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# Embryo rescue of crosses between diploid and tetraploid grape cultivars and production of triploid plants

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**Five cross combinations Jumeigui×Xinghua No.1, 87-1×Kyoho, Kyoho×Muscat Hamburg, Jumeigui× Hongqitezao and Red globle×Kyoho were used as the testing materials. Factors that affect embryo rescue from crossed seeds between diploid and tetraploid grape were studied applying $L_{25}(5^5)$ orthogonal experiment design. The effect of inoculation time, medium type and phytohormone concentration was investigated. The chromosome numbers of the progenies were identified by conventional squash method. The results show that inoculation time was the key factor that affects embryo rescue. Other factors' effects differed according to the combination. The best inoculation time was 55 to 80 days after pollination, which lead to the highest emergence rate of 39.13% to 73.08%. The generation of triploid plants was proved by chromosome number measurement.**

**Key words:** Grape, diploid, tetraploid, embryo rescue, triploid.

## INTRODUCTION

Crossing between cultivars with different ploidy is one of the effective methods to create new germplasms. The advantage lies in its hugeness: vigorous growth, thick cane, thick leaf, large fruit, seedlessness, high yield, adaptability and stress resistance. Most of the triploid fruit trees are highly sterile and fruit-seedless. Compared with the food crop (aiming to get seeds), the fruits' seedlessness can be a fine trait rather than a disadvantage. Because of the vegetative propagation habit, the triploid fine traits could be fixed and steadily inherited. Triploid breeding exploited a new path for seedless grape breeding, we could take full advantage of the highly sterilization and parthenocarpy of the triploid to obtain seedless grape cross between diploid and tetraploid grapes can be a good way to obtain new triploid germplasm. However, there exists a severe mating obstacle in crosses between diploid and tetraploid grape. The embryo rescue technique may prevent the early stage abortion of triploid young embryