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Establishment and Optimization of Micropropagation System for Southern Highbush Blueberry

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Abstract: The optimization of micropropagation for blueberries is crucial due to the growing blueberry industry and demand for plantlets. This study focused on four stages: explant sterilization, in vitro establishment, in vitro proliferation, and ex vitro rooting, aiming to establish an efficient in vitro propagation system for southern highbush blueberry cultivar 'ZY09'. The most effective explant sterilization method was a 60 s treatment with 75% ethanol and a 5 min treatment with 4% NaClO. During the establishment of the in vitro culture, the modified woody plant medium was found to be suitable. The replacement of NH_4NO_3 in woody plant medium with $(NH_4)_2SO_4$ facilitated the proliferation of blueberry microshoots. The optimal combination of plant growth regulators for the in vitro proliferation of blueberry microshoots was indole-3-butyric acid (0.1 mg·L^{-1}), thidiazuron (0.0005 mg·L^{-1}), and zeatin (1 mg·L^{-1}). Perlite was the most suitable substrate for ex vitro rooting. The best ex vitro rooting performance was observed without immersion in growth regulators. Indole-3-butyric acid enhances root formation and suppresses root elongation in blueberries. The findings of this study can be applied to large-scale in vitro propagation of southern highbush blueberry and provide a reference for the genetic transformation of blueberries.

Keywords: blueberry; micropropagation; woody plant medium; $(NH_4)_2SO_4$; growth regulators; ex vitro rooting

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1. Introduction

Blueberry (*Vaccinium* spp.) is a perennial shrub belonging to the Ericaceae family. Blueberry fruits are known for their high anthocyanin and vitamin content [1]. These compounds exhibit anti-inflammatory and antioxidant properties, and their consumption is associated with a reduced risk of cardiovascular diseases and type 2 diabetes [2]. In recent years, there has been a rapid increase in the global blueberry cultivation area and production, with China exhibiting the fastest growth, with its cultivated land area expanding from 681 ha in 2006 to 66,400 ha in 2020 [3]. The rapid growth of the blueberry market has generated an increased demand for high-quality plantlets.

Southern highbush blueberry (*Vaccinium corymbosum* L.) is a hybrid species derived from crossing the northern highbush blueberry and low-chill *Vaccinium* species [4]. It is well adapted to subtropical regions and serves as an important commercial crop [5]. Blueberry propagation via stem cuttings is challenging, whereas tissue culture allows for the rapid production of a large number of plantlets [6]. Furthermore, blueberries propagated through tissue culture exhibit enhanced rooting capacity and higher yields [7,8]. Although numerous studies have reported on blueberry in vitro propagation systems, the special genetic characteristics of southern highbush blueberries necessitate the optimization

of tissue culture protocols for individual varieties, considering their specific responses to different culture media and growth regulator treatments [9,10].

Plant tissue culture refers to the aseptic cultivation of any plant part (cells, tissues, or organs) under controlled laboratory conditions to produce plant clones [11]. The establishment of a plant tissue culture system typically involves four stages: explant induction, proliferation, rooting, and domestication [12]. Among these stages, the sterilization of explants, formulation of basal culture media, types and combinations of plant growth regulators, and induction of rooting are critical components of plant tissue culture systems.

Sterilization is the initial step of in vitro propagation, and inadequate sterilization can lead to the contamination and death of explants, particularly field-grown plants [13]. To ensure effective sterilization and enhance the induction rate, the type, concentration, and sterilization duration of the disinfectants must be adjusted according to the type of explant [14–16]. Quinoa seeds soaked in a 20% sodium hypochlorite solution for 5 min showed the highest seed germination rate, while no contamination was observed for sterilization durations of 10 and 15 min [17]. For blueberry 'Red Button' stem segments, the lowest contamination and highest survival rates were achieved by sequentially immersing the segments in 70% ethanol for 30 s, 2% NaClO for 10 min, and 0.1% $HgCl_2$ for 10 min [18].

Commonly used basal media in plant tissue culture include Murashige and Skoog (MS), Gamborg's B5 medium, and Woody Plant Medium (WPM), among others [19]. WPM and Modified Woody Plant Medium (M-WPM) are widely used in blueberry tissue cultures [20–22]. Both WPM and M-WPM contain NH_4NO_3 as a macronutrient. However, NH_4NO_3 is prone to explosion when exposed to fire, high temperatures, or severe impacts, leading to potential safety hazards during production, transportation, storage, and usage [23]. Therefore, identifying inorganic salts that can replace NH_4NO_3 is of considerable importance. In *Hydrocotyle sibthorpioides* tissue culture, substituting $(NH_4)_2SO_4$ for NH_4NO_3 in the modified MS medium not only enhanced safety but also significantly increased plant height and proliferation compared to the control [24].

Plant growth regulators play crucial roles in plant tissue culture by promoting growth, increasing branching, and stimulating rooting [25,26]. Commonly used growth regulators in blueberry tissue culture include zeatin (ZT), N6-(2-isopentyl)adenine (2iP), thidiazuron (TDZ), and 6-benzylaminopurine (BAP) [20,26,27]. Inducing shoot cultures from stem segments of rabbit-eye blueberry 'Delite' using different concentrations of four cytokinins, ZT, 2iP, BAP, and kinetin (KIN) revealed that lower concentrations of ZT yielded better results, with a survival rate of 89.7% and a shoot formation rate of 81.3%. In the primary culture, supplementing the 20 mM zeatin medium with 1 mM 1-naphthaleneacetic acid (NAA) facilitated the differentiation of explants of 'Red Pearl' and 'Ozarkblue' lingonberry [6]. Furthermore, the addition of indole-3-butyric acid (IBA) at concentrations below 1 $mg \cdot L^{-1}$ to MAN medium supplemented with zeatin enhanced the proliferation efficiency of highbush blueberry microshoots [26].

