Basal Medium with Modified Nitrogen Source and other Factors Influence the Rooting of Banana

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Abstract. MS is the most commonly used basal medium for the micropropagation of banana (*Musa* spp.), in which inorganic N source consists of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, and $\text{NH}_4^+:\text{NO}_3^- = 1:2$. In the present study, basal medium for rooting culture was modified by supplying $\text{NO}_3^-\text{-N}$ as the sole N source at the concentration of 17.80 to 19.78 $\text{mmol}\cdot\text{L}^{-1}$. Not only was the percentage of qualified plantlets higher than that of MS or 1/2 MS, but the cost for medium preparation (per liter) was about \$0.6 lower than that of MS based on local retail price, for the concentration of N decreased from about 60 $\text{mmol}\cdot\text{L}^{-1}$ to nearly 20 $\text{mmol}\cdot\text{L}^{-1}$. The effects of four factors: sucrose (25 to 35 $\text{g}\cdot\text{L}^{-1}$), NAA (0.2 to 1.0 $\text{mg}\cdot\text{L}^{-1}$), IBA (0.2 to 1.0 $\text{mg}\cdot\text{L}^{-1}$), and basal medium with modified N source on the percentage of qualified plantlets were studied by using orthogonal design. The variance analysis of data showed that all the four factors significantly affected the process of rooting culture. Among them, sucrose was the most important factor, followed by revised basal medium, IBA, and NAA. The optimal medium for rooting culture was BM II supplemented with 30 $\text{g}\cdot\text{L}^{-1}$ sucrose, 0.5 $\text{mg}\cdot\text{L}^{-1}$ NAA, and 0.5 $\text{mg}\cdot\text{L}^{-1}$ IBA. During the following acclimation phase, >90% of plantlets survived. Chemical name used: 6-benzylaminopurine (BA); indole-3-butyric acid (IBA); α -naphthaleneacetic acid (NAA).

Since Ma and Shii first reported the shoot-tip culture of banana (*Musa* spp.) and the regeneration of plantlets through the formation of adventitious buds (1972, 1974), the in vitro multiplication of banana by shoot-tip culture or meristem culture has been successfully established (Bower and Fraser, 1982; Dore Swamy et al., 1983; Cronauer and Krikorian, 1984; Wong, 1986; Acuña Chinchila, 1993; Dhumale et al., 1997; Bekheet and Saker, 1999; Arinaitwe et al., 2000). Compared with the conventional method of propagation, suckers produced at the base of each mother plant, micropropagated plantlets have many advantages: 1) The rate of proliferation is high and there are no season limits for the production of planting material; 2) The field performance of plantlets is characterized by growing fast and regularly, and the fact that banana fruit can be harvested almost at the same time makes field management easier (Hwang et al., 1984; Vuylsteke and Ortiz, 1996); and 3) Micropropagation procedure including virus eliminating treatment (Berg and Bustamante, 1974) and virus-free status confirmation by molecular indexing methods (Samuel et al., 1999) is helpful to control virus disease effectively.

So far, the most commonly used basal medium for the micropropagation of banana is MS (Murashige and Skoog, 1962), in which inorganic nitrogen source consists of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, and $\text{NH}_4^+:\text{NO}_3^- = 1:2$. On the other hand, reports comparing the effects of different

nitrogen forms are lacking, despite that the formation of adventitious bud is affected by the ratio of $\text{NH}_4^+:\text{NO}_3^-$ in the micropropagation of some other fruit trees (Fasolo et al., 1989; Leblay et al., 1991; Li and Han, 2003).

The objective of this paper was to modify basal medium for banana rooting culture on the basis of studying the effects of different nitrogen sources on the percentage of qualified plantlets; moreover, the effects of revised basal medium and other several selected factors (sucrose, NAA and IBA) on rooting culture were evaluated by using orthogonal design which had already been applied in banana tissue culture lately (Wang et al., 2001). Since the influence of factors selected and their interactions can be investigated by using orthogonal design, it is very useful for discovering and selecting optimal experimental treatment.

