Simultaneously, the rhizomes' availability as planting materials is restricted due to the high demand for fresh rhizomes in the market. Moreover, ginger propagation using its rhizome is accompanied by several types of soil-borne diseases. Plant tissue culture techniques have been applied to produce disease-free planting materials of ginger to overcome these problems. Hence, the in vitro-induced microrhizomes are considered as alternative disease-free planting materials for ginger cultivation. On the other hand, Bentong ginger has not been studied for its microrhizome induction. Therefore, this study was conducted to optimize sucrose and plant growth regulators (PGRs) for its microrhizome induction. Microrhizomes were successfully induced in Murashige and Skoog (MS) medium supplemented with a high sucrose concentration ($>45 \text{ g L}^{-1}$). In addition, zeatin at 5–10 μM was found more effective for microrhizome induction than 6-benzylaminopurine (BAP) at a similar concentration. The addition of 7.5 μM 1-naphthaleneacetic acid (NAA) further enhanced microrhizome formation and reduced sucrose's required dose that needs to be supplied for efficient microrhizome formation. MS medium supplemented with 60 g L^{-1} sucrose, 10 μM zeatin and 7.5 μM NAA was the optimum combination for the microrhizome induction of Bentong ginger. The in vitro-induced microrhizomes sprouted indoors in moist sand and all the sprouted microrhizomes were successfully established in field conditions. In conclusion, in vitro microrhizomes can be used as disease-free planting materials for the commercial cultivation of Bentong ginger.

Keywords: *Zingiber officinale* Rosc. var Bentong; microrhizome; sucrose; cytokinin; 1-naphthaleneacetic acid



Citation: Zahid, N.A.; Jaafar, H.Z.E.; Hakiman, M. Alterations in Microrhizome Induction, Shoot Multiplication and Rooting of Ginger (*Zingiber officinale* Roscoe) var. Bentong with Regards to Sucrose and Plant Growth Regulators Application. *Agronomy* **2021**, *11*, 320. <https://doi.org/10.3390/agronomy11020320>

Academic Editor: Mukund R. Shukla

Received: 4 January 2021

Accepted: 8 February 2021

Published: 11 February 2021

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1. Introduction

The Bentong variety of ginger (*Zingiber officinale* Roscoe) is well-known in Malaysia. It has bigger rhizomes with lower fibrous pulp compared to other Malaysian cultivars of ginger [1]. Due to the unique characteristics of Bentong ginger, there is a high demand for its rhizomes in the domestic and international markets [2]. Since the rhizomes are economically utilized parts of the plant, using a high proportion of ginger rhizomes as planting materials for cultivating the plant in the next growing season negatively affects its supply in the market. Besides, most of the diseases are easily transmitted through vegetative reproduction by fragmentation of rhizomes [3]. Therefore, micropropagation could be a suitable alternative option for the effective production of Bentong ginger.

The efficiency of micropropagation techniques depends on establishing the in vitro-raised plants in ex vitro conditions. Micropropagation through in vitro shoot regeneration requires a proper rooting and acclimatizing of the in vitro-raised plantlets to be successfully established in field conditions. Rooting and acclimatizing the in vitro-raised shoots adds extra cost and time to prepare them for field transplantation. Hence, microrhizomes have been considered as alternative disease-free planting materials. Microrhizomes produced under in vitro conditions are modified stems or storage organs of the rhizomatous plant species. They are the same as the mature rhizomes of these plant species and they can sprout and develop into a whole plant. Using microrhizomes as planting materials minimize the cost and time of acclimatization and they can be planted directly in the field without the process of acclimatization [4,5]. Microrhizomes can be easily stored and they are less vulnerable during transportation. Thus they are more suitable for international shipping and germplasm exchange [6]. Microrhizomes could also be used as a source of secondary metabolites [7]. Hence, microrhizome production is one of the most useful techniques for rhizomatous plant micropropagation.

Many researchers have attempted microrhizome induction of Zingiberaceae species. A higher sucrose concentration than the control for standard micropropagation systems (20–30 g L⁻¹) was found as the primary factor for microrhizome induction [7–9]. Ginger microrhizome initiation starts with a swelling at the shoot base when they are cultured on medium supplemented with a high concentration of sucrose [8]. The swelling part gradually increases in size and appears as a yellow-orange colored structure with an aromatic odor like the mature rhizome [10]. Since the high concentration of sucrose is the primary metabolic signal for the in vitro microrhizome induction, the optimum concentration of sucrose for microrhizome induction of ginger has been varying reported in different studies. Abbas et al. [10] reported that the addition of 60 g L⁻¹ sucrose to MS medium was the optimum concentration for microrhizome induction of ginger. Mehaboob et al. [8] found that 80 g L⁻¹ was the optimum concentration of sucrose for microrhizome induction of ginger. On the other hand, Swarnathilaka et al. [5] obtained the best result for microrhizome induction when 90 g L⁻¹ sucrose was supplemented into MS culture medium.

Besides, auxin and cytokinin have also affected in vitro microrhizome induction of ginger [11]. David et al. [4] obtained a maximum number of microrhizomes with the highest biomass with 26.64 µM BAP in MS medium supplemented with 60 g L⁻¹ sucrose. Abbas et al. [10] also reported that a high concentration of BAP (39.96 µM) was more effective for microrhizome induction of ginger. However, in other studies, high concentration of cytokinin was not effective for microrhizome production of ginger. Increasing the BAP concentration of more than 17.76 µM caused a reduction in the number and biomass of the microrhizomes [5,9].

The previous studies' varying results indicated that sucrose and PGRs need to be optimized in the culture medium to achieve a better response of the ginger in vitro microrhizome induction. Bentong ginger is the most expensive and popular variety of *Zingiber officinale* in Malaysia, and it has not been studied yet for its in vitro microrhizome induction. Therefore, this study was conducted to optimize sucrose and PGRs for microrhizome induction of Bentong ginger. The main objectives of the study were to determine the suitable type and optimum concentration of cytokinin for microrhizome induction and establish the best combination of cytokinin, sucrose and auxin for improving the microrhizome induction of Bentong ginger. Besides the microrhizome induction, the effects of sucrose, cytokinins and auxin were also evaluated for shoot multiplication and rooting of Bentong ginger.

2. Materials and Methods

2.1. Plant Materials and Experimental Conditions

In this study, the in vitro microrhizome induction of Bentong ginger was evaluated by conducting two separate experiments. The in vitro-induced microrhizomes were assessed

for their sprouting capability and ex vitro establishment. Fresh mature rhizomes of Bentong ginger were collected from the Bentong ginger growing area located in Bukit Tinggi, Bentong a western part of Pahang in Malaysia. The rhizomes were incubated in moist sterilized sand in the dark at 25 ± 2 °C for three weeks to produce sprouting buds. The sprouted buds of rhizomes (1–1.5 cm) were used as explants for the culture initiation (Figure 1A). The rhizome sprouted buds were washed thoroughly under running tap water with a few drops of liquid detergent and 2–4 drops of Tween 20 for 30 min to remove all the adhering soil particles. After washing thoroughly under running tap water, the sprouted buds as explants were surface sterilized with 70% (*v/v*) ethanol for 1 min [12]. This was followed by immersing in 70% (*v/v*) Clorox (5.25% NaOCl) with two drops of Tween 20 and agitated by shaker at 300 rpm for 30 min. Finally washed for 4–5 times with sterilized distilled water to remove the traces of Clorox. The sterilized rhizome sprouted buds were then aseptically trimmed to 0.5–0.8 cm long [13] and cultured in 35 mL vials containing 12 mL MS basal medium.

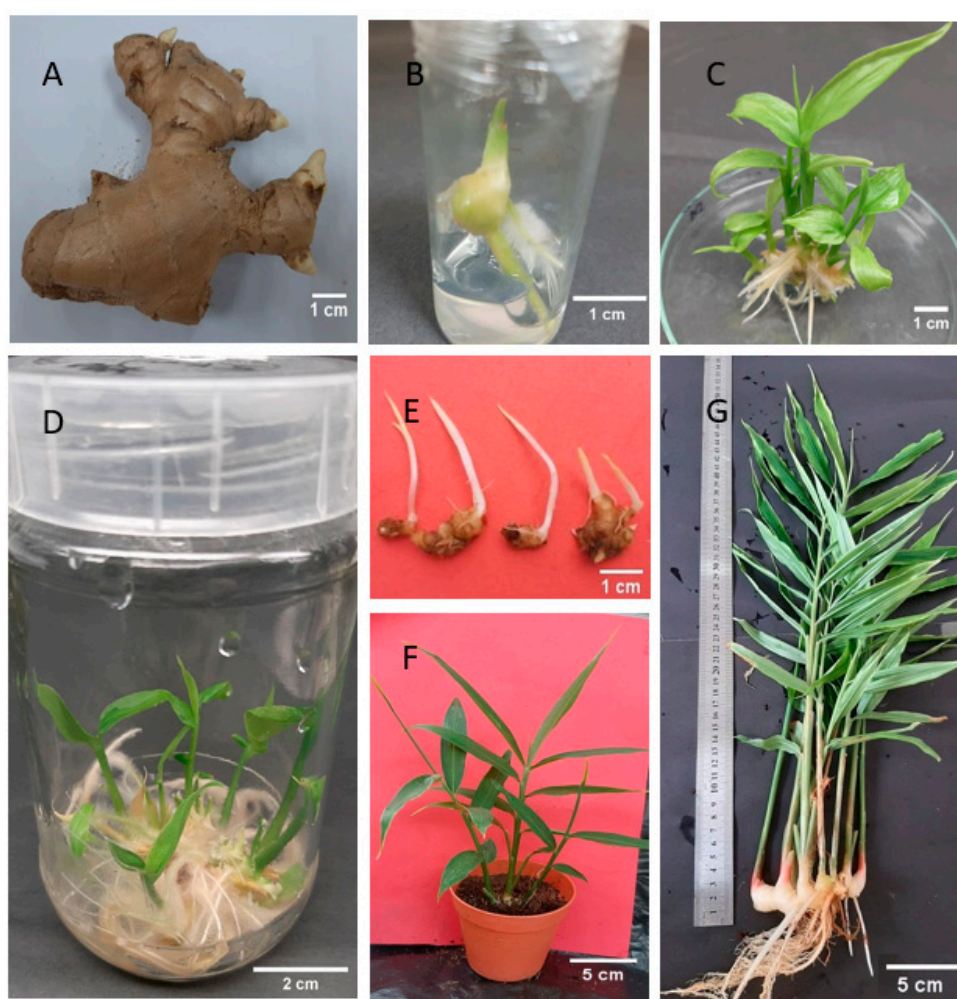


Figure 1. Different stages of Bentong ginger microrhizome induction from culture initiation until ex vitro establishment. (A) Mature rhizome with sprouted buds as a source of explants, (B) initiated culture from rhizome sprouted bud of Bentong ginger after four weeks of inoculation, (C) in vitro-raised shoots of Bentong ginger obtained from MS medium supplemented with 30 g L^{-1} sucrose, $10 \mu\text{M}$ zeatin and $2.5 \mu\text{M}$ NAA, and these shoots were used as explant for microrhizome induction, (D) in vitro-induced microrhizomes with multiple shoots and a bunch of roots after 12 weeks of inoculation, (E) microrhizome sprouted in moist sand after two weeks of sowing, (F) Bentong ginger plant raised from sprouted microrhizome after one month of growing, and (G) Bentong ginger plant with young rhizomes harvested from polybag three months after transplanting.

After four weeks of culture initiation, the *in vitro* raised shoots (Figure 1B) were multiplied by subculturing on MS medium supplemented with 30 g L⁻¹ sucrose, 10 µM zeatin and 2.5 µM NAA, which was found to be the best culture medium for shoot multiplication in our previous study (not published). After four weeks of shoot multiplication, the *in vitro* raised shoots of Bentong ginger of about 3–4 cm length were used as explants for microrhizome induction (Figure 1C). All the roots were removed from the shoots before culturing into 300 mL culture jars containing MS medium solidified with 2.5 g L⁻¹ Gelrite. The pH of the medium was adjusted to 5.8 by using 0.1 N NaOH or HCl solutions before adding the Gelrite. The media were heated to boil in a microwave oven to homogenize the Gelrite before dispensing into the culture jars. The culture jars were tightly closed by transparent polypropylene caps and then autoclaved at 121 °C and 104 kPa pressure for 20 min. All the cultures were incubated in the culture room at 25 ± 2 °C, under 16 h photoperiod with a light intensity of 35 µmol m⁻² s⁻¹ provided by Philips cool white fluorescent tubes. All the chemicals required for MS medium were of analytical grade purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. Zeatin and Gelrite were purchased from Duchefa, Haarlem, The Netherlands. BAP and NAA were purchased from R & M Chemicals, Essex, UK.

2.2. Effects of Different Cytokinins on Microrhizome Induction, Shoot Multiplication and *In Vitro* Rooting

In the first experiment, the addition of zeatin and BAP at 5, 10, 15 and 20 µM into MS medium supplemented with 80 g L⁻¹ sucrose, which was found to be the optimum for microrhizome induction in the previous studies [8], and 2.5 µM NAA, which was optimum for shoot multiplication of Bentong ginger in our previous study, were evaluated for microrhizome induction, shoot multiplication and rooting of Bentong ginger. Cytokinin-free MS medium was considered as a control treatment. Each treatment was replicated three times with six explants per replication cultured in an individual jar. Data related to the number of microrhizomes per explant, the biomass of microrhizome per explant (g), microrhizome diameter (mm), number of shoots per explant, shoot length (cm) and the number of roots per explant were recorded after 14 weeks of culture.

2.3. Effects of Different Concentrations of Sucrose and NAA on Microrhizome Induction, Shoot Multiplication and *In Vitro* Rooting

In the second experiment, MS medium supplemented with 10 µM zeatin, which resulted in the highest number, biomass and diameter of microrhizome in the first experiment, was further studied for microrhizome induction, shoot multiplication and rooting of Bentong ginger by the addition of sucrose at 30, 45, 60, 75 and 90 g L⁻¹ with the combination of NAA at 0, 2.5, 5 and 7.5 µM. Each combination was replicated three times with six explants per replication cultured in an individual jar. Data related to the number of microrhizomes per explant, the biomass of microrhizome per explant (g), microrhizome diameter (mm), number of shoots per explant, shoot length (cm) and number of roots per explant was recorded after 12 weeks of culture.

2.4. Microrhizome Sprouting and *Ex Vitro* Establishment

The 85 day-old *in vitro*-induced microrhizomes were taken out from the culture jars and their shoots and roots were trimmed out from each microrhizome. The microrhizomes were thoroughly washed under running tap water to remove the residue of Gelrite from the microrhizomes. A total of 100 microrhizomes, obtained from MS medium supplemented with 60 g L⁻¹ sucrose, 10 µM zeatin and 7.5 µM NAA, were cultured in a tray filled with moist river sand and maintained indoor in the dark at 27 ± 2 °C. The moisture of the sand was maintained by spraying with water every three days. After two weeks, the sprouting rate of the microrhizomes was recorded and 90 sprouted microrhizomes were transplanted into small pots (200 mL) containing coco peat (Figure 1E). A single sprouted microrhizome was planted per pot. The pots containing sprouted microrhizomes were kept outside under direct sunlight in field conditions at an average of 33 °C daytime temperature. After one month, their survival rate was recorded. The one-month-old well-

rooted plantlets produced from microrhizomes (Figure 1F) were transplanted in polybags (30 × 30 cm) filled with 6 L coco peat and kept in a shade house under 50% black shade net at $33 \pm 2/25 \pm 2$ °C day/night temperature and $235\text{--}250 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. After three months of transplanting, the weight of the young rhizomes (g) was measured.

2.5. Experimental Design and Statistical Analysis

A completely randomized design (CRD) was applied for this study. The data were analyzed by analysis of variance (ANOVA) using statistical analysis software (SAS) (version 9.4, SAS Institute Inc., North Carolina 27513, USA) and means were separated by using Tukey's Studentized Range (HSD) test at $p < 0.05$.

3. Results

3.1. Effects of Different Types and Concentrations of Cytokinins on Microrhizome Induction of Bentong Ginger

Microrhizome formation started after 7–8 weeks of culture by initial swelling at the base of the shoot. Both types of cytokinin (zeatin and BAP) significantly increased ($p < 0.05$) the number, biomass and diameter of microrhizomes compared to the control treatment (cytokinin-free MS medium) (Table 1). The effects of cytokinins on microrhizome induction were dependent on the type and concentration of cytokinins. Based on the number, biomass and diameter of microrhizomes, zeatin at all concentrations was more effective for microrhizome induction than BAP. Addition of zeatin at $10 \mu\text{M}$ to the MS culture medium which was supplemented with 80 g L^{-1} sucrose and $2.5 \mu\text{M}$ NAA, resulted in the highest number of microrhizomes per explant (4.50 ± 0.29) with the maximum biomass ($3.61 \pm 0.29 \text{ g}$) and diameter (7.82 ± 0.28) of microrhizome but it was not significantly different from 5 and $15 \mu\text{M}$ of zeatin. On the other hand, BAP showed the highest response of microrhizome induction at $15 \mu\text{M}$ concentration which was significantly lower than that of zeatin at $5\text{--}15 \mu\text{M}$ concentration. Further increase of both zeatin and BAP of more than $15 \mu\text{M}$ caused a significant reduction in the number, biomass and diameter of microrhizomes. The lowest number of microrhizomes per explant (2.28 ± 0.15) with the lowest biomass ($1.43 \pm 0.05 \text{ g}$) and diameter ($6.01 \pm 0.12 \text{ mm}$) was induced under the control treatment but it was not significantly different from all concentrations of BAP.

Table 1. Effects of different types and concentrations of cytokinins on microrhizome induction of Bentong ginger in MS medium supplemented with 80 g L^{-1} sucrose and $2.5 \mu\text{M}$ NAA after 14 weeks of inoculation.

Cytokinin	Cytokinin Concentration (μM)	Number of Microrhizomes/Explant	Microrhizome Biomass/Explant (g)	Microrhizome Diameter (mm)
Control	0	$2.28 \pm 0.15 \text{ c}$	$1.43 \pm 0.05 \text{ d}$	$6.01 \pm 0.12 \text{ d}$
BAP	5	$2.39 \pm 0.06 \text{ c}$	$1.76 \pm 0.14 \text{ cd}$	$6.05 \pm 0.13 \text{ d}$
BAP	10	$3.00 \pm 0.19 \text{ c}$	$2.40 \pm 0.10 \text{ c}$	$6.45 \pm 0.29 \text{ cd}$
BAP	15	$3.17 \pm 0.17 \text{ bc}$	$2.54 \pm 0.14 \text{ bc}$	$6.55 \pm 0.33 \text{ bcd}$
BAP	20	$2.56 \pm 0.11 \text{ c}$	$1.90 \pm 0.09 \text{ cd}$	$6.29 \pm 0.09 \text{ d}$
Zeatin	5	$4.11 \pm 0.29 \text{ a}$	$3.29 \pm 0.25 \text{ ab}$	$7.67 \pm 0.19 \text{ ab}$
Zeatin	10	$4.50 \pm 0.29 \text{ a}$	$3.61 \pm 0.29 \text{ a}$	$7.82 \pm 0.28 \text{ a}$
Zeatin	15	$4.17 \pm 0.17 \text{ ab}$	$3.37 \pm 0.18 \text{ a}$	$7.59 \pm 0.22 \text{ abc}$
Zeatin	20	$3.06 \pm 0.24 \text{ c}$	$2.11 \pm 0.09 \text{ cd}$	$6.43 \pm 0.34 \text{ cd}$

Values are means \pm standard error (SE) of $n = 18$. Means followed by different letters in each column are significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test. BAP = 6-benzylaminopurine.

3.2. Effects of Different Types and Concentrations of Cytokinins on Shoot Multiplication and Rooting of Bentong Ginger

The number of shoots per explant, shoot length and number of roots per explant were significantly affected at $p < 0.05$ in different concentrations of zeatin and BAP (Table 2). Shoot multiplication on MS medium supplemented with 80 g L^{-1} sucrose and $2.5 \mu\text{M}$ NAA

was positively affected by the addition of BAP compared to zeatin. The highest number of shoots (14.28 ± 0.86) was produced in 10 μM of BAP which was not significantly different with 5, 15 and 20 μM of BAP. The lowest number of shoots (6.5 ± 0.29) was recorded with 20 μM of zeatin, which was not significantly different from the control treatment. The shoot length was drastically reduced in applying all concentrations of BAP and zeatin at 5 μM or higher into the culture medium compared to the control treatment. The most extended shoot length (6.2 ± 0.31 cm) was recorded in the control treatment. The shoot multiplication rate and shoot growth on culture medium with a high sucrose concentration were inhibited for the first two months of the inoculation. After two months, the shoots' stress symptoms gradually reduced, and new shoots were induced along with microrhizomes in all of the treatments. The induction of shoots that appear after microrhizome initiation was more enhanced in BAP treatments compared to that of zeatin.

Table 2. Effects of different types and concentrations of cytokinins on shoot multiplication and rooting of Bentong ginger cultured on MS medium supplemented with 80 g L⁻¹ sucrose and 2.5 μM NAA after 14 weeks of inoculation.

Cytokinin	Cytokinin Concentration (μM)	Number of Shoots/Explant	Shoot Length (cm)	Number of Roots/Explant
Control	0	8.00 ± 0.51 cd	6.2 ± 0.31 a	27.78 ± 2.00 bc
BAP	5	11.83 ± 0.60 ab	3.89 ± 0.2 b	27.33 ± 1.20 bc
BAP	10	14.28 ± 0.86 a	4 ± 0.19 b	31.22 ± 1.13 bc
BAP	15	14.17 ± 0.93 a	3.75 ± 0.14 b	27.67 ± 1.45 bc
BAP	20	13.5 ± 0.76 a	3.58 ± 0.08 b	26.39 ± 1.22 bc
Zeatin	5	8.72 ± 0.49 cd	5.2 ± 0.19 a	41.50 ± 1.61 a
Zeatin	10	9.67 ± 0.38 bc	3.56 ± 0.22 b	33.78 ± 2.38 b
Zeatin	15	9.39 ± 0.20 bcd	3.33 ± 0.22 b	33.50 ± 1.32 b
Zeatin	20	6.50 ± 0.29 d	3.36 ± 0.18 b	23.67 ± 1.20 c

Values are means \pm standard error (SE) of $n = 18$. Means followed by different letters in each column are significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test. BAP = 6-benzylaminopurine.

In addition, numerous roots per explant were also induced in all treatments of cytokinins and the control treatment. The addition of zeatin at 5–15 μM significantly increased the number of roots compared to the control treatment. The highest number of roots per explant (41.5 ± 1.61) was recorded in 5 μM of zeatin. Increasing zeatin concentration of more than 15 μM caused a significant reduction in the number of roots and the lowest number of roots per explant (23.67 ± 1.2) was recorded with 20 μM of zeatin. The addition of BAP did not significantly increase the number of roots per explant over the control treatment.

3.3. Effects of Different Concentrations of Sucrose and NAA on Microrhizome Induction of Bentong Ginger

Microrhizome induction of Bentong ginger was effectively enhanced by optimizing PGRs and sucrose concentration in MS culture medium. The analysis of variance showed that the number, biomass and diameter of microrhizomes were significantly affected at $p < 0.001$ in both factors of sucrose and NAA concentrations and their interaction (Table 3). Microrhizome induction did not occur in all NAA concentrations when the culture medium was supplemented with 30 g L⁻¹ sucrose. Microrhizomes were induced when the culture medium was supplemented with 45 to 90 g L⁻¹ sucrose. The addition of auxin to the culture medium significantly enhanced microrhizome induction. Microrhizome number, biomass and diameter were significantly increased with increasing NAA concentration from 0 to 7.5 μM . In comparison to the auxin-free MS medium, microrhizomes number and biomass were increased more than 1.5- and 2.5-fold, respectively, with the supplementation of 7.5 μM NAA.

Table 3. Effects of different sucrose and NAA concentrations on microrhizome induction of Bentong ginger in MS medium supplemented with 10 μM of zeatin after 12 weeks of inoculation.

NAA (μM)	Sucrose (g L^{-1})	Number of Microrhizome	Microrhizome Biomass (g)	Microrhizome Diameter (mm)
0	30	0.00 \pm 0.00 g	0.00 \pm 0.00 j	0.00 \pm 0.00 h
	45	1.58 \pm 0.08 f	0.68 \pm 0.02 i	4.88 \pm 0.23 g
	60	1.92 \pm 0.17 ef	0.83 \pm 0.06 i	5.43 \pm 0.23 fg
	75	2.00 \pm 0.14 ef	0.94 \pm 0.04 i	5.56 \pm 0.01 efg
	90	2.19 \pm 0.10 def	1.06 \pm 0.07 hi	5.64 \pm 0.16 d-g
	Mean	1.54	0.70	4.30
2.5	30	0.00 \pm 0.00 g	0.00 \pm 0.00 j	0.00 \pm 0.00 h
	45	2.11 \pm 0.18 def	1.46 \pm 0.07 gh	5.87 \pm 0.09 c-f
	60	2.81 \pm 0.10 bcd	1.71 \pm 0.09 efg	6.24 \pm 0.03 b-f
	75	2.92 \pm 0.08 c	2.07 \pm 0.12 cde	6.53 \pm 0.23 bcd
	90	3.25 \pm 0.14 ab	2.24 \pm 0.11 bcd	6.66 \pm 0.15 abc
	Mean	2.22	1.50	5.06
5	30	0.00 \pm 0.00 g	0.00 \pm 0.00 j	0.00 \pm 0.00 h
	45	2.17 \pm 0.08 def	1.53 \pm 0.04 g	6.10 \pm 0.21 b-f
	60	3.25 \pm 0.14 ab	2.45 \pm 0.10 bc	6.43 \pm 0.16 b-e
	75	3.39 \pm 0.20 ab	2.61 \pm 0.10 b	6.92 \pm 0.12 ab
	90	3.00 \pm 0.14 bc	2.06 \pm 0.06 cde	6.46 \pm 0.21 b-e
	Mean	2.36	1.73	5.18
7.5	30	0.00 \pm 0.00 g	0.00 \pm 0.00 j	0.00 \pm 0.00 h
	45	2.33 \pm 0.08 cde	1.65 \pm 0.08 fg	6.31 \pm 0.30 b-f
	60	3.83 \pm 0.25 a	3.03 \pm 0.10 a	7.50 \pm 0.27 a
	75	3.17 \pm 0.17 ab	2.24 \pm 0.12 bcd	6.50 \pm 0.26 b-e
	90	2.92 \pm 0.17 bc	2.00 \pm 0.07 def	6.38 \pm 0.25 b-f
	Mean	2.45	1.78	5.34
F-value				
NAA		48.42 ***	226.09 ***	32.88 ***
Sucrose		355.44 ***	514.30 ***	967.87 ***
NAA \times Sucrose		6.83 ***	29.22 ***	4.65 ***
CV (%)		10.74	9.02	6.25

Values are means \pm standard error (SE) of $n = 18$. Means followed by different letters in each column are significantly different at $p < 0.05$. The means separation was analyzed using Tukey's Studentized Range (HSD) test. F value represented *** = $p < 0.001$. NAA = 1-naphthaleneacetic acid and CV = coefficient of variation.

The effects of sucrose and NAA on the number, biomass and diameter of microrhizomes depended on their concentrations in the culture medium. In auxin-free MS medium and MS medium supplemented with 2.5 μM NAA, microrhizome number, biomass and diameter were gradually increased by increasing sucrose concentration up to 90 g L^{-1} . On the other hand, in MS medium supplemented with 5 and 7.5 μM NAA, microrhizome number, biomass and diameter were increased when sucrose concentration elevated up to 75 and 60 g L^{-1} , respectively. A further increase of sucrose concentration of more than 60 and 75 g L^{-1} in the presence of 7.5 and 5 μM NAA, respectively, reduced the measurements of all these recorded parameters. The increment in these parameters' indices by increasing sucrose up to 60 and 75 g L^{-1} in the presence of 7.5 and 5 μM NAA were significantly higher compared to 0 and 2.5 μM NAA in the culture medium. The highest number of microrhizomes per explant (3.83) with the maximum biomass (3.03 g) and diameter (7.5 mm) was recorded with the combination of 7.5 μM NAA and 60 g L^{-1} sucrose and this was followed by the combination of 5 μM NAA and 75 g L^{-1} sucrose in the culture medium. Hence, the required level of sucrose supply for a successful microrhizome induction was reduced by applying 5 and 7.5 μM NAA compared to 0 and 2.5 μM NAA in the culture medium.