The two common methods of root induction in blueberry micropropagation are in vitro and ex vitro rooting. Ex vitro rooting exhibits a higher rooting rate than in vitro rooting and the acclimatization is also completed during ex vitro rooting, which can save a significant amount of time [7]. In a previous study, blueberry 'woodard' and 'Georgiagem' microshoots, after a 10 min immersion in a 250 $mg \cdot L^{-1}$ IBA solution, were inserted into five different substrates, and medium-sized vermiculite and pine sawdust were identified as the optimal culture substrates [27]. However, in a substrate screening experiment for 'Snowchaser' blueberry seedling growth, peat and coir substrates emerged as highly suitable [28]. For bilberry and lingonberry microshoots, preculturing in a 2.07 mM IBA solution (potassium salt of IBA) followed by insertion into peat resulted in higher rooting efficiency compared to direct peat insertion [13]. Moreover, even in the absence of growth regulators, blueberry shoots can root on shoot proliferation medium [29,30]. The optimal growth regulators and substrates vary for the ex vitro rooting of blueberries, highlighting the crucial need for a systematic evaluation of the effects of growth regulators and substrates on the ex vitro rooting of individual blueberry cultivars.

In this study, a micropropagation system for the southern highbush blueberry cultivar 'ZY09' was established using stem segment tissues. The optimization process involved determining the optimal sterilization time for 75% ethanol and 4% NaOCl, replacing NH_4NO_3 with $(NH_4)_2SO_4$ to optimize the composition of the basal medium, screening different combinations of IBA, TDZ, and ZT at various concentrations, and selecting the optimal substrate and different concentrations of IBA and NAA for ex vitro rooting. These optimizations were conducted to enhance various aspects of micropropagation in southern highbush blueberry. These optimization strategies were developed based on the practical challenges encountered in production, aiming to enhance safety, save time, and improve efficiency. They provide valuable technical support and theoretical guidance for the large-scale and rapid production of micropropagated southern highbush blueberry plantlets.

2. Materials and Methods

2.1. Plant Materials and Micropropagation Steps

In March 2022, apical shoots measuring 10 cm in length were collected as explants from annual southern highbush blueberry 'ZY09' plants grown in the Solar Greenhouse located at the Baihua Garden in the College of Horticulture, South China Agricultural University. The explants were inoculated into 300 mL culture bottles, with each bottle containing 50 mL of culture medium. Each bottle was inoculated with five explants. Except for the basic medium formulation experiments, all experiments were conducted using WPM [31]. The culture media were supplemented with 6 $g \cdot L^{-1}$ agar and 20 $g \cdot L^{-1}$ sucrose. The pH of the medium was adjusted to 5.4, and the medium was autoclaved at 120 °C for 20 min. ZT and TDZ were filter-sterilized through 0.22 μm filters and added to the cooled medium. IBA was added to the medium prior to sterilization and sterilized with the medium. The culture room was maintained at 25 ± 2 °C, with an air humidity of 50–75%. The light intensity was set at 88 μmol m^{-2} s^{-1}, and the photoperiod was set at 16 h light/8 h dark.

The micropropagation procedure of this experiment is illustrated in Figure 1. In the first step, 10 cm long top segments of one-year-old shoots from southern highbush blueberry were used as explants. In the second step, the sterilized explants were inserted into the primary culture medium to induce bud formation, completing the in vitro establishment. In the third step, the induced shoots were cut into approximately 2 cm segments and inserted into the subculture medium for proliferation. Finally, in the fourth step, the proliferated microshoots were cut into approximately 3 cm segments and inserted into rooting substrates for ex vitro rooting.

Figure 1. Micropropagation steps of southern highbush blueberry 'ZY09'. (Step 1) Annual blueberry shoots used as explant sources; (Step 2) In vitro establishment; (Step 3) In vitro proliferation; (Step 4) Ex vitro rooting and acclimatization.

2.2. Explant Sterilization

The explants devoid of leaves were thoroughly washed under tap water for 30 min. They were subsequently subjected to nine disinfection treatments using 75% ethanol and 4% NaOCl on a clean bench (Table 1). After each sterilization treatment, the explants were rinsed three times with sterile water, with each rinse lasting for 2 min. The surface moisture of the explants was absorbed using sterile filter paper, the apical portion was removed, and approximately 5 mm of the basal portion was excised. The resulting explants were cut into

2 cm long stem segments with 1–2 axillary buds and inoculated onto WPM supplemented with 2 mg·L^{-1} ZT. The cultures were then incubated in a growth chamber. After 6 weeks, the contamination situation stabilized, and the numbers of contaminated and induced explants were observed and recorded to calculate the contamination and induction rates.

Table 1. Ethanol and sodium hypochlorite treatments with different disinfection durations.

Treatment	Time of Ethanol (70%) Sterilization (s)	Time of NaOCl (4%) Sterilization (min)
1	0	5
2	0	5
3	0	5
4	30	10
5	30	10
6	30	10
7	60	15
8	60	15
9	60	15

2.3. Optimization of Basal Culture Media

To identify the optimal basal culture medium for in vitro establishment and proliferation of southern highbush blueberry, various basal culture media were selected, including WPM [31], M-WPM [21], WPM with $(NH4)_2SO_4$ replacing NH_4NO_3 (WPM1), M-WPM with $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ replacing NH_4NO_3 (WPM2), and WPM2 supplemented with 1.5 times calcium salts (WPM3), as outlined in Table 2. The inorganic salts used in this experiment were obtained from Guangzhou Chemical Reagent Factory (China), while the vitamins and plant growth regulators were sourced from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). All primary basal media were supplemented with 2 mg·L^{-1} ZT, and all subculture media were supplemented with 1 mg·L^{-1} ZT. After 6 weeks, the number of shoots, length of the longest shoot, and fresh and dry weights were recorded for each treatment.

Table 2. Composition of WPM, M-WPM, WPM1, WPM2, and WPM3 media.

