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INFLUENCING FACTORS OF EMBRYO RESCUE IN SEEDLESS GRAPE

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Abstract

In this study, we investigated the impact of inoculating stage, medium type and concentration of plant growth regulators on embryo rescue effectiveness by $L_{25}(5)^6$ orthogonal design using selfed ovules of 'Venus Seedless' as the testing material. The main results were as follows. The most important factor influencing ovule germination was inoculating stage. Ovule germinating rate gradually increased as inoculating being postponed. The highest germinating rate appeared when inoculation was done 55d after flowering. Other influencing factors were IBA concentration, exogenous amino acid, 6-BA concentration, GA_3 concentration and medium type in descending order. The best embryo rescue result was based on Nitsch medium including 1.0 mg/L IBA, 0.1 mg/L GA_3, 0.7 mg /L 6-BA and 2.0 mmol/L glutamine using ovules inoculated 55d after flowering. The highest germinating rate reached 41.25%, and a batch of seedlings was also obtained.

Key words: Venus Seedless grape; Embryo rescue; Influencing factors.

Introduction

Seedlessness, as a good character for table grapes, is widely appreciated by consumers, and it also has a promising prospect in fresh, raisin and market making. Selection of seedless grape with great quality has been an important breeding objective. Conventionally, seedless cultivars were only obtained through the cross by using seeded cultivars as female parent and seedless cultivars as male parent. However, this method takes a long time and produce only 0-15.9% seedless seedlings were (Ledbetter *et al*.,1994; Ramming *et al*., 2000). The usage of seedless cultivar as female parent was restricted in breeding practice, because viable seeds can not be formed due to the abortion of fertilized ovule during their developing process. Fortunately, the in vitro embryo rescue technique provides a possibility of saving the aborted or vestigial ovules in their early developing stage (Yi *et al*., 2001; Usman *et al*., 2012; Xie *et al*., 2013; Zhang *et al*., 2013). Embryo rescue technique allows a wider parent selecting range-seedless cultivars can be used as female parent in breeding. This technique has been extensively used since its first use in 1982 (Ramming *et al*., 1982). The cross "Seedless × Seedless" could be successfully carried out using embryo rescue. Ramming *et al*. (1990) obtained a batch of hybrid seedlings (82% was seedless) by rescuing the embryos (Seedless × Seedless) before their abortion took place. At least 5 years will be saved when a seedless cultivar is bred through embryo rescue technique rather than conventional method, which pushed forward seedless grape breeding (Ramming *et al*., 1995). Since the 1980s, embryo rescue study of seedless grape has been implemented in China. Embryo rescue technique has been improved, and lots of hybrid seedlings have also been obtained (Dong & He 1991; Zhang *et al*., 1991; Meng *et al*., 1992, 1993; Wang *et al*., 1997; Qi *et al*., 2001; Wang *et al*., 2001; Pan *et al*., 2005; Guo *et al*., 2007; Tang *et al*., 2008, 2009). However, there are still some problems in embryo rescue technique such as low survival rate and complicated operation. So this technique needs improvement. We cultured the ovules of natural selfed'Venus Seedless'*in vitro*, and proper medium and optimal inoculating time was discussed, so as to provide some support for embryo rescue in seedless grape.

Materials and Methods

Materials (ovules of selfed 'Venus Seedless') were collected from vineyard of Shenyang Agriculture University. Inflorescences were bagged 3-5d before flowering. Then clusters of different stages were flushed for several times. Clusters were dipped in 70% ethanol for 30s, and were washed by sterile water for 3 times, then were dipped in 0.1% $HgCl_2$ for 8-10min, and were washed again by sterile water for 3-5 times. Berries were cut open, and ovules were taken out. The ovules were inoculated in conical flask of 100ml filled with 50ml medium. 10 ovules were inoculated into 1 flask with 8-10 flasks per treatment. $L_{25}(5^6)$ orthogonal experiment was carried with the following 6 factors: inoculating stages (days after flowering), medium type, IBA concentration, GA_3 concentration, 6-BA concentration and amino acid type (Table 1).

All media was supplemented with 6% cane sugar, 0.6% agar and 0.1% activated charcoal. 60 days later, ovules were cut horizontally and transferred into germination medium (1/2 MS + 2% cane sugar + 0.6% agar + 0.1% activated charcoal + BA0.5 mg/L+IBA1.5 mg/ L+GA_3 0.5 mg/L). germination rate was investigated 30d later. Culture conditions were as follows: temperature (25±1)^oC, light intensity 2000Lx and 12-14h illumination per day.

Data was analyzed using software SPSS.

Table 1. Result of orthogonal tests.

No. of Treatment	Combined factors						Rate of Emergence (%)
	A Days after blooming(d)	B Medium	C IBA (mg/L)	D GA3 (mg/L)	E 6-BA (mg/L)	F Amino acid (2.0mmol/L)	
1	40□ 1□	MS(1□	0.5(1)	0.1(1)	0.1(1)	cysteine(1)	0.00
2	40	B5(2)	1.0(2)	0.3(2)	0.3(2)	glutamine(2)	0.00
3	40	NN(3)	1.5(3)	0.5(3)	0.5(3)	proline(3)	0.00
4	40	ER(4)	2.0(4)	0.7(4)	0.7(4)	phenylalanine(4)	0.00
5	40	Nitsch(5)	2.5(5)	0.9(5)	0.9(5)	gap(5)	0.00
6	45□ 2□	MS	1.0	0.5	0.7(4)	gap	3.00
7	45	B5	1.5	0.7	0.9(5)	cysteine	5.00
8	45	NN	2.0	0.9	0.1	glutamine	1.00
9	45	ER	2.5	0.1	0.3	proline	3.33
10	45	Nitsch	0.5	0.3	0.5	phenylalanine	2.00
11	50□ 3□	MS	1.5	0.7	0.3	phenylalanine	10.00
12	50	B5	2.0	0.9	0.5	gap	13.00
13	50	NN	2.5	0.1	0.7	cysteine	6.25
14	50	ER	0.5	0.3	0.9	glutamine	8.00
15	50	Nitsch	1.0	0.5	0.1	proline	3.00
16	55□ 4□	MS	2.0	0.3	0.9	proline	34.12
17	55	B5	2.5	0.5	0.1	phenylalanine	12.50
18	55	NN	0.5	0.7	0.3	gap	31.25
19	55	ER	1.0	0.9	0.5	cysteine	40.00
20	55	Nitsch	1.5	0.1	0.7	glutamine	41.25
21	60□ 5□	MS	2.5	0.7	0.5	glutamine	7.50
22	60	B5	0.5	0.9	0.7	proline	6.67
23	60	NN	1.0	0.1	0.9	phenylalanine	6.25
24	60	ER	1.5	0.3	0.1	gap	13.00
25	60	Nitsch	2.0	0.5	0.3	cysteine	11.00
K1	0.85	10.92	9.58	13.89	5.90	11.31	
K2	2.86	7.43	9.31	11.07	11.11	12.68	
K3	8.05	8.95	14.98	6.90	11.36	9.42	
K4	31.82	11.73	11.82	9.35	12.56	6.15	
K5	8.88	12.58	5.91	10.40	10.67	12.05	
R	315.80	8.80	17.30	12.00	13.10	13.70	

Note: The □ 1□ ~□ 5□ represent the A□ B□ C□ D□ E□ F factors'five level in table 1