3.4. Effects of Different Concentrations of Sucrose and NAA on Shoot Multiplication and Rooting of Bentong Ginger

The analysis of variance showed that along with microrhizome induction, the number of shoots per explant, shoot length and number of roots per explant were also significantly affected at $p < 0.001$ by different concentrations of sucrose and NAA (Table 4). The interaction effects of different concentrations of sucrose and NAA were also significant at $p < 0.001$ for the number of shoots and shoot length but there were no interaction effects on the number of roots per explant. In MS medium devoid of NAA or supplemented with 2.5 μM NAA, increasing sucrose concentration up to 90 g L^{-1} caused an increment in the number of shoots per explant. However, the increasing rate of the shoots was only significant when sucrose concentration increased from 30 to 45 g L^{-1} . By adding 5 and 7.5 μM NAA, the number of shoots increased with increasing sucrose concentration just up to 60 g L^{-1} and the highest number of shoots (13.67 ± 0.33) was recorded in the combination of 60 g L^{-1} sucrose and 7.5 μM NAA. The increasing rate of shoot multiplication with the high concentration of NAA (5–7.5 μM) was significantly higher than that with the lower concentration of NAA (0–2.5 μM).

Table 4. Effects of different sucrose and NAA concentrations on shoot multiplication and rooting of Bentong ginger after 12 weeks of culture.

NAA (μM)	Sucrose (g L^{-1})	Number of Shoots/Explant	Shoot Length (cm)	Number of Roots/Explant
0	30	5.33 ± 0.33 g	6.00 ± 0.29 bcd	18.00 ± 0.69 k
	45	7.22 ± 0.40 efg	4.39 ± 0.18 efg	23.67 ± 1.09 h–k
	60	7.78 ± 0.40 efg	3.98 ± 0.13 fgh	26.5 ± 1.04 d–i
	75	8.00 ± 0.38 ef	3.13 ± 0.03 h	25.5 ± 0.87 e–i
	90	8.22 ± 0.4 def	3.15 ± 0.14 h	23.5 ± 1.53 ijk
	Mean	7.31	4.13	23.43
2.5	30	7.33 ± 0.44 efg	6.50 ± 0.14 bc	18.39 ± 0.96 jk
	45	9.00 ± 0.38 de	5.36 ± 0.23 de	27.67 ± 0.88 c–i
	60	9.17 ± 0.25 de	4.50 ± 0.29 efg	32.56 ± 1.24 a–d
	75	9.44 ± 0.29 de	3.83 ± 0.30 fgh	30.06 ± 1.27 b–1
	90	9.67 ± 0.44 cde	3.67 ± 0.08 gh	24.17 ± 1.30 g–k
	Mean	8.92	4.77	26.57
5	30	6.06 ± 0.34 fg	8.25 ± 0.29 a	23.17 ± 1.09 ijk
	45	9.44 ± 0.40 de	6.78 ± 0.24 bc	32.00 ± 1.9 a–f
	60	13.00 ± 0.76 ab	4.92 ± 0.20 efd	36.67 ± 1.17 ab
	75	12.00 ± 0.58 abc	4.17 ± 0.17 fgh	34.33 ± 1.42 abc
	90	8.83 ± 0.60 de	3.75 ± 0.14 gh	30.67 ± 1.54 b–h
	Mean	9.87	5.57	31.37
7.5	30	6.06 ± 0.34 fg	8.38 ± 0.32 a	25.17 ± 1.17 f–j
	45	10.56 ± 0.73 bcd	6.83 ± 0.25 b	32.33 ± 1.92 a–e
	60	13.67 ± 0.33 a	5.64 ± 0.10 cd	37.89 ± 1.42 a
	75	13.17 ± 0.60 a	3.98 ± 0.19 fgh	33.17 ± 1.59 a–d
	90	9.33 ± 0.33 de	3.55 ± 0.15 gh	31.17 ± 1.36 a–g
	Mean	10.56	5.68	31.94
F-value				
NAA		46.89 ***	58.92 ***	47.85 ***
Sucrose		66.95 ***	213.76 ***	48.86 ***
NAA \times Sucrose		8.26 ***	4.48 ***	0.85 ns
CV (%)		8.67	7.29	8.02

Values are means \pm standard error (SE) of $n = 18$. Means followed by different letters in each column are significantly different at $p < 0.05$. The means separation was analyzed Tukey's Studentized Range (HSD) test. F value represented *** = $p < 0.001$ and ns = not significant. NAA = 1-naphthaleneacetic acid and CV = coefficient of variation.

Increasing sucrose concentration in the culture medium reduced the length of shoots in all concentrations of NAA. The highest shoot length (8.38 cm) was observed in the

combination of 30 g L⁻¹ sucrose and 7.5 µM NAA, but it was not significantly different from the combination of 30 g L⁻¹ sucrose and 5 µM NAA. At 30–45 g L⁻¹ sucrose, the shoot length at high concentration of NAA (5–7.5 µM) was significantly higher than that with the lower concentration of NAA (0–2.5 µM). However, at 90 g L⁻¹ sucrose, the shoot length was statistically the same with all concentrations of NAA and at this high level of sucrose, shoot length was reduced to the minimum height. Besides, numerous roots were simultaneously induced in all the treatments of sucrose and NAA. Increasing sucrose concentration up to 60 g L⁻¹ increased the number of roots per explant at all concentrations of NAA. At the same time, increasing NAA concentration up to 7.5 µM significantly increased the number of roots per explant. The highest number of roots per explant (37.89 ± 1.42) was recorded at the combination of 60 g L⁻¹ sucrose and 7.5 µM NAA in MS medium supplemented with 10 µM zeatin.