Components	WPM	M-WPM	WPM1	WPM2	WPM3
Macronutrients	**Final Concentration in the Culture Medium (mg·L^{-1})**				
$NH_4H_2PO_4$	-	-		99.98	99.8
$(NH_4)_2SO_4$	-	-	330.18	247.65	247.65
NH_4NO_3	400.00	400.00	-	-	-
KNO_3	-	190.00	505.8	695.8	190
K_2SO_4	990.00	-	554.57	-	-
KH_2PO_4	170.00	170.00	170	-	-
$CaCl_2$	72.5	-	72.5	-	-
$Ca(NO_3)_2{\cdot}4H_2O$	556.00	684.00	834.00	684.00	924.42
$MgSO_4{\cdot}7H_2O$	370.00	370.00	370.00	370.00	370.00
Micronutrients					
$FeSO_4{\cdot}7H_2O$	27.80	55.60	27.80	55.60	55.60
Na_2-EDTA	37.30	74.6	37.3	74.6	74.6
H_3BO_3	6.20	6.20	6.20	6.20	6.20
$MnSO_4{\cdot}H_2O$	22.30	22.30	22.30	22.30	22.30
$ZnSO_4{\cdot}7H_2O$	8.60	8.60	8.60	8.60	8.60
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.25	0.25	0.25	0.25	0.25
Vitamins					
Glycine	2.00	2.00	2.00	2.00	2.00
Inositol	100.00	100.00	100.00	100.00	100.00
Vitamin B1	0.10	0.10	0.10	0.10	0.10
Vitamin B6	0.50	0.50	0.50	0.50	0.50
Nicotinic acid	0.50	0.50	0.50	0.50	0.50

2.4. Screening of Growth Regulator Combinations

In this study, we investigated the effects of three plant growth regulators, namely IBA, TDZ, and ZT, on the proliferation of blueberry plantlets in vitro. Each growth regulator was tested at four different concentrations using an orthogonal array L16(4^3), as shown in Table 3. Uniform and consistent 'ZY09' in vitro plantlets were selected and cut into approximately 2 cm long stem segments on a clean bench. These segments were inoculated into culture media containing different combinations of plant growth regulators. After a 6-week incubation period, the number of shoots, length of the longest shoot, and fresh and dry weights were recorded.

Table 3. L16(4^3) Orthogonal Array Design with Three Factors (IBA, TDZ, ZT) at Four Levels Each.

Hormone Treatment	IBA Concentration ($mg \cdot L^{-1}$)	TDZ Concentration ($mg \cdot L^{-1}$)	ZT Concentration ($mg \cdot L^{-1}$)
1	0	0	0
2	0	0.0005	0.5
3	0	0.001	1
4	0	0.005	2
5	0.01	0	0.5
6	0.01	0.0005	0
7	0.01	0.001	2
8	0.01	0.005	1
9	0.005	0	1
10	0.005	0.0005	2
11	0.005	0.001	0
12	0.005	0.005	0.5
13	0.1	0	2
14	0.1	0.0005	1
15	0.1	0.001	0.5
16	0.1	0.005	0

2.5. Substrate Screening for Rooting

To identify the optimal substrate for the ex vitro rooting of blueberry plantlets, six treatments were designed: (1) sphagnum moss, (2) peat, (3) perlite, (4) peat:perlite (2:1), (5) sphagnum moss:peat (2:1), and (6) perlite:peat:vermiculite (3:3:1). Shoots were inserted into covered seedling trays filled with the respective substrates. In order to prevent water loss from the shoot and prolong their viability before rooting, a high humidity level is required. During the initial two weeks, daily watering was performed, and a transparent plastic cover was utilized to maintain the humidity at approximately 99%. Subsequently, in the following two weeks, watering was reduced to once every two days, while gradually opening the ventilation holes of the transparent cover to maintain the humidity at around 80%. After 4 weeks, the lids were removed. Prior to root initiation, the cuttings were irrigated with tap water, and a nutrient solution containing macronutrients from the WPM formulation was applied after root initiation. After 6 weeks, the rooting percentage, number of roots, longest root length, and fresh and dry root weight were recorded.

2.6. Screening of Growth Regulators for Root Induction

A total of 16 treatments were established by combining IBA and NAA at four different concentrations (Table 4). Uniform and consistent 'ZY09' micropropagated plantlets were selected and cut into approximately 3 cm long segments. These segments were dipped in the respective growth regulator solutions for 20 s and inserted into covered seedling trays containing a substrate composed of a mixture of perlite, peat, and vermiculite in a ratio of 3:3:1. The management procedures were consistent with those described in Section 2.5. After a 6-week period, the rooting percentage, number of roots, longest root length, and fresh and dry root weight were measured.

Table 4. Experimental growth factor levels for *Vaccinium* ex vitro rooting.

Level	Experimental Factor	
	1h-Indole-3-Butanoic Acid (mg·L^{-1})	1-Naphthylacetic Acid (mg·L^{-1})
1	0	0
2	500	500
3	1000	1000
4	1500	1500

2.7. Statistical Analysis

Contamination rate (%) = the number of contaminated explants/the number of total initial explants × 100%;

Induction rate (%) = the number of induced explants/the number of total initial explants × 100%;

Rooting rate (%) = the number of the rooted plantlets/the number of total shoots × 100%;

All treatments were replicated three times, with 15 plantlets per replicate for explant sterilization, in vitro establishment, and in vitro proliferation experiments, and 24 plantlets per replicate for the ex vitro rooting experiment. Ten plantlets were randomly selected from each replicate, resulting in a total of 30 plants analyzed for each treatment. The variance analysis (ANOVA) was performed using Microsoft Excel 2019 and SPSS Statistics 22.0 program. Duncan's multiple range test was used to assess the significance of differences ($p < 0.05$). Column charts were generated using Origin 2022 software.

3. Results

3.1. Effect of Different Ethanol and NaClO Sterilization Times on the Contamination and Induction Rates of Explants

Different sterilization times for the explants resulted in varying contamination and induction rates (Figure 2). With a constant NaClO disinfection time, an increase in the ethanol disinfection time led to a significant reduction in the contamination rate and a significant increase in the induction rate. The lowest contamination rate of 0.00% was observed when the ethanol treatment time was 30 s and the NaClO treatment time was 15 min (Figure 2a). Conversely, the highest induction rate of 93.33% was achieved when the ethanol treatment time was 60 s and the NaClO treatment time was 5 min (Figure 2b).

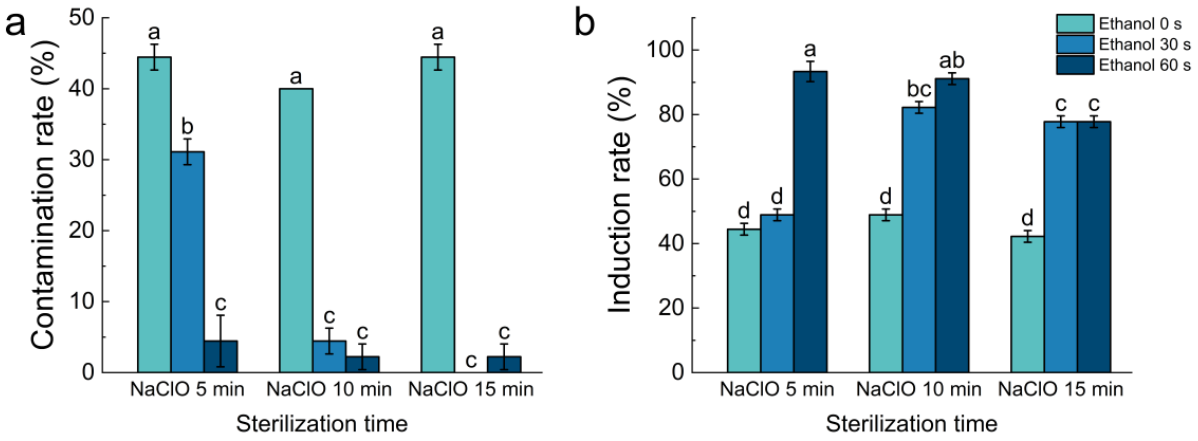

Figure 2. Effects of sterilization treatments on southern highbush blueberry explants. (**a**) Contamination rate of explants; (**b**) Induction rates of explants. According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

3.2. Effects of Different Basal Medium Formulations on Blueberry In Vitro Establishment

The different ammonium salts and nitrates in the five culture basal media resulted in varying induction responses in blueberry explants. In vitro plantlets induced from the ex The explants cultured in M-WPM achieved optimal performance, with an induction rate of 62.22%, an average of 2.10 induced shoots per explant, a shoot length of 40.48 mm, a fresh weight of 343.81 mg, and a dry weight of 41.07 mg (Figure 3). The explants grown in WPM exhibited the highest induction rate and the highest number of shoots (Figure 3a,c). Compared with WPM, WPM1 led to an increased shoot fresh weight but decreased shoot number in cultured blueberry explants (Figure 3a,d). Considering overall performance, M-WPM was the most suitable basal medium for blueberry shoot induction during the establishment phase of in vitro culture.

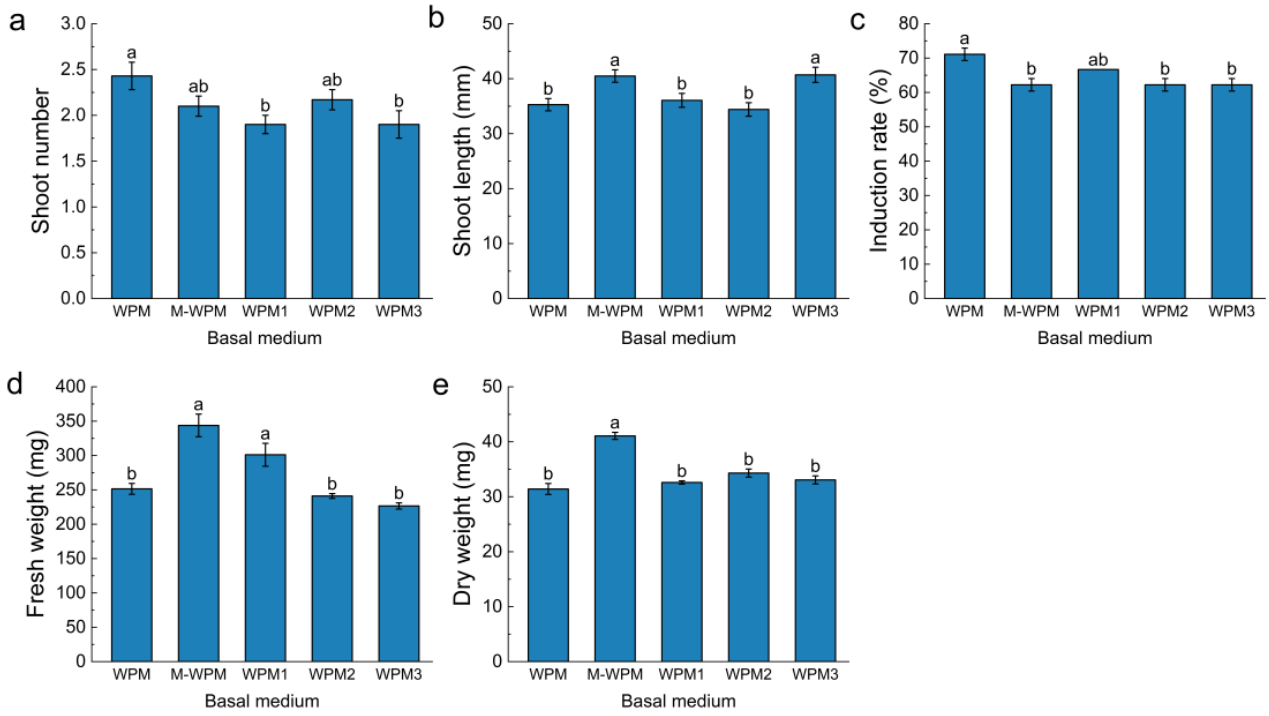

Figure 3. Effects of different basal media formulations on the induction of blueberry in vitro establishment. (**a**) Shoot number of explants; (**b**) Shoot length of explants; (**c**) Induction rate; (**d**) Fresh weight of shoot; (**e**) Fresh weigh of shoot. According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

3.3. Effects of Different Basal Medium Formulations on In Vitro Blueberry Proliferation

The growth of blueberry micropropagules varied across different basal medium formulations during the in vitro proliferation phase (Figure 4). Shoots cultured on WPM1 exhibited optimal levels of shoot quantity (12.13), shoot length (56.29 mm), and dry weight (81.04 mg), showing significant differences compared with other medium formulations (Figure 4a,b,d). However, the highest fresh weight of blueberry micropropagules was observed in WPM (Figure 4c). Compared to WPM, plantlets cultured on WPM1 showed a significant increase in shoot number and shoot length, whereas fresh weight decreased (Figure 4a–c). The poorest performance in terms of shoot number, shoot length, fresh weight, and dry weight was observed in WPM3 (Figure 4). Overall, WPM1 was identified as the most suitable basal medium for the in vitro blueberry propagation proliferation phase.

