Pak. J. Bot., 53(3): 1037-1043, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-3(7)

EFFICIENT *IN-VITRO* MICROPROPAGATION OF AN ENDANGERED MARSH SPECIES *RANALISMA ROSTRATUM* THROUGH ORGANOGENESIS OF BUDS AND STOLONS

JIAYAO YANG[1,3], CONGGUANG SHI[2,3], QIANQIAN XIANG[1,3], DAJIN WANG[1], HONGXIN ZHAO[1] AND WANLI GUO[1*]

[1]*College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China*
[2]*Zhejiang Academy of Forestry, Hangzhou 310023, China,* [3]*These authors contributed equally to this work*
[*]*Corresponding author e-mail: gwl1016@aliyun.com*

Abstract

Ranalisma rostratum, a marsh plant species, is considered to be extinct in China because the wild population had not been discovered till 1930s. In 1993, *R. rostratum* was rediscovered in Chaling, Hunan Province, and is under people's supervision and preservation. In this study, an efficient system was established to preserve this species through *In vitro* micropropogation through an orthogonal analysis (L_{16} (4^4)) with four factors (6-benzylaminopurine, BA; kinetin, KT; zeatin, ZT; and indolebutyric acid, IBA) at four levels. Four indexes (bud induction rate (BIR), shoot proliferation (SP), shoot height (SH), and shoot color (SC)) were recorded to select the optimum combinations of the hormones. The results showed that: (1) Buds are only induced from stem segments. (2) Three cytokinins (BA, KT and ZT) and appropriate IBA promote the bud occurrence and shoot growth. (3) The growth vigor is improved by ZT, but inhibited by high concentrations of BA and KT. (4) The plantlets produce stolons with novel rooted plants in MS medium without any hormone. Therefore, the optimum bud-induction, shoot recovery, and shoot-proliferation mediums are 2 mg·L^{-1} BA + 2 mg·L^{-1} KT +0.2 mg·L^{-1} ZT + 0.5 mg·L^{-1} IBA, 1 mg L^{-1} BA + 0.4 mg L^{-1} ZT+ 0.25 mg L^{-1} IBA and 3 mg·L^{-1} BA +3 mg·L^{-1} KT + 0.4 mg·L^{-1} ZT, respectively, and the optimum root-induction and stolon proliferation medium is MS without hormone.

Key words: *Ranalisma rostratum*, Micropropagation, Hormones, Bud, Stolon

Introduction

Ranalisma rostratum, a small marsh herb, belongs to *Alismataceae* (*Ranalisma*), and historically distributed in Asian, including Vietnam, Malaysia, and China (Wang *et al*., 1998; Yang *et al*., 2017). In 1930, *R. rostratum* plants were first disovered in Lishui (N 28º 27´, E 119º 55´), Zhejiang Province, China, but those small populations were dispeared and had not been discovered in the following 6 decades. In 1990, *R. rostratum* plants were rediscovered in Dongxiang (N28º14´, E116º36´), Jiangxi Province, China, and soon disappeared again. Fortunately, 3 years later, a small population with 37 plants of *R. rostratum* was discovered in Chaling (N 26º 50´, E 113º 40´), Hunan Province, China, and some smaller populations remain in Chaling today (Wang & Chen, 1994; Wang *et al*., 1993, 1997, 1998). However, the scales of the wild populations have still being downscaled (Chen, J.K. & Chen, 1999; Chen, Z.Y. & Chen, 1999; Yang *et al*., 2017) and *R. rostratum* was listed as the first national protection species with extremely small population size in China (Yi, 1999).

Some reasons lead to near extinction of *R. rostratum*. The survival rate of young seedlings is very low (2% - 6%) (Wang *et al*., 1993). The seeds of *R. rostratum* usually mature from mid-August to early December, and germinate from next Spring to Autumn. *R. rostratum* plants also propagate through vegetative organ stolons (a metamorphosis inflorescence organ), developed from axillary meristems in short stem (Wang *et al*., 1993; Chen & Chen, 1997). However, the seed-germination rate and young-seedling survival always suffer from the long-term submergence in the water in Spring, the excessive crowding individuals at a point, and lower temperature in Autumn and Winter (Chen & Chen, 1997). Hu *et al*., (1999) proved that *R. rostratum* has lower population genetic diversity, and lacks the genetic basis for adapting to complex heterogeneous habitats. Lower ability of interspecific competition was discovered in *R. rostratum*, that *Oryza rufipogon* and *Isachne globosa* could reduce *R. rostratum* plant biomass, seed proliferation, photosynthesis, and led to the death of smaller plants (Zhou *et al*., 2000). *I. globosa* and *Polygonum praetermissum* significantly inhibited young-plant growth and seed germination (Li & Feng, 2000). Besides, human activities also led to the disappearance of some plants and even the population of *R. rostratum*, such as pesticide pollution, digging ponds and fish farming (Yang *et al*., 2017). For example, both the rising water level for aquaculture and the shrink of marshes in Dongxiang, Jiangxi Province resulted in the rapid extinction of *R. rostratum* (Chen, 2016).