Results and analysis

Intuitive analysis of ovule germination rate: Table 1 shows the germination rate of 25 treatments. It can been seen that different factors and different levels lead to different ovule germination rate ranging from 0 to 41.25%. The best factor and level combination was A4B5C2D1E4F2 (Treatment 20), being Nitsch medium including 1.0 mg/L IBA, 0.1 mg/L GA_3, 0.7 mg /L 6-BA and glutamine using ovules inoculated 55d after flowering. Theoretically from K value, the best combination should be A4B5C3D1E4F2, which is Nitsch medium including 1.5 mg/L IBA, 0.1 mg/L GA_3, 0.7 mg /L 6-BA and glutamine using ovules inoculated 55d after flowering. However, this combination did not appear. That is, the rate under the combination A4B5C3D1E4F2 should be higher than that (41.25%) under the combination A4B5C2D1E4F2. From range value (R) in this study, the sequence was inoculating stage (A)>IBA concentration (C)>amino acid type (F)>6-BA concentration (E)>GA_3 concentration (D) >medium type (B). The larger the value is, the more important it is. So the determination of inoculating stage and hormone concentration has an significant effect on germination rate.

Variance analysis of germination rate influencing factors: We can see from Table 2 that, inoculating stage generated extremely significant differences (Sig=0.002<0.01) in germinating rate. Further study of inoculating stage showed that, as the stage was postponed, the rate reached to the top when inoculation was done 55d after flowering, and the rate dropped when it is done 60d after flowering. Statistically, there is no significant difference when inoculation was done 40d, 45d or 50d after flowering. 60d after flowering resulted in a significant difference (Sig=0.040<0.05), while 55d after flowering resulted in an extremely significant difference (Sig=0.000<0.01). No significant difference of the effect on germinating rate existed in medium type, 6-BA concentration, GA3 concentration, IBA concentration and amino acid type. But practically, factors of different levels have different effect on germination rate.

Table 2. Variance analysis of orthogonal test result from Table 1.

Source of variance	Square sum of deviation	Free degree	Mean squar	F-value	Sig	A factor level	A factor Sig
A	3158.50	4	789.62	35.93	0.002	1	0.792
B	88.00	4	22.37	1.12	0.564	2	0.388
C	177.14	4	44.28	2.02	0.257	3	0.053
D	119.54	4	29.88	1.36	0.386	4	0.000
E	130.28	4	32.57	1.38	0.356	5	0.040
F	136.48	4	34.12	1.55	0.34		
Error	87.88	4	21.97				
Total variance	6459.5	25					

Discussion

In this study, we used orthogonal design, which could take into account more factors and levels compared with single factor experiment. What's more, accuracy and efficiency were both increased. We hereby designed 6 factors and 5 levels based on former study (Guo *et al*., 2006a), and studied embryo rescue efficient. We got the highest germination rate of 41.25%. In our study, the most important influencing factor was inoculating stage, which is the same with Guo *et al*.'s study (2007). Different seedless cultivars have different embryo development and embryo aborting stage. Generally, the best inoculating stage appears when the ovule reaches the highest development degree but without abortion (Amaral *et al*., 2001). In this study, ovule germination rate increased as the inoculating stage was postponed, and it reached to the top when inoculating 55d after flowering. This maybe because that the ovules of 'Venus Seedless'develop to a high degree at 55d after flowering but haven't abort yet, while ovules starts to abort at 60d after flowering.Exogenous hormones in the medium as well as their concentrations play an important role in seedless grape embryo rescue (Neal *et al*., 1985). Some researchers believed that adding proper exogenous hormones into the medium could improve the development of the ovules (Jiang *et al*., 2002; Li *et al*., 2001;Gribaudo *et al*., 1993; Spiegle *et al*., 1985). GA3 and IAA has a better effect, and their common concentrations are GA_3 10^{-6}mol/L and IAA10^{-5}mol/L. During the embryo germination stage, cytokinin, such as 6-BA are often added to push the embryo's germination (Gray *et al*., 1990; Bharathy *et al*., 2005). Guo *et al*. (2006a, 2006b) considered that IBA of high concentration and GA_3 as well as 6-BA of low concentration were needed for the young embryos' development. High seedling rate can be gained under proper hormones. Li *et al*. (2001) thought that adding 0.5mg/L IAA, 1.5mg/L BA and 0.5mg/L GA_3 into Nitsch medium is suitable for the development of Thompson Seedless ovules. Zhang *et al*. (1992) report showed that the sensibility of different seedless cultivars to hormones is different from that of a same cultivar to different hormones. This is mainly because of genotype and the degree of embryo development.In this study, the effect of the hormones and their concentrations had no significant difference statistically, but intuitively there existed some effect on ovule germination rate, whose reasons still need to be discussed. The development of young embryo and their germination could be co-regulated by a variety of hormones. Meanwhile, amino acid is necessary for the development and germination of the embryos. Different amino acids have different effect on embryo development. Generally, cysteine, serine, glutamine and asparagine would improve the development of the embryos (Bridgen ,1994; Emershad *et al*., 1984,1989). Pan (2005) and Wang (2010) believed that adding glycine and proline was benefit for embryo development and seedling establishment. Tian *et al*. (2008) cultured young embryos from the cross Emerald Seedless×Beichun by adding 8 different amino acids into ER medium, and their results showed that adding 2.0mmol/L asparagine, glycine, arginine and glutamine had a better effect on ovule germination than control; while adding 2.0mmol/L phenylalanine, serine, proline and methionine restrained the ovule germination. Here we added 4 kinds of amino acids, among which glutamine showed the best effect. However, the adding of 4 amino acids generated no significant difference. The functions of exogenous hormones on germination rate of 'Venus Seedless' is still to be studied. At present, some rescued seedlings have been transplanted into the field (Fig. 1), and their observation is under way.

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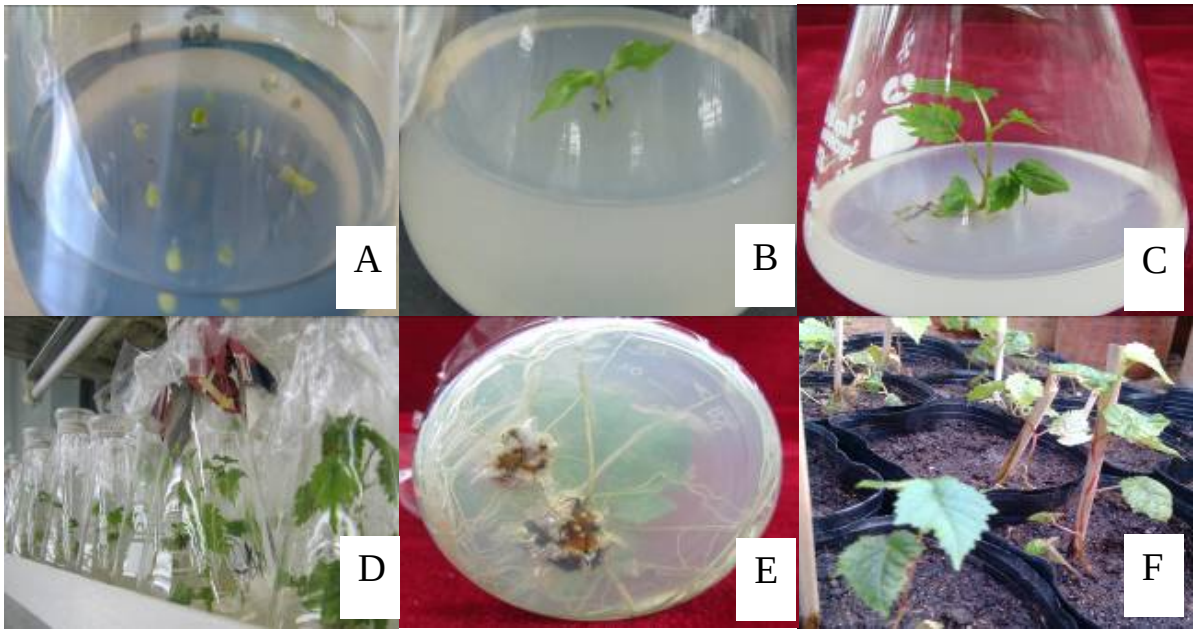

Fig. 1. Embryo rescue and plant regeneration of Venus seedless grape.
Note: A. Ovules cultured; B. Embryo germination; C, D. Seeding survival; E. Rooting; F. Transplanted into the greenhouse

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