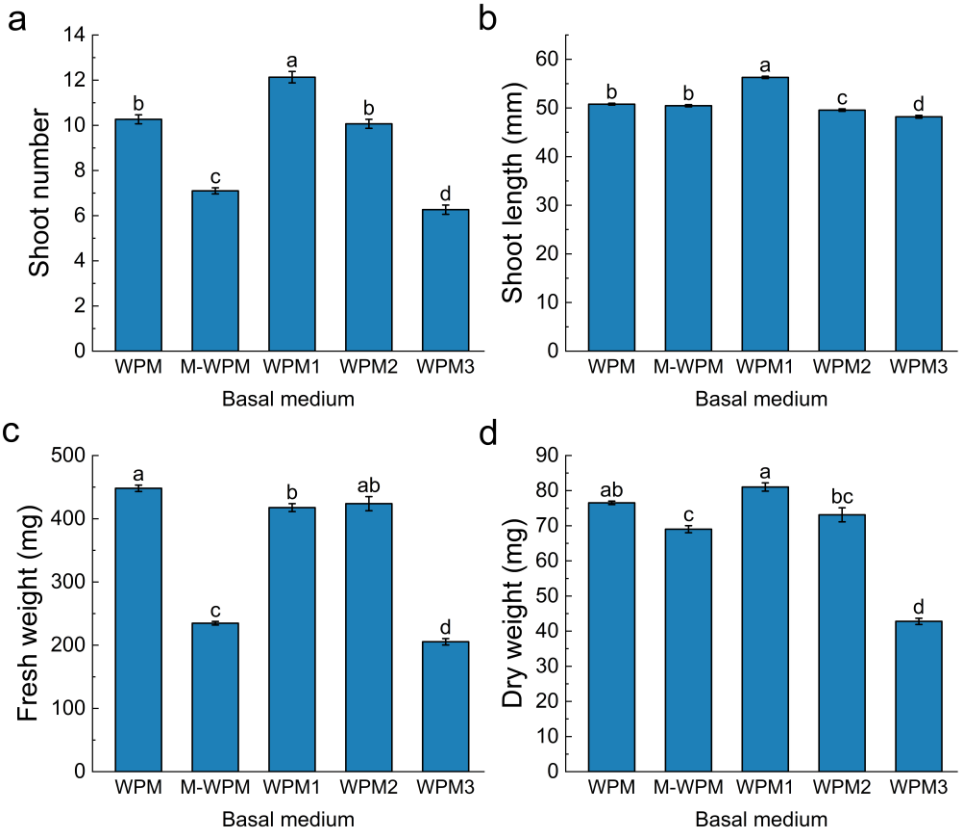

Figure 4. Effects of different basal media formulations on the in vitro proliferation of blueberry. (**a**) Shoot number; (**b**) Shoot length; (**c**) Fresh weight of shoot; (**d**) Dry weigh of shoot. According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

3.4. Effects of Different Growth Regulator Combinations on In Vitro Proliferation of Blueberry

The orthogonal array design is an efficient method for screening the optimal combination of multiple factors [32]. Variance analysis of the experimental treatments revealed significant differences among the average values of the four levels of ZT for the parameter of shoot number (Table 5). However, there were no significant differences in shoot length among the different concentrations of IBA, TDZ, or ZT (Table 5). In terms of fresh weight, significant differences were observed among the four levels of IBA and the four levels of ZT, with ZT exhibiting the highest F-ratio (Table 5). The different concentrations of TDZ did not significantly affect the three indicators of blueberry micropropagation process (Table 5).

Table 5. Effects of various factors on blueberry proliferation in terms of shoot number, plant height, and fresh weight determined using analysis of variance and L16(4^3) orthogonal design.

Sources	Shoot Number Lant (n °)		Shoot Length (mm)		Fresh Weight (mg)	
	F-Ratio	Prob. F	F-Ratio	Prob. F	F-Ratio	Prob. F
IBA	4.557	0.122	3.883	0.147	9.834	0.046 *
TDZ	1.467	0.380	1.001	0.499	0.655	0.632
ZT	55.202	0.004 *	6.492	0.079	38.948	0.007 *

The asterisk (*) indicates significant differences ($p < 0.05$) among the levels of the factors in terms of blueberry proliferation indicators.

Among the in vitro plantlets (Figure 5a), Treatment 13 with an IBA concentration of 0.1 mg·L^{-1}, TDZ concentration of 0 mg·L^{-1}, and ZT concentration of 2 mg·L^{-1} (Table 3) significantly outperformed other treatments in terms of shoot number (Figure 5b). Treatment 11, containing 0.005 mg·L^{-1} IBA, 0.01 mg·L^{-1} TDZ, and 0 mg·L^{-1} ZT, exhibited significantly

greater shoot length than other treatments (Figure 5c). Treatment 14, with 0.1 mg·L^{-1} IBA, 0.0005 mg·L^{-1} TDZ, and 1 mg·L^{-1} ZT, exhibited the highest shoot fresh weight (Figure 5d). Subsequent proliferation aims to obtain robust in vitro plantlets with high multiplication rates, thus requiring a balance between shoot number, shoot length, and shoot fresh weight. In conclusion, the optimal growth regulator combination was Treatment 14, containing an IBA concentration of 0.1 mg·L^{-1}, TDZ concentration of 0.0005 mg·L^{-1}, and ZT concentration of 1 mg·L^{-1}. The blueberry micropropagation plantlets from Treatment 14 exhibited a shoot length of 50.98 mm, an average of 17.17 shoots per plantlets, and a fresh weight of 304.21 mg (Figure 5b–d).