Materials and Methods

Initiation and maintenance of in vitro multiplication. Micropropagated shoot clusters (6th to 8th subculture) for rooting were originated from shoot-tip culture of small suckers of 'Baxi Xiangjiao' banana (AAA Group), which is one of leading commercial varieties in China. Medium for explant culture and subcultures was MS supplemented with 3.0 $\text{mg}\cdot\text{L}^{-1}$ BA,

0.3 $\text{mg}\cdot\text{L}^{-1}$ NAA, 30 $\text{g}\cdot\text{L}^{-1}$ sucrose and 6 $\text{g}\cdot\text{L}^{-1}$ agar; and the pH adjusted to 5.8. The cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16-h photoperiod of 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from cool-white fluorescent lamps.

Root differentiation. The basal media of rooting culture included MS (control treatment) and those with modified nitrogen source: basal medium (BM) I = 1600 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , no NH_4NO_3 , 440 $\text{mg}\cdot\text{L}^{-1}$ $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, the rest of components were the same as MS; BM2 = 1800 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM1; BM3 = 1900 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM1; BM4 = 2000 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM1; BM5 = 2100 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM1; BM6 = 2600 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM1; BM I = 1213 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , no NH_4NO_3 , 706 $\text{mg}\cdot\text{L}^{-1}$ $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, no $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, the rest of components were the same as MS; BM II = 1314 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM I; BM III = 1415 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , the rest of components were the same as BM I.

All basal media were supplemented with 0.2 to 1.0 $\text{mg}\cdot\text{L}^{-1}$ NAA, 0.2–1.0 $\text{mg}\cdot\text{L}^{-1}$ IBA, 2 $\text{g}\cdot\text{L}^{-1}$ activated charcoal, 25 to 35 $\text{g}\cdot\text{L}^{-1}$ sucrose and 6 $\text{g}\cdot\text{L}^{-1}$ agar; and the pH adjusted to 5.8. The cultures maintained at the same condition. The culture vessels were polypropylene bags (12.5×14 cm) rather than glass bottles (6×9.5 cm) that had been used at previous stage. Each treatment included 150 to 200 plantlets and was repeated three times.

Orthogonal design for rooting culture. Factors that affected the process of in vitro rooting were evaluated individually in the preliminary experiments. On the basis of initial result obtained, four main factors (sucrose, NAA, IBA, and basal medium with modified nitrogen source) and their three different levels were determined respectively. The orthogonal table of $L_9(3^4)$ was selected for studying the effects of four factors on the percentage of qualified plantlets (Table 1). Nine treatments were arranged according to the design and each treatment was repeated three times (Table 4).

Measurement and observation of rooted plantlets. The rooted plantlets were measured when adventitious buds had been inoculated onto rooting culture medium for 30 days according to following morphological standards (Lin et al., 2004).

- 1) Diameter of pseudostem: measuring the diameter of pseudostem where 2 cm away from the base of pseudostem (including the part growing roots), those ≥ 0.3 cm, light green in color were marked as qualified.
- 2) Height of pseudostem: measuring the dis-

Table 1. The design form of the orthogonal test $L_9(3^4)$.

Levels	Factor			
	A Sucrose ($\text{g}\cdot\text{L}^{-1}$)	B NAA ($\text{mg}\cdot\text{L}^{-1}$)	C IBA ($\text{mg}\cdot\text{L}^{-1}$)	D Basal medium (BM)
1	25	0.2	0.2	I
2	30	0.5	0.5	II
3	35	1.0	1.0	III

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Table 2. Effect of inorganic nitrogen form on the percentage of qualified plantlets.

Basal medium (BM) ^z	Inorganic N (m mol·L ⁻¹)		No. of investigated plantlets	Qualified plantlets (%) ^y
	[NO ₃ ⁻]	[NH ₄ ⁺]		
MS	39.41	20.62	440	61.1 bc
1/2 MS	19.71	10.31	546	57.9 c
1	15.83	---	462	61.5 b
2	17.80	---	349	72.2 a
3	18.79	---	517	72.8 a
4	19.78	---	538	70.3 a
5	20.77	---	464	64.1 b
6	25.72	---	509	31.1 d

^zBasal media were all supplemented with 0.2 mg·L⁻¹ NAA, 0.5 mg·L⁻¹ IBA, 25 g·L⁻¹ sucrose.^yValues followed by different letters were significantly different according to LSD test ($p = 0.05$).

Table 3. Effect of the type of calcium on the percentage of qualified plantlets.

Basal medium (BM) ^z	Type of calcium salt	Total [NO ₃ ⁻] (mmol·L ⁻¹)	Total [Ca ²⁺] (mmol·L ⁻¹)	No. of investigated plantlets	Qualified (%) ^y
III	Ca(NO ₃) ₂ ·4H ₂ O	19.98	2.99	523	81.1 a
4	CaCl ₂ ·2H ₂ O	19.78	2.99	538	70.3 b

^zBasal media were both supplemented with 0.2mg·L⁻¹ NAA, 0.5 mg·L⁻¹ IBA, 25 g·L⁻¹ sucrose^yValues followed by different letters were significantly different according to LSD test ($p = 0.05$)

tance from the base of pseudostem (including the part growing roots) to the crossing point of the newest spreading leaf and pseudostem, those ≥ 4 cm, grow normally and no variation were marked as qualified.

3) Number of leaves spreading naturally: measuring the width of leaf which has leaf stalk at the middle of leaf, recording the number of leaves which are more than or equal to 0.8 cm in width, those ≥ 1 , deep green in color, grow normally and no variation were marked as qualified.

4) Root system: measuring the length of white roots, recording the number of roots which are more than or equal to 2 cm in length, those ≥ 3 , and plantlets with profuse root system were marked as qualified.

The percentage of qualified plantlets (%) = the number of rooted plantlets that meet four morphological standards mentioned above/the total number of rooted plantlets (not including those contaminated by fungi or bacteria) $\times 100\%$. The data collected from all treatments were arcsine transformed for statistical analysis.

Results and Discussion

Effect of inorganic nitrogen form on the percentage of qualified plantlets. At rooting stage culture, 1/2 MS is a commonly used

basal medium besides MS. As shown in Table 2, although the concentration of N is half decreased, the ratio of NH₄⁺:NO₃⁻ unchanged and the percentage of qualified plantlets was close to that of MS. When supplying NO₃⁻-N as the sole nitrogen source, the growth of rooted plantlets could be significantly improved in a narrow range of concentration (17.80 to 19.78 mmol·L⁻¹). The percentage decreased when the concentration of NO₃⁻ higher or lower than this range. On the other hand, although 1/2 MS and BM4 were almost equally supplied with NO₃⁻-N, the percentage were 57.9% and 70.3% respectively. It seemed that the difference between them was attributed to the existence of NH₄⁺-N.

For most corps, the biomass of plants which applied with mixture nitrogen source are higher than those supplied with NH₄⁺-N or NO₃⁻-N as the sole nitrogen source. This phenomenon has been called the combination effect of NH₄⁺-N and NO₃⁻-N by some authors (Abbès et al., 1995). So the reason why the revised medium with NO₃⁻-N as the sole nitrogen source could improve the growth of rooted plantlets deserves further study. On the other hand, the adjustment of inorganic nitrogen source in the micropropagation of banana has not been previously reported.

Effect of calcium salt form on the percentage of qualified plantlets. When CaCl₂·2H₂O in BM4 which was one of the basal media with the highest percentage of qualified plantlets shown in Table 2, was substituted by Ca(NO₃)₂·4H₂O, and the total [NO₃⁻] and [Ca²⁺] were unchanged at the same time, the percentage can be improved significantly (Table 3). It has already verified that CaCl₂ was one of the factors that affected the pH value of MS during the process of autoclaving (Wang et al., 1997). On the other hand, supplying Ca(NO₃)₂·4H₂O instead of CaCl₂·2H₂O decreased the concentration of K indirectly, while some authors suggested that MS should be enriched with P and K in order to optimize the growth and development of in vitro cultured plantlets (Marchal et al., 1988). Another significant observation was that the substitution also deferred the darkening of root system.