3.5. Sprouting and Ex Vitro Establishment of In Vitro-Induced Microrhizomes of Bentong Ginger

The in vitro-produced microrhizomes were successfully sprouted and used as planting materials similar to mature rhizome sets for Bentong ginger cultivation. Some 93% of the microrhizomes were sprouting after two weeks of sowing in the moist river sand. The microrhizome-produced plantlets were established at a 100% survival rate under field conditions. Three months after transplanting, an average of 8 tillers and 58.6 g young rhizomes were produced per plant raised from the microrhizome (Figure 1G).

4. Discussion

Storage organ formation is a complex process that is affected by multiple internal and external factors. Among these factors, the availability of high sucrose and hormonal balance are the key contributors in the in vitro storage organ formation [14]. The high concentration of sucrose is considered the first metabolic signaling molecule. It provides the source of carbon and energy that influences the in vitro storage organ formation in many plant species [15–17]. A high sucrose concentration regulates multiple physiological processes involved in the storage organ formation [14,15,18]. García et al. [19] observed that a high concentration of sucrose increased the expression of protein phosphatases type 2A gene in potatoes (StPP2Ac2b) and it was accompanied by the upregulation of the gibberellin 2-oxidase gene (StGA2ox1) and finally it enhanced tuberization in potatoes. GA 2-oxidases (GA2ox) is a catabolic enzyme of gibberellic acid (GA) which is responsible for the deactivation of the bioactive GA [20]. Reduction in the bioactive GA levels in the storage organ formation zone leads to a change in the cell division plane, which triggers that zone to swell and form storage organs [21]. A high concentration of sucrose also activates a significant number of genes related to starch and storage protein synthesis [14,15,22]. As a result, sucrose is a critical regulator of storage organ formation by mediating all these physiological and structural processes during storage organ formation [23]. Hence, an optimum concentration of sucrose is the most critical factor for in vitro microrhizome formation.

In addition, PGRs also significantly enhanced in vitro microrhizome induction. The addition of 5–7.5 µM NAA was the optimum level for an effective microrhizome induction of Bentong ginger. This is in agreement with the findings of Singh et al. [11], who found that 5.37 µM NAA was more effective than a lower concentration of NAA in microrhizome induction of ginger. The promoting role of auxin in the storage organ formation could be due to its involvement in regulating several cellular processes. Auxins stimulate cell division, expansion and change the orientation of the plane of cell division [14,24]. Besides, auxin increases starch content and the size of starch granules, which consequently increases the sink capacity, which leads to storage organ induction by swelling the storage organ formation zone [23,25,26]. Moreover, auxin enhances the efficiency of transcription and translation of protein synthesis and mediates the accumulation of storage proteins in the storage organ [27]. Hence, auxin is also one of the influencing factors in microrhizome induction.

The role of auxin and sucrose and their interaction effects on storage organ formation are widely studied in potato tuberization. Similarly with the current study data, Romanov et al. [28] found that potato tuber response to exogenous auxin was highly dependent on the sucrose level in the culture medium. At 1 and 5 g L⁻¹ sucrose, potato tuber biomass was markedly increased with 5.71 µM indole-3-acetic acid (IAA) compared to auxin-free medium, but at 8 g L⁻¹ sucrose, 5.71 µM IAA application drastically repressed microtuber biomass accumulation, while in an auxin-free medium, the biomass accumulation of the microtuber was still enhanced by elevating sucrose concentration up to 8 g L⁻¹. Kolachevskaya et al. [29] also found that the tuberization rate and tuber biomass of a transformed potato (harboring agrobacterial auxin synthesis gene *tms1* fused to B33-promoter of the patatin class I gene (B33-*tms1*)) was significantly higher compared to the non-transformed potato. In the following study by Kolachevskaya et al. [18], in the presence of 3 and 5 g L⁻¹ sucrose in the culture medium, the tuber biomass of both *tms1*-transformed and non-transformed potato plants was significantly increased by the exogenous application of 5.71 µM IAA compared to the auxin-free medium. Whereas, at 8 g L⁻¹ sucrose, 5.71 µM IAA treatment caused a reduction in the tuber biomass of *tms1*-transformed plants. However, in the non-transformed plants, tuber biomass was still increased by IAA treatment. These findings indicated that sucrose and auxin affect each other's efficiency in the culture medium for storage organ formation. Similar interaction effects of sucrose and NAA were observed in the current study. The increment in the concentration of one of them reduces the required dose of another for microrhizome induction.

The interaction effects of NAA and sucrose level could be due to the upregulation of genes related to auxin synthesis, signaling and transporting by sugar [30,31]. High sugar increases the indigenous auxin level in the plant [32]. Moreover, exogenous treatment with a high concentration of auxin and cytokinin further accumulates auxin in the plants. As a result, the total concentration of endogenous auxin in the plant may rise beyond the physiological optimum. Thus it could negatively affect the growth and development of the plant [18]. That could be the reason for the reduction of microrhizome number and biomass in the presence of 7.5 µM NAA when sucrose concentration was increased by more than 60 g L⁻¹.

Besides auxin, cytokinins also significantly influenced the microrhizome induction, shoot multiplication and rooting of Bentong ginger. Cytokinins play an essential role in stimulating cell proliferation. Cell division and radial cell growth during the initial stage of storage organ formation depend on the local activation of cytokinins in the storage organ formation zone [33,34]. Cytokinins also activate starch biosynthesis enzymes during storage organ initiation, which accumulate starch and enhance the sink capacity of storage organs [23,35]. Application of both types of cytokinin (zeatin and BAP) to the culture medium, which was supplemented with the high concentration of sucrose (80 g L⁻¹) and 2.5 µM NAA, positively influenced microrhizome induction of Bentong ginger. Zeatin and BAP gave the highest response to microrhizome induction at 10 and 15 µM, respectively. It is in agreement with the findings of An et al. [9], who reported larger microrhizomes of ginger by using 17.76 µM BAP in the culture medium containing 80 g L⁻¹ sucrose. They also indicated a significant reduction in the biomass and size of microrhizomes with a further increment of BAP concentration of more than 17.76 µM. Similarly, Swarnathilaka et al. [5] also reported that MS medium containing 17.76 µM BAP with 0.54 µM NAA showed the highest response to microrhizome induction of ginger and increasing BAP concentration of more than 17.76 µM caused a significant reduction in the number and biomass of microrhizomes.