The protection of *R. rostratum* plants was carried out at some small scales through ex-situ cultivation (Wang *et al*., 2006). In 1999, the individual number of *R. rostratum* population increased 80 times through ex-situ cultivation in Yujiang, Jiangxi Province (Li *et al*., 1999; Chen Z.Y. & Chen, 1999). In 2015, the individual number increased 3 times through ex-situ cultivation in Lishui, Zhejiang Province (Chen, 2016). One of the ways of protecting the threatened plants is to increase individual numbers in a short time by micropropagation through shoot / bud organogenesis (Chee *et al*., 2015; Wang *et al*., 2018). However, the study of the *In vitro* micropropagation of *R. rostratum* was known little. Zhou & Tian (1994) found that combination of 6-BA (1 mg·L^{-1}) + NAA (0.2 mg·L^{-1}) in the medium could improve the shoot growth of *R. rostratum*, but offerred little of the intact micropropagation system. Herein, an efficient micropropagation system was established to protect the endangered species *R. rostratum*.

Materials and Methods

Plant materials: *R. rostratum* plants in pots had been cultured in greenhouse at 27℃ with natural light for two months to promote the development and growth of young stems and leaves. Young leaves, petioles, and stems were then selected as the explants.

Culture mediums and conditions: MS (Murashige & Skoog, 1962) was used as the basic medium with adding phytohormones 6-benzylaminopurine (BA), kinetin (KT), zeatin (ZT), and indolebutyric acid (IBA) (Table 1), and orthogonal array L16 (4^4) (Table 2) was formed using software SPSS 22 (SPSS, Inc.). In addition, 4 mediums with or without antibiotics were used for explants sterilization: AM1, MS + Timentin 200 mg·L^{-1} + cefotaxime sodium (cef) 200 mg·L^{-1}; AM2, MS + Timentin 200 mg·L^{-1}; AM3, MS + cef 200 mg·L^{-1}, AM4, MS without antibiotic. All the 19 mediums were added with sucrose 30 g·L^{-1}, agar 5.4 g·L^{-1}, and the pH was adjusted to 5.8-6.0 with 1M KOH before adding agar, and the mediums were then autoclaved at 115℃ for 15 min. Antibiotics were added to AM1-AM3 after cooling of the mediums below 60℃. The cultivation was carried out in the culture chamber with temperature 25 ± 1℃, 60% - 80% relative humidity, and 14 h light with 25 μmol m^{-2} s^{-1} light intensity and 10 h darkness.

Table 1. Factors and levels of orthogonal test L_{16} (4^4).

Levels	Factors (mg L^{-1})			
	(A) BA	(B) KT	(C) ZT	(D) IBA
1	0	0	0	0
2	1.0	1.0	0.1	0.25
3	2.0	2.0	0.2	0.50
4	3.0	3.0	0.4	1.00

Note: BA, 6-benzylaminopurine; KT, kinetin; ZT, zeatin; IBA, indolebutyric acid

Surface sterilization of the explants: Mud and old organs of young and health plants without roots taken out from the mire were removed clearly, and the explants were rinsed for 2 h with flowing tap water and then soaked with detergent solution for 5 min with shake constantly. The cleaned explants were transferred into the superclean bench. The surface sterilization of explants was carried out with 70% ethanol for 15 sec and mercuric chloride (0.1%, wt/vol) for 8 min, then the explants were washed with sterile water for 4 times (5 min each time), and finally, water on the explants was absorbed by sterile filter papers.

Cultivation of aseptic plantlets: The sterile plantlets were directly inoculated in the mediums AM1-AM4 and changed new mediums with interval of 7 d for 3 times. Then, the plantlets were moved into medium MS without antibiotics, and number of aseptic plantlets was recorded within 2 weeks.

Callus induction: Stems, young leaves and petioles were cut into segment 0.5-1.0 cm or 0.5 × 0.5 cm, separately, and those explants were inoculated in the mediums M1-M16 (Table 2). Ten explants in each dish, five dishes of one medium, and 50 explants totally in each medium. And the explants morphological changes were recorded in every 3 d for 20 d.

Bud induction and shoot cultivation: The sterile explants (stems) were cut with two times as cross along the physiological direction and the stem segments were inoculated into the mediums M1-M16 (Table 2). 5 stem segments were in each bottle, 6 bottles for each medium with 30 explants in total, and repeated 3 times. The explants were observed in every 7 d, and 4 indexes (bud induction rate, BIR, shoot height, SH, shoot color, SC, and shoot proliferation, SP) were recorded in 30 days. The shoot cultivation was the same as that of the bud induction.

Rooting and transplanting: The young shoots with height 1.5-3 cm were cut and transferred into MS medium without hormone. Bottles with intact plantlets having adventitious roots (0.5 cm) after culture for 10 d were moved into greenhouse; The rooted shoots (plantlets), removed from the medium after hardening for 3 d, were washed thoroughly to remove medium and were transferred to pots containing sterilized vermiculite and loamy soil in a ratio of 1:1. The pots were maintained in greenhouse with temperatures 25 ± 2℃, disperse light, and 70-80% relative humidity for 4 d. Then the erect plants were put in the greenhouse with 14 h light with 40 μmol m^{-2} s^{-1} light intensity and 10 h darkness. The survival rate was recorded in 20 days.

Data analysis: Multivariate in the general liner model and analysis of variance (ANOVA) in software SPSS 22 was employed to analyze the different effects of four hormones on the micropropogation of *R. rostratum*.

Results

Explants sterilization: It was difficult to remove microorganisms totally from the silt explants, the explants appeared microbial contaminations totally after 3 d in the mediums without antibiotic (AM4), or with one antibiotic Timentin (AM2) or cef (AM3), and turned into brown and death in 7 days after inoculation. However, the medium AM1 with both antibiotics Timentin and cef could eliminate pollution source efficiently (survival rate was 85%, 153 aseptic plantlets out of 180 inoculated explants totally) (Fig. 1A). Meanwhile, new leaves (Fig. 1A) appeared on the explants in AM1 in 6 d of their inoculation; the shoots grew well in 60 d (Fig. 1B). Interestingly, stolons with new adventitious shoots and roots grew well (Figs. 1C, E), and some plantlets had flowers (Fig. 1D). Those results indicated that MS medium without hormones may be one option for *R. rostratum* organogenesis of stolons from explants.

No callus induced from explants: Confusingly, no-callus was induced from explants stems, leaves, and petioles in all 16 mediums (Table 2), even in the medium M3 with the highest concentrations of cytokinins BA, KT, and ZT (Figs. 1F, G). However, the induction, growth, and development of the buds and stolons were influenced by those four hormones BA, KT, ZT, and IBA.

Table 2. The analysis of orthogonal array ($L_{16}(4^4)$) of bud induction and shoot growth induced from *Ranalisma rostratum* stems.

Medium code		Factors (mg/L)				BIR (%)	SH (cm)	SC	SP (number)
		(A)BA	(B)KT	(C)ZT	(D)IBA				
M1		3.0	2.0	0.4	0	60 ± 4	2.88 ± 0.18	3.43 ± 0.51	6.27 ± 1.50
M2		1.0	1.0	0	0.25	72 ± 1	3.73 ± 0.28	3.65 ± 0.49	3.22 ± 1.10
M3		3.0	3.0	0	1.0	80 ± 4	1.52 ± 0.19	1.56 ± 0.89	3.45 ± 1.33
M4		2.0	0	0.1	1.0	76 ± 9	2.84 ± 0.16	3.79 ± 0.42	4.26 ± 1.13
M5		1.0	2.0	0.2	1.0	64 ± 7	2.83 ± 0.15	3.92 ± 0.28	3.00 ± 1.26
M6		0	2.0	0.1	0.25	68 ± 8	3.33 ± 0.25	3.81 ± 0.40	2.94 ± 1.25
M7		3.0	0	0.2	0.25	76 ± 9	2.40 ± 0.16	2.89 ± 0.37	4.95 ± 1.39
M8		2.0	3.0	0.4	0.25	88 ± 7	2.47 ± 0.15	2.77 ± 0.31	5.00 ± 1.12
M9		0	3.0	0.2	0.5	72 ± 8	3.20 ± 0.19	3.44 ± 0.51	4.39 ± 1.15
M10		3.0	1.0	0.1	0.5	88 ± 8	1.78 ± 0.10	2.14 ± 0.13	4.77 ± 1.22
M11		2.0	2.0	0	0.5	84 ± 7	2.42 ± 0.15	2.29 ± 0.19	3.57 ± 0.52
M12		1.0	0	0.4	0.5	84 ± 2	3.40 ± 0.18	3.57 ± 0.51	3.48 ± 0.83
M13		0	0	0	0	60 ± 2	3.02 ± 0.19	3.77 ± 0.44	2.20 ± 0.79
M14		1.0	3.0	0.1	0	64 ± 6	3.09 ± 0.19	3.09 ± 0.30	5.40 ± 1.20
M15		0	1.0	0.4	1.0	76 ± 7	2.99 ± 0.16	3.84 ± 0.37	3.21 ± 0.74
M16		2.0	1.0	0.2	0	56 ± 9	2.85 ± 0.11	2.71 ± 0.07	5.31 ± 0.75
BIR	$\overline{K}_{j1}$	276	296	296	240				
	$\overline{K}_{j2}$	284	291	296	304				
	$\overline{K}_{j3}$	304	286	268	328				
	$\overline{K}_{j4}$	304	304	308	296				
	OL	A_3 or A_4	B_4	C_4	D_3				
	OC	$A_3/A_4B_3C_3D_3$ (2.0/3.0 mg L^{-1} BA +2.0 mg L^{-1} KT + 0.2 mg L^{-1} ZT+ 0.5 mg L^{-1} IBA)							
SP	$\overline{K}_{j1}$	12.78	14.89	12.44	19.18				
	$\overline{K}_{j2}$	15.1	16.51	17.37	16.11				
	$\overline{K}_{j3}$	18.14	15.78	17.65	16.21				
	$\overline{K}_{j4}$	19.44	18.24	17.96	13.92				
	OL	A_4	B_4	C_4	D_1				
	OC	$A_4B_4C_4D_1$ (3.0 mg L^{-1} BA +3.0 mg L^{-1} KT + 0.4 mg L^{-1} ZT)							
SH	$\overline{K}_{j1}$	12.54	11.66	10.69	11.84				
	$\overline{K}_{j2}$	13.05	11.35	11.04	11.93				
	$\overline{K}_{j3}$	10.58	11.46	11.28	10.8				
	$\overline{K}_{j4}$	8.58	10.28	11.74	10.18				
	OL	A_2	B_1	C_4	D_2				
	OC	$A_2B_1C_4D_2$ (1.0 mg L^{-1} BA + 0.4 mg L^{-1} ZT+ 0.25 mg L^{-1} IBA)							
SC	$\overline{K}_{j1}$	14.86	14.02	11.27	13				
	$\overline{K}_{j2}$	14.23	12.34	12.83	13.12				
	$\overline{K}_{j3}$	11.56	13.54	12.96	11.44				
	$\overline{K}_{j4}$	10.02	10.86	13.61	13.11				
	OL	A_1	B_1	C_4	D_2				
	OC	$A_1B_1C_4D_2$ (0.4 mg L^{-1} ZT+ 0.25 mg L^{-1} IBA)							

Note: BA, 6-benzylaminopurine; KT, kinetin; ZT, zeatin; IBA, indolebutyric acid; BIR, bud induction rate, the number of survival explants with buds / total explants; SP, shoot proliferation (number), the number of shoots of an explants; SH, shoot height; SC, shoot color, the color intensity of the shoot: the white is 1, the yellow is 2, the yellow-green is 3, and the green is 4; OL, optimal level; OC, optimum combination

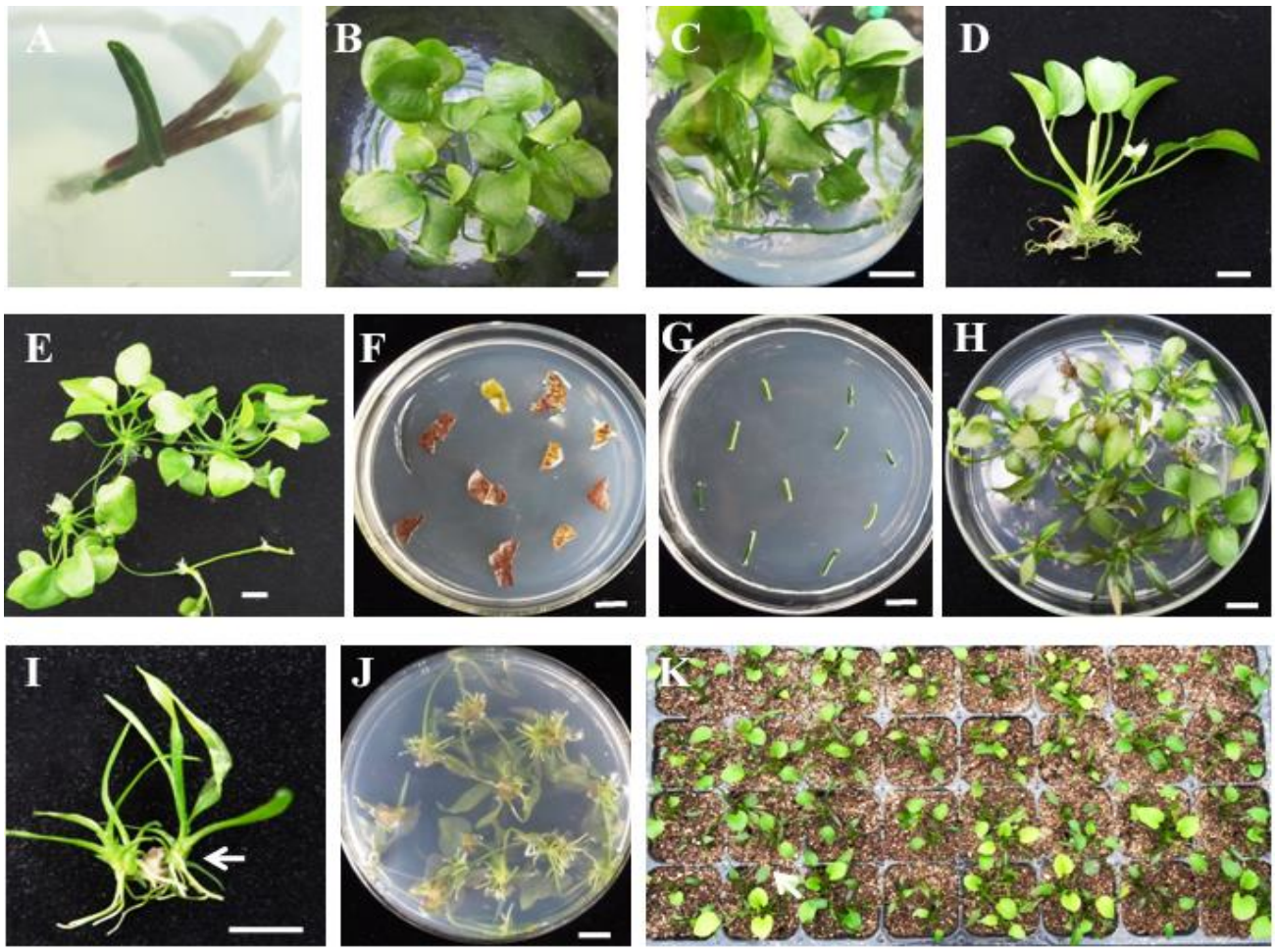

Fig. 1. Efficient micropropagation of *Ranalisma rostratum* plants. A, innoculated shoots in hormone-free MS medium for 6 days; B-D, aseptic plantlets have been cultured in hormone-free MS medium for 60 days; E, a stolon dissected from C; explants from leaves (F) and petioles (G) had been innoculated in medium M3 for 20 days; H-I, shoots in subculture medium for 30 days, the white arrow indicates adventitious shoots in I; J, rooting in the medium MS without hormone for 10 days; K, plantlets growing in pots containing roseite and humus soil (1:1) for 20 days.

Bud induction and shoot sub-cultivation: To select optimal mediums for bud induction and shoot growth, orthogonal test (L16 (4^4)) was used to explore the effects of the hormones on the induction and growth of buds, including 4 factors (BA, KT, ZT, and IBA) and 4 levels (different concentrations of hormones) (Table 1), and produced 16 mediums with different hormone combinations (Table 2). According to the statistical results (Table 2), higher concentrations of cytokinins (BA, KT, and ZT) inhibited the occurrence of stolon but promoted the occurrence and development of buds. The highest BIR (bud induction rate) was M8 and M10 (88%, average), the lowest one was M16 (56%). M13 is a MS medium without hormone, its BIR was 60%. The indexes of shoot quality are SH (shoot height) and SC (shoot color), the growth potential of shoots is better when those two indexes are higher. M2 had the highest SH value (3.73 cm), M3 had the minimum value (1.52 cm), and M13 has a value of 3.02 cm. For SC, M3 also had the lowest value (1.56), M5 had the highest value (3.92), and the value of M13 was 3.77. Those results indicated that the high concentration of cytokinin combinations inhibit bud growth. For shoot proliferation (SP), the maximum was M1 (6.27), and the lowest was M13 (2.20), indicating that the combination of 4 hormones improve shoot proliferation and growth to some extent.

The different effects of 4 hormones on the induction and growth of buds were obtained through the range analysis, also called intuitive analysis (Table 2). The mean values of the sum of the evaluation indexes of all levels ($\overline{K}_{ji}$, i = 1, 2, 3, 4; j = A, B, C, and D) in each treatment were calculated to determine the optimum levels and factors (Table 2), and positive correlation is between $\overline{K}_{ji}$ and quality due to the level of a factor. For example, to estimate the effect of factor A (BA, 6-benzylaminopurine) on the SP, the value of $\overline{K}_{j4}$ was the largest among the four $\overline{K}_{ji}$ values. That indicated that A_4 (3.0 mg L^{-1} BA) had the most significant effect on the SP. A_4, B_4, C_4, and D_1 were the optimum levels of BA, KT (kinetin), ZT (zeatin), and IBA (indolebutyric acid), respectively. Thus, $A_4B_4C_4D_1$ was determined to be the optimum combination for shoot proliferation, $A_3/A_4B_3C_3D_3$ for bud induction, $A_2B_1C_4D_2$ for shoot height, and $A_1B_1C_4D_2$ for shoot color (Table 2). The results of variance (ANOVA) (Table 3) presented that factor D (IBA) is the most important factor for bud induction from explants (BIR, $p<0.01$) among A, B, C, and D. IBA significantly ($p<0.05$) promoted the BIR at

lower concentration (< 0.5 mg·L^{-1}), but inhibited significantly ($p<0.05$) with high concentration (>0.5 mg·L^{-1}), and high content of BA (3 mg·L^{-1}) decreased BIR (Table 4). Thus, medium with 2.0 mg L^{-1} BA +2.0 mg L^{-1} KT + 0.2 mg L^{-1} ZT+ 0.5 mg L^{-1} IBA is suitable for bud induction and shoot subculture. In fact, young buds were induced from the stem segments (Fig. 1I, indicated by the arrow) in the subculture medium, and some plantlets also had some stolens (Fig. 1H). For indexes of shoot quality, factor A was the most negative effect ($p<0.05$) on shoot quality (Tables 2, 3). However, ZT promoted the SH and maintained the SC, although not significantly. Thus, medium with 1.0 mg L^{-1} BA + 0.4 mg L^{-1} ZT+ 0.25 mg L^{-1} IBA is suitable for shoot recovery. For SP, although no significance was discovered among four factors, high concentrations of three cytokinines (BA, KT, and ZT) improved SP, and high concentration of IBA inhibited the SP (Table 4). Therefore, medium (3.0 mg L^{-1} BA +3.0 mg L^{-1} KT + 0.4 mg L^{-1} ZT) is suitable for SP in short time, because explants in medium with high concentrations of cytokinins for longtime decreased plantlet quality. For rooting, *R. rostratum* plantlets not only grew well in MS medium without any hormone (AM4; M13, Table 2), including BIR (60%), SH (3.02 cm) and SC (3.77), and also rooted well (Fig. 1J). And the most importance was that plantlets could have stolons and adventitious buds spontaneously. Thus the cultivation of stolons in hormone-free MS medium is also an efficient way for *R. rostratum* micropropogation, although the propagation coefficient is lower (SP was 2.20 and BIR was 60%, separately, Table 2) and the sub-cultivation cycle is longer (45-60 d). According to those results, the optimum bud-induction (subculture medium), shoot-recovery, and shoot-proliferation (primary culture) mediums are 2 mg·L^{-1} BA + 2 mg·L^{-1} KT +0.2 mg·L^{-1} ZT + 0.5 mg·L^{-1} IBA, 1.0 mg L^{-1} BA + 0.4 mg L^{-1} ZT+ 0.25 mg L^{-1} IBA, and 3 mg·L^{-1} BA +3 mg·L^{-1} KT + 0.4 mg·L^{-1} ZT, respectively.