Orthogonal experiment in rooting culture.

The results of extreme deviation indicated that the factor that most influenced the results was sucrose, followed by revised basal medium, IBA and NAA. The results of calculation also showed that the optimal combination of rooting culture medium was A₂B₂C₂D₂: BM II supplemented with 30 g·L⁻¹ sucrose, 0.5 mg·L⁻¹ NAA, 0.5 mg·L⁻¹ IBA (Table 4). This combination was not included in 9 combinations of the orthogonal experiment. In the following verification test, although the percentage of qualified plantlets from combination A₂B₂C₂D₂ was slightly higher than those from combination 6, which had highest percentage in Table 4, the difference between them was not significant. One of the explanations was that the present study was focused on the in vitro rooting from micropropagated shoots, but rooting stage is closely relative to its previous process: the formation and proliferation of adventitious buds. From this viewpoint, the concentration of growth regulators in rooting culture medium can be varied with those in proliferation stage and the times of subculture.

The results of analysis of variance of the percentage of qualified plantlets among the four selected factors were presented in Table 5. The percentage of qualified plantlets was highly significantly ($p < 0.01$) influenced by sucrose, revised basal medium, IBA and NAA.

In recent years, the commercial production of banana plantlets is developing mainly

Table 4. Results of orthogonal experiment in rooting culture and analysis.

Combination no.	Factors and their codes				The percentage of qualified plantlets (%)		
	A Sucrose (g·L ⁻¹)	B NAA (mg·L ⁻¹)	C IBA (mg·L ⁻¹)	D Basal medium (BM)	Replication 1	Replication 2	Replication 3
1	25	0.2	0.2	I	63.9	53.2	55.3
2	25	0.5	0.5	II	80.3	75.1	76.9
3	25	1.0	1.0	III	60.4	63.3	65.8
4	30	0.2	0.5	III	71.0	80.0	81.5
5	30	0.5	1.0	I	69.7	65.0	66.5
6	30	1.0	0.2	II	84.1	81.6	83.1
7	35	0.2	1.0	II	53.0	54.5	55.4
8	35	0.5	0.2	III	70.2	68.8	69.7
9	35	1.0	0.5	I	54.8	57.9	58.8
K ₁	490.85	475.72	514.32	460.26			
K ₂	547.17	519.50	517.73	524.22			
K ₃	459.31	502.11	465.28	512.85			
Range	29.29	14.59	17.48	21.32			

Table 5. Result of one-way analysis of variance (ANOVA).

Sources of variance	Sum of squares	df	Mean square	F value	F _{0.01}
A) Sucrose	440.226	2	220.113	57.218*	6.23
B) NAA	107.983	2	53.992	14.035*	
C) IBA	191.391	2	95.696	24.876*	
D) Basal medium	258.736	2	129.368	33.629*	
Error	69.244	16	4.328		

*Significantly different at $p < 0.01$ among A, B, C, and D.

towards the goals of improving quality and reducing production cost at the same time (Alvard et al., 1993; Vinh et al., 1997; Matsumoto and Brandão, 2002). When supplied with proper concentration of sucrose and growth regulators (NAA, IBA), modified basal medium with NO_3^- -N as the sole nitrogen source at the concentration of 17.80 to 19.78 $\text{mmol}\cdot\text{L}^{-1}$ could significantly improve the quality of rooted plantlets. Not only was the percentage of qualified plantlets higher than that of MS or 1/2 MS, but the cost for medium-preparation (per liter) was about \$0.6 lower than that of MS based on local retail price, for the concentration of N decreased from about 60 $\text{m mol}\cdot\text{L}^{-1}$ to nearly 20 $\text{m mol}\cdot\text{L}^{-1}$. During the following acclimation phase, over ninety percent of plantlets survived.

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