In the current study, zeatin was more effective than BAP for microrhizome induction of Bentong ginger. It is in agreement with the previous report that zeatin was more effective for the in vitro tuber formation of *Serapias vomeracea* than BAP, kinetin, thidiazuron (TDZ) and 2-isopentenyl adenine (2-iP) [36]. It stated that cytokinin enrichment in the storage organ formation zone stimulates storage organ initiation and favors the growth of young storage organs by stimulating cell division during the early stages of growth, but they do

not regulate further phases of storage organ growth [23]. However, cytokinin accumulation in leaves and growing shoots of the storage organ inducible plants responds oppositely, in which the growth of shoots is induced, but storage organ initiation and growth is inhibited [33]. Similarly, the same effects of cytokinin were observed in the current study, in which the growth of shoots was inhibited during the first two months of inoculation. The shoot growth and new shoot induction were improved after two months of the culture. In comparison to zeatin treatment, the new shoot induction rate was significantly higher and rhizome growth was reduced with BAP treatments, which indicates the high stability and accumulation of BAP in the culture compared to zeatin. However, in our previous study (data not shown), BAP was less effective for shoot multiplication of Bentong ginger than zeatin, which shows that BAP is biologically less active than zeatin. The lower biological activity and high stability of BAP in the culture medium could probably be the reason for its lower efficiency in microrhizome induction [14].

On the other hand, the shoot multiplication rate and rooting were also dependent on microrhizome induction and growth. After the primary microrhizome induction in the presence of high sucrose, the growth of the primary microrhizome was accompanied by a secondary microrhizome induction and continuously, new buds emerged on the microrhizomes. Almost all these emerged buds on the primary and secondary microrhizomes produced new shoots and the number of shoots was substantially increased after microrhizome induction. Besides the highest microrhizome induction and biomass accumulation, the maximum numbers of shoots and roots were also recorded at 60 g L^{-1} sucrose in the medium. This is in confirmation with the previous studies that a high number of shoots were produced at the optimum level of sucrose required for the maximum microrhizome induction compared to the standard dose of sucrose (30 g L^{-1}) used for shoot multiplication [7,8]. Oppositely, the shoot length was gradually reduced by increasing sucrose concentration in the culture medium and the growth of the shoots was inhibited before the microrhizome initiation. The reduced growth of shoots could be due to osmotic stress induced by a high concentration of sucrose [37].

Zeatin, biologically more active than BAP [38], efficiently stimulated microrhizome induction. On the other hand, the high oxidative cleavage of zeatin reduces its effects in the cultures with a prolonged incubation [39]. After two months of culture where microrhizomes were already initiated, the reduced effects of zeatin on new shoots induction compared to BAP treatment could be the cause of the improvement in the growth of microrhizomes. Therefore, zeatin is highly effective for rapid shoot multiplication within a short duration and microrhizome induction, requiring a long duration for its development. To the best of our knowledge zeatin application has not been studied for ginger microrhizome induction and this is the first report that shows the high efficiency of zeatin for microrhizome induction of Bentong ginger. As the *in vitro*-induced microrhizomes were able to be sprouted and established in *ex vitro* conditions, they could be successfully used as planting materials for commercial cultivation of Bentong ginger.

5. Conclusions

This study proposes an efficient procedure for *in vitro* microrhizome formation and field establishment of Bentong ginger. Cytokinin, auxin and sucrose were the affecting factors for microrhizome induction, shoot multiplication and rooting of Bentong ginger. Cytokinins enhanced microrhizome induction, and in this regard, zeatin at $5\text{--}10 \mu\text{M}$ was more effective than BAP at the same concentration. A high concentration of sucrose ($>45 \text{ g L}^{-1}$) was the prerequisite for microrhizome initiation in the culture medium. The effects of sucrose and NAA on microrhizome induction were dependent on the concentration of each other. Increasing the concentration of one of them reduced the required concentration of the other for successful microrhizome induction. Finally, the results of the study showed that MS medium supplemented with 60 g L^{-1} sucrose, $10 \mu\text{M}$ zeatin and $7.5 \mu\text{M}$ NAA was the optimum combination for microrhizome induction of Bentong ginger.

Although sucrose, cytokinins and auxin significantly influenced microrrhizome induction and shoot multiplication of Bentong ginger, their mechanism is not well understood. Hence, further studies are required to detect sucrose and PGRs' effects on the biochemical changes during microrrhizome induction. Moreover, the expression of genes related to microrrhizome induction also needs to be studied to establish a simple, time- and cost-effective protocol for microrrhizome induction of ginger. Besides, it is also recommended to study the conventionally propagated and microrrhizome-induced plants of Bentong ginger to determine if there are any changes in the yield and phytochemical contents of their rhizomes.

Author Contributions: Conceptualization, N.A.Z. and M.H.; methodology, N.A.Z.; resources, H.Z.E.J.; data curation, N.A.Z.; writing—original draft preparation, N.A.Z.; writing—review and editing, N.A.Z., H.Z.E.J. and M.H.; supervision, H.Z.E.J. and M.H.; funding acquisition, H.Z.E.J. and M.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Higher Education Malaysia, grant number 5526700 (Translational Research @ MOHE (TR@M)), Universiti Putra Malaysia, grant number 9629000 (Putra Grant—Putra Young Initiative) and Industry Grant, grant number 6300919 and the APC was funded by Universiti Putra Malaysia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article.

Acknowledgments: Authors would like to thank the Higher Education Development Project (HEDP) for Afghanistan and Afghanistan National Agricultural Sciences and Technology University for the scholarship.

Conflicts of Interest: The authors declare no conflict of interest.

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