Rooting and transplanting: Interestingly, plantlets might have root occurrences in different mediums, excluding some mediums with high concentrations of cytokinins, such as M1, M3, and M8. Roots grew well (rooting rate was more than 95%) in medium M13 (Fig. 1J) without hormone, and plantlets also had stolon occurrences. Thus, MS medium with no hormone was chosen as rooting and stolon happening medium. The plantlets with roots were transplanted in pots and cultured in the greenhouse, the seedling leaves stood upright after 2 d and returned to green soon. Then the plants grown well (Fig. 1K) and the survival rate was about 90% (230 survival individuals / 256 plantlets) in 20 d.

Discussion

Two micropropagation ways to preserve the endangered species *R. rostratum* have been established through increasing individuals in a short time. Two reproductive types of *R. rostratum* plants are in nature: asexual or sexual ways, the former is vegetative propagation and forming a series of new plants through adventitious shoots and stolons happening on the short stems (Wang *et al*., 1998). Similarly, the stolons with novel plants arranged as rosary necklace were also occurred in our experiments (Figs. 1C, E, & J). Those results suggested the mimic method according to the natural vegetative propagation is feasible through producing stolons from the stems *In vitro*, such as the micropropogation by stolon in *Glycyrrhiza glabra* (Gupta *et al*., 2013) and *G. uralensis* (Kojoma *et al*., 2010), but proliferation time may take longer. On the other hand, the bud induction from stems overcomes the problem of longer-proliferation time, and raises the induction rate of shoots and reproduction coefficient, separately (Table 2), but the operation procedure is more complicated comparing with the propagation using stolons.

Table 3. Analysis of variance (ANOVA) of 4 indexes of *Ranalisma rostratum* plantets (* < 0.05, ** <0.01).

Items	Source of variance	Degree of freedom	Sum of squares of deviations	F value	p value
BIR (%)	A	3	152.000	0.425	0.739
	B	3	104.000	0.281	0.838
	C	3	216.000	0.632	0.609
	D	3	1040.000	7.647	0.004**
SP	A	3	6.834	2.294	0.130
	B	3	1.515	0.352	0.789
	C	3	5.140	1.511	0.262
	D	3	3.485	0.913	0.464
SH	A	3	3.125	6.890	0.006**
	B	3	0.285	0.245	0.864
	C	3	0.147	0.123	0.945
	D	3	0.533	0.484	0.700
SC	A	3	3.878	4.182	0.030*
	B	3	1.448	0.943	0.450
	C	3	0.747	0.437	0.731
	D	3	0.509	0.288	0.833

Note: BIR, bud induction rate, the number of survival explants with buds / total explants; SP, shoot proliferation (number), the number of shoots of an explants; SH, shoot height; SC, shoot color, the color intensity of the shoot: the white is 1, the yellow is 2, the yellow-green is 3, and the green is 4

Table 4. Different effects on the bud induction and shoot growth of *Ranalisma rostratum* among different levels of the factors through paired comparison analysis (* < 0.05).