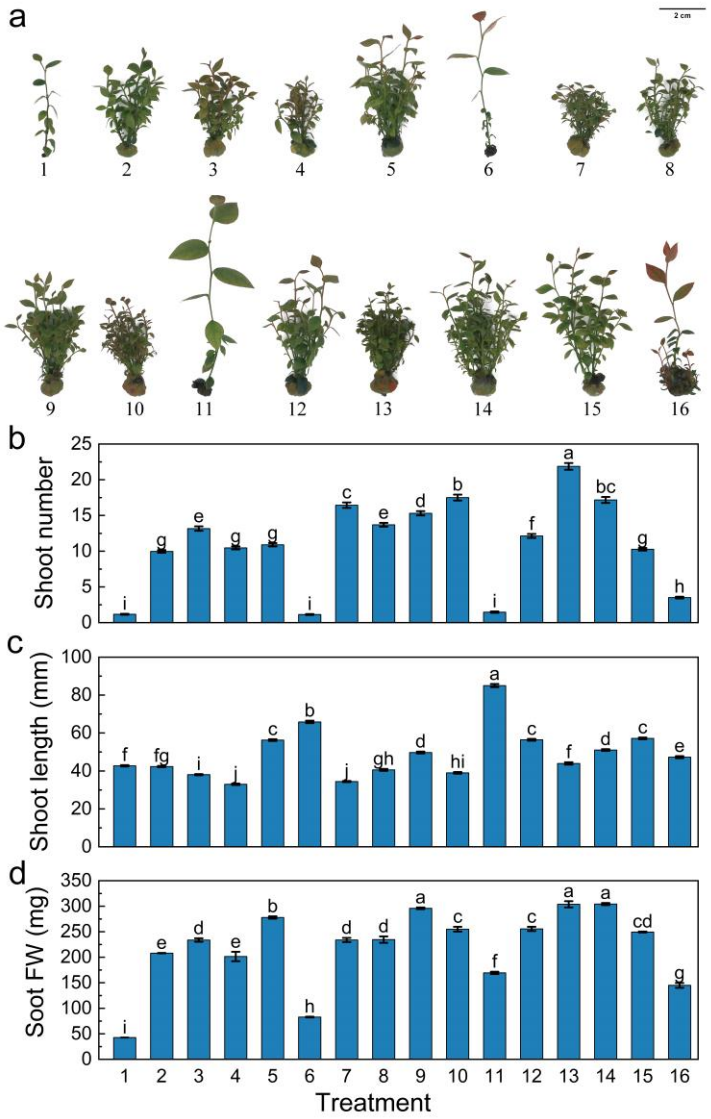

Figure 5. Effects of 16 growth regulator combinations from an L16(4^3) orthogonal design on blueberry micropropagation. (**a**) Microshoots were cultured for 6weeks on proliferation media containing different combinations of growth regulators; (**b**) Shoot number; (**c**) Shoot length; (**d**) Fresh weight of shoot. According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

3.5. Effects of Different Substrates on Ex Vitro Rooting of Blueberry

The influence of the different substrates on ex vitro rooting varied (Figure 6). Treatments 3 (perlite), 4 (peat:perlite 2:1), and 6 (perlite:peat:vermiculite 3:3:1) exhibited the highest rooting percentages, reaching 91.67% (Figure 6c). Treatment 1 (sphagnum moss) resulted in the highest average number of roots (18.77), which significantly exceeded that

of the other treatments (Figure 6a). Treatment 4 (peat:perlite 2:1) resulted in a significantly longer average root length than the other treatments (Figure 6b). Treatment 3 (perlite) resulted in significantly higher fresh root weight and a dry root weight than those of the other treatments (Figure 6d,e). Overall, blueberry plants grown in treatment 3, using perlite as the substrate, exhibited the best growth performance, with an optimal rooting percentage (91.67%), fresh weight (11.53 mg), and dry weight (1.68 mg) (Figure 6).

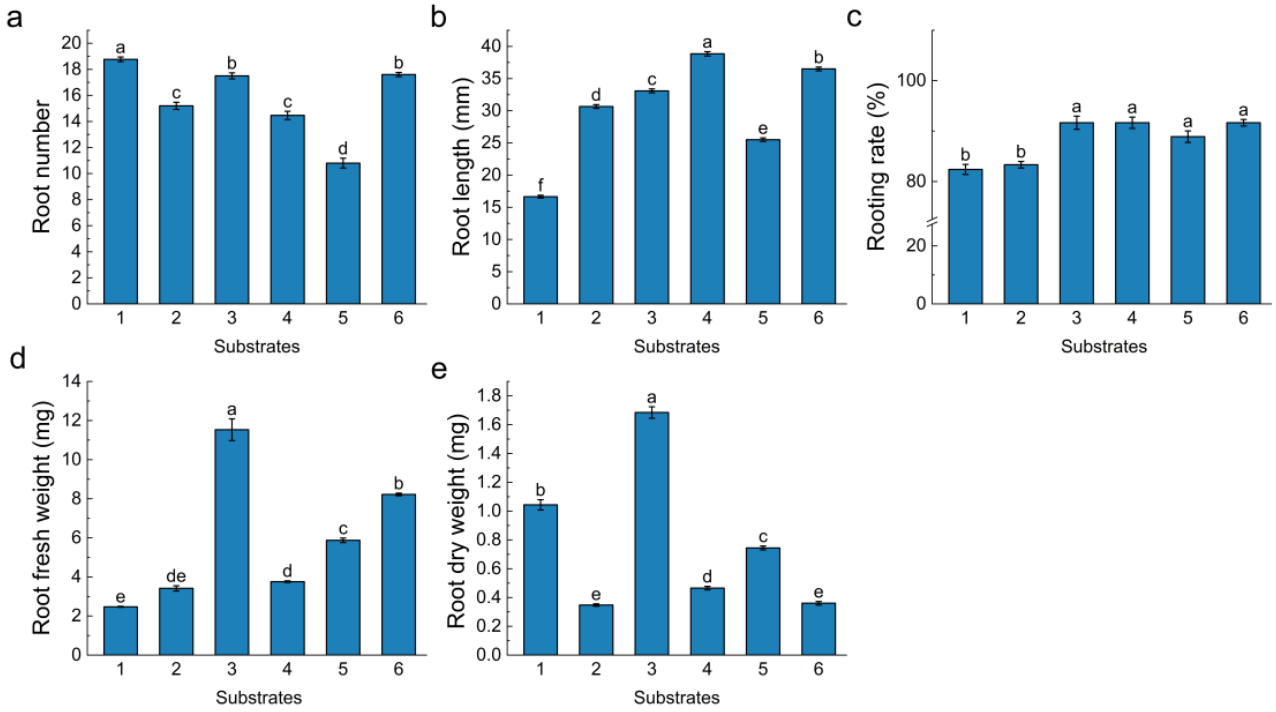

Figure 6. Effects of different substrates on rooting of blueberry cultivar 'ZY09'. (**a**) Root number; (**b**) Root length; (**c**) Rooting rate; (**d**) Fresh weight of root; (**e**) Fresh weigh of root. (1) sphagnum moss, (2) peat, (3) perlite, (4) peat:perlite (2:1), (5) sphagnum moss:peat (2:1), and (6) perlite:peat:vermiculite (3:3:1). According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

3.6. Effects of Different Growth Regulators and Their Combinations on Ex Vitro Rooting

The treatment without growth regulator immersion exhibited the highest ex vitro rooting efficiency in blueberry, with a rooting rate of 100%, average number of roots of 14.37, longest root length of 59.51 mm, fresh root weight of 24.72 mg, and dry root weight of 3.11 mg (Figure 7). IBA immersion alone resulted in a significant increase in root number with increasing IBA concentration, accompanied by a significant decrease in root length, fresh root weight, and dry root weight (Figure 7a,b,d,e). The highest average number of roots (24.33) was observed with an NAA concentration of 0 and an IBA concentration of 1500, which was significantly different from the other treatments (Figure 7a). Immersion in NAA alone led to a decrease in root number, root length, and fresh and dry root weight with increasing NAA concentration (Figure 7). At low IBA concentrations (≤500), an increase in NAA concentration decreased the rooting rate, root length, and fresh and dry root weights (Figure 7b,c,e). At high IBA concentrations (≥1000), an increase in NAA concentration initially resulted in a decrease, and, subsequently, an increase in rooting rate, root length, and fresh and dry root weights (Figure 7b,c,e).

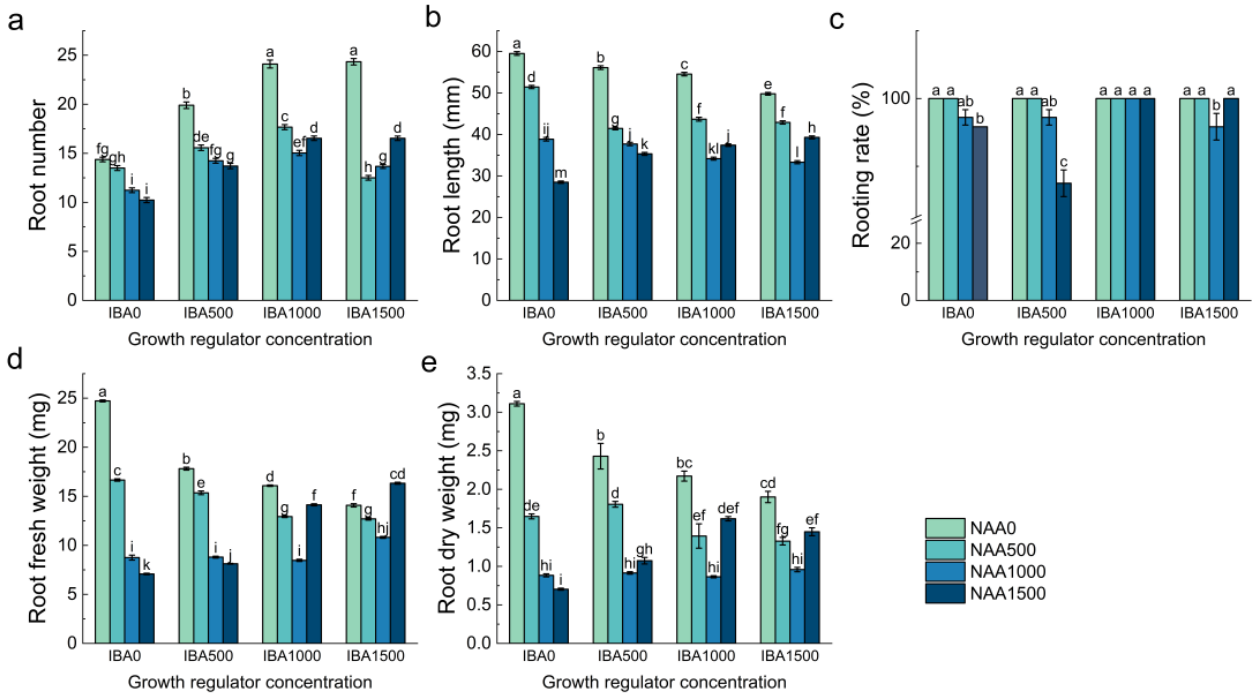

Figure 7. Effects of different concentrations of IBA and NAA immersion treatments on rooting of blueberry cultivar 'ZY09'. (**a**) Root number; (**b**) Root length; (**c**) Rooting rate; (**d**) Fresh weight of root; (**e**) Fresh weigh of root. The concentration of growth regulators is expressed in $mg \cdot L^{-1}$, for example, IBA 1500 indicates a concentration of 1500 $mg \cdot L^{-1}$ of IBA. According to Duncan's multiple range test ($p < 0.05$), the means ± standard errors ($n = 30$) represented by different letters are significantly different.

4. Discussion

The blueberry industry in China is rapidly expanding with extensive prospects [3]. Therefore, optimizing tissue culture systems for the rapid propagation of blueberries is of great importance for the sustainable development of the industry. Common methods for disinfecting explants include mercuric chloride ($HgCl_2$) sterilization or a combination of ethanol and sodium hypochlorite (NaClO) sterilization [18,33]. However, because of the toxic nature of $HgCl_2$, this study employed a disinfection approach using 75% ethanol and 4% sodium hypochlorite to sterilize blueberry stem segments. By optimizing the sterilization time with ethanol and sodium hypochlorite, the sterilization efficiency and induction rate of the southern highbush blueberry explants were improved.

The composition of the base medium can influence the induction, elongation, and weight accumulation of microshoots. M-WPM medium contains higher levels of NO_3^- and $C_{10}H_{13}FeN_2NaO_8$ compared to WPM medium, while K+ and SO_4^{2-} are reduced, exhibiting the best performance during the in vitro establishment of southern highbush blueberry. The in vitro establishment and multiplication stages exhibited different responses to the base medium formulations. In this study, WPM1 was developed by replacing equal amounts of NH_4^+ and NO_3^- from NH_4NO_3 in WPM with $(NH_4)_2SO_4$ and KNO_3. The increase in shoot elongation, shoot number, and dry weight during the in vitro proliferation stage may be attributed to the higher concentrations of K^+ and SO_4^{2-} in WPM1 compared to WPM. This finding is consistent with those of previous studies in tissue culture media for *Malus*, strawberry, grape, and *Hydrocotyle sibthorpioides*, where the replacement of NH_4NO_3 with $(NH_4)_2SO_4$ was beneficial for plant growth [24,34]. The medium WPM1, in which $(NH_4)_2SO_4$ replaces NH_4NO_3, demonstrates excellent performance during the proliferation stage of southern highbush blueberry. Notably, this substitution not only reduces the risks associated with ammonium nitrate usage but also alleviates the challenges in procuring the raw materials, signifying significant practical implications for large-scale production.

The combination of auxins and cytokinins can modulate plant growth, and a high proportion of cytokinins to auxins can induce shoot regeneration [35]. Our research findings are in line with this observation, as the lower concentration of IBA combined with a higher concentration of ZT favored an increase in the number of shoots. Among the three growth regulators, ZT had the greatest impact, followed by IBA, whereas TDZ had the least influence. ZT exhibited a more pronounced promoting effect on blueberry micropropagation, which is consistent with the results of hormone screening in some blueberry tissue culture studies [36–38]. However, when the concentration of ZT is relatively high (2 mg·L^{-1}), it negatively affects the elongation of shoots, while it does not influence the number of shoots and their fresh weight. The lack of decrease in fresh weight might be attributed to the increase in the number of shoots. In lingonberry and southern highbush blueberry tissue cultures, ZT was found to be more effective than TDZ in promoting adventitious shoot formation [6]. Consistent with these findings, our study demonstrated that ZT had a greater promoting effect on bud differentiation than did TDZ.

Blueberries have a fibrous root system without root hairs, and root formation is closely related to substrate aeration and water-holding capacity. Substrates with good aeration and water-holding capacities include moss, perlite, and vermiculite. Root fresh weight, root dry weight, and rooting rate were optimal when rooting in perlite, although root length was slightly shorter. The longest root length was observed in the mixture of peat and perlite at a ratio of 2:1. In the ex vitro rooting of *Clematis*, perlite exhibited the highest rooting percentage and the greatest number of roots, whereas a peat-perlite mixture produced the greatest total root length, similar to the results of this study [39]. Perlite, with its porous structure, good drainage, and excellent aeration, exhibited the best performance in this experiment, making it suitable as a substrate for the ex vitro rooting of blueberry.

Ex vitro rooting showed excellent results, with root initiation starting at 12 days and the development of robust plantlets with 6 weeks. Furthermore, compared to in vitro rooting, there was no need for additional acclimatization steps, allowing for time savings and improved production efficiency. The rooting efficiency of blueberry (Vaccinium corymbosum) and lingonberry (Vaccinium vitis-idaea) cultured without any growth regulators under fog was comparable to that of in vitro rooting [6]. Similarly, our ex vitro rooted plantlets reached their optimal level without the use of any growth regulators. However, this differs from the conclusion that IBA enhances the rooting rate of blueberries, which may be attributed to the varying sensitivities of different blueberry cultivars to growth regulators [40]. When IBA was used alone, an increase in concentration led to an increase in the number of roots, but a decrease in root length, fresh weight and dry weight. This is consistent with findings in *Arabidopsis thaliana*, where exogenous IBA inhibited root elongation and induced the formation of adventitious roots [41,42]. In many plant species, IBA has been shown to be more effective in promoting lateral root and adventitious root formation compared to indole-3-acetic acid or synthetic auxins [43]. Similarly, in this study, IAA exhibited a less favorable effect on ex vitro rooting compared to IBA. When NAA was used alone, an increase in its concentration led to a reduction in root number, root length, rooting rate, fresh weight, and dry weight.

5. Conclusions

In this study, we optimized the explant sterilization, in vitro establishment, in vitro proliferation, and ex vitro rooting of the rapid micropropagation system for southern highbush blueberry 'ZY09'. The most effective treatment for sterilizing blueberry stem explants involved a 60 s ethanol treatment, followed by a 5 min sodium hypochlorite treatment. WPM was identified as the optimal basal medium for in vitro establishment, whereas for the proliferation stage, the optimal medium was formulated by replacing NH_4NO_3 in WPM with $(NH_4)_2SO_4$ (referred to as WPM1). The optimal combination of growth regulators was determined to be 0.1 mg·L^{-1} IBA, 0.0005 mg·L^{-1} TDZ, and 1 mg·L^{-1} ZT during the in vitro proliferation stage. Perlite was the most suitable substrate for ex vitro rooting. Treatment without exogenous growth regulators resulted in the best ex vitro

rooting performance, with optimal rooting percentage, root length, and root weight. The substitution of $(NH_4)_2SO_4$ for NH_4NO_3 in WPM was found to be beneficial for in vitro proliferation of blueberry 'ZY09', with the added advantage of lower toxicity compared to NH_4NO_3. This finding can serve as a reference for optimizing other culture media. These optimized protocols provide valuable technical support and theoretical guidance for large-scale and rapid production of micropropagated plantlets of the southern highbush blueberry cultivar.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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