Items	BA				KT				ZT				IBA			
	(I)	(J)	AD (I-J)	p	(I)	(J)	AD (I-J)	p	(I)	(J)	AD (I-J)	p	(I)	(J)	AD (I-J)	p
BIR	0	1	-0.020	0.604	0	1	0.010	0.792	0	0.1	0.000	1.000	0	0.25	-0.160	0.019*
		2	-0.070	0.137		2	0.050	0.245		0.2	0.070	0.137		0.5	-0.220	0.008*
		3	-0.070	0.137		3	-0.020	0.604		0.4	-0.030	0.450		1.0	-0.140	0.027*
	1	2	-0.050	0.245	1	2	0.040	0.332	0.1	0.2	0.070	0.137	0.25	0.5	-0.060	0.182
		3	-0.050	0.245		3	-0.030	0.450		0.4	-0.030	0.450		1.0	0.020	0.604
	2	3	0.000	1.000	2	3	-0.070	0.137	0.2	0.4	-0.100	0.063	0.5	1.0	0.080	0.104
SH	0	1	-0.127	0.759	0	1	0.077	0.851	0	0.1	-0.087	0.833	0	0.25	-0.023	0.955
		2	0.494	0.280		2	0.050	0.903		0.2	-0.146	0.724		0.5	0.257	0.544
		3	0.991	0.078		3	0.344	0.428		0.4	-0.264	0.534		1.0	0.414	0.351
	1	2	0.621	0.197	1	2	-0.027	0.946	0.1	0.2	-0.060	0.884	0.25	0.5	0.280	0.511
		3	1.118	0.059		3	0.266	0.530		0.4	-0.177	0.670		1.0	0.437	0.329
	2	3	0.497	0.278	2	3	0.294	0.492	0.2	0.4	-0.117	0.775	0.5	1.0	0.158	0.704
SC	0	1	0.159	0.724	0	1	0.421	0.379	0	0.1	-0.391	0.410	0	0.25	-0.031	0.944
		2	0.827	0.137		2	0.144	0.749		0.2	-0.428	0.373		0.5	0.391	0.410
		3	1.212	0.060		3	0.789	0.150		0.4	-0.588	0.247		1.0	-0.029	0.949
	1	2	0.668	0.201	1	2	-0.278	0.546	0.1	0.2	-0.037	0.934	0.25	0.5	0.422	0.378
		3	1.053	0.082		3	0.367	0.436		0.4	-0.196	0.664		1.0	0.002	0.996
	2	3	0.385	0.416	2	3	0.645	0.213	0.2	0.4	-0.160	0.723	0.5	1.0	-0.420	0.381
SP	0	1	-0.589	0.358	0	1	-0.407	0.509	0	0.1	-1.233	0.108	0	0.25	0.766	0.254
		2	-1.350	0.089		2	-0.223	0.709		0.2	-1.300	0.097		0.5	0.741	0.266
		3	-1.674	0.054		3	-0.838	0.221		0.4	-1.377	0.085		1.0	1.313	0.095
	1	2	-0.761	0.256	1	2	0.183	0.758	0.1	0.2	-0.067	0.910	0.25	0.5	-0.025	0.967
		3	-1.085	0.140		3	-0.431	0.485		0.4	-0.144	0.808		1.0	0.547	0.389
	2	3	-0.324	0.594	2	3	-0.615	0.340	0.2	0.4	-0.077	0.896	0.5	1.0	0.571	0.370

Note: BA, 6-benzylaminopurine; KT, kinetin; ZT, zeatin; IBA, indolebutyric acid; BIR, bud induction rate, the number of survival explants/total explants; SP, shoot proliferation (number), the number of shoots of an explants; SH, shoot height; SC, shoot color, the color intensity of the shoot: the white is 1, the yellow is 2, the yellow-green is 3, and the green is 4; AD, average deviation

No callus was induced although the indefinite shoots could be induced from stem segments (Figs. 1F, G), and was not accord to the general roles. That the ratio of of auxin and cytokinin determines the explants state of differentiation, and a high ratio of auxin-to-cytokinin or cytokinin-to-auxin generally induces root and shoot regeneration, respectively (Skoog & Miller, 1957; Ikeuchi *et al*., 2013). The reasons may due to that the types and concentrations of the hormones selected are not suitable for the induction of callus, or the control abilities of the endogenous hormones are stronger, the stimulation of exogenous hormones have little interference with the endogenous phytohormones, and difficult to induce callus. But the possibility needs to be studied further.

The induction and growth of *R. rostratum* buds and shoots were influenced by cytokinins 6-BA, KT and ZT, and auxin IBA. Higher concentrations of cytokinins could promote the happening of buds, but restrain the shoot growth and worsen the shoot health (fading shoot color and dwarfing shoot height, Tables 2, 3, and 4). And lower concentrations of cytokinins could promote the shoot growth but decrease the bud occurrence. Zhou and Tian (1994) also had the similar results that the shoot growth was inhibited in the mediums with high BA levels. Contrary, higher IBA levels promoted the shoot growth but inhibited the bud happening. ZT could not only play the cytokinin roles, such as promoting shoot growth, but also maintain shoot's normal development; similar results were discovered in soybean (Neumann *et al*., 1983) and *Pseudosasa japonica* cv. *Akebonosuji* (Yang *et al*., 2010). However, the ZT's roles in promoting health and normal development of shoots remain to be investigated next.

Conclusion

In this study, the optimum bud-induction, shoot-recovery, and shoot-proliferation mediums were selected, respectively, and organogenesis of buds and stolons could be happened on explants in those mediums. In addition, plantlets on MS medium without hormone could not only have roots, but also have stolons. Thus, the efficient micropropogation system of the endangered species *R. rostratum* was established, including slow one (MS medium without hormone for stolon induction) or rapid one (MS medium with hormones for bud induction).

Acknowledgements

This work was supported by the Biodiversity Conservation Project of Zhejiang Provincial Wildlife Conservation Management Station ((2015) 30) and support from China Scholarship Council (award to Wanli Guo for 1 year's study abroad at the University of North Carolina at Greensboro).

References

Chee, F.M., X. Rathinam, M. Danial, C.K. Lam, M.H. Qui and S. Subramaniam. 2015. Effect of methyl-jasmonate on 9-methoxycanthin-6-one content in *Eurycoma longifolia* (Tongkat Ali) root culture. *Pak. J. Bot.*, 47(3): 897-904.

Chen, B.J. 2016. Miniature crown grass *Ranalisma rostratum* in water. *China Nature*, 2: 74-75.

Chen, J.K. and Z.Y. Chen. 1999. Population dynamics of *Ranalisma rostratum*, an endangered species growing in different habitats. *Acta Phytoecol. Sinica*, 23: 8-13.

Chen, Z.Y. and J.K. Chen. 1997. The endangered causes and protection methods about *Ranalisma rostratum*. *J. Hubei Agri. College*, 17: 28-32.

Chen, Z.Y. and J.K. Chen. 1999. The spatial pattern of *Ranalisma rostratum* population and interspecific association in the community. *Acta Phytoecol. Sinica*, 23: 56-61.

Gupta, S., P. Pandotra, A.P. Gupta, M.K. Verma, A. Ahuja and R.A. Vishwakarma. 2013. Direct rhizogenesis, *In vitro* stolon proliferation and high-throughput regeneration of plantlets in *Glycyrrhiza glabra*. *Acta Physiol. Planta*., 35: 2699-2705.

Hu, X., J. Chen, J. Wang, G. He and S. Ge. 1999. Genetic diversity and its relationship with breeding system of *Ranalisma rostratum. Acta Botanica Yunnanica*, 21: 232-238.

Ikeuchi, M., K. Sugimoto and A. Iwase. 2013. Plant callus: mechanisms of induction and repression. *Plant Cell*, 25: 3159-3173.

Kojoma, M., K. Ohyama, H. Seki, Y. Hiraoka, S.N. Asazu, S. Sawa, H. Sekizaki, S. Yoshida and T. Muranaka. 2010. *In vitro* proliferation and triterpenoid characteristics of licorice (*Glycyrrhiza uralensis* Fischer, *Leguminosae*) stolons. *Plant Biotech.*, 27(1): 59-66.

Li, J. and B. Feng. 2000. Research on allelopathic effect of weeds on endangered plant *Ranalisma Rostrata*. *J. Hubei Normal Uni. (Natural Science)*, 20: 9-11.

Li, J., J.K. Chen, J. Zhou and G.Q. He. 1999. Dynamics and evaluation of *Oryza rufipogon* and *Ranalisma rostratum* population in ex-situ conservation of communities. *Acta Phytoecol. Sinica*, 3: 275-282.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.

Neumann, P.M., A.T. Tucker and L.D. Nooden. 1983. Characterization of leaf senescence and pod development in soybean explants. *Plant Physiol.*, 72: 182-185.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *In vitro*. *Symp. Soc. Exp. Biol*., 11: 118-130.

Wang, D., X. Li, Z. Cheng and C. Long. 2018. *In vitro* preservation and micropropagation of *Oreocharis mileense* (W.T. Wang) M. Möller & A. Weber (*Gesneriaceae*) through shoot organogenesis. *In Vitro Cell. Dev-Pl.*, 54: 606-611.

Wang, J.B., J.K. Chen and R.Q. Li. 1997. Characters of seed storage and germination in *Ranalisma rostratum* and *Sagittaria guyanensis* subusp. lapp ula. *Plant Physiol. Comm*., 33(2): 119-122.

Wang, J.B., J.K. Chen, R.Q. Li and G.Q. He. 1998. Life history traits and mechanisms of endangerment in *Ranalisma rostratum. Chinese Biodiv.*, 6: 167-171.

Wang, J.B., X.F. Wang, J.K. Chen, R.Q. Li and W.Q. Wang. 1993. A preliminary study on reproductive traits of *Ranalisma rostratum*. *J. Wuhan Uni. (Natural Science)*, 6: 130-132.

Wang, X., Y. Tan, J. Chen and Y. Lu. 2006. Pollen tube reallocation in two preanthesis cleistogamous species, *Ranalisma rostratum* and *Sagittaria guyanensis* ssp. lappula (*Alismataceae*). *Aqu. Bot.*, 85: 233-40.

Wang, X.F. and J.K. Chen. 1994. A survey on the habitats of rare and endangered aquatic plants in Huan Province. *Chinese Biodiv.*, 2(4): 193-198.

Yang, H., X. Wang, N. Zhang, X. Lin and W. Fang. 2010. Tissue culture and leaf color variation of *Pseudosasa Japonica* cv. *Akebonosuji*. *J. Bamboo Res.*, 29: 15-20.

Yang, J.Y., C.G. Shi, Q.Q. Xiang and W.L. Guo 2017. Review on breeding property of *Ranalisma rostratum* and researches of propagation techniques. *J. Zhejiang Forest SCI & TECH.*, 37: 107-110.

Yi, M. 1999. List of wild plants of national priority protection (first batch). *Life World*, 5: 4-11.

Zhou, C. and H. Tian. 1994. Tissue culture of endangered plant *Ranalisma rostratum. Plant Physiol. Comm.*, 4: 277.

Zhou, J., Z. Chen and J. Chen. 2000. The effects of competition among the mixed crop populations of *Oryza rufipogon*, *Ranalisma rostrata* and *Isachne globosa. Acta Ecologica Sinica*, 20: 685-91.

(Received for publication 3 June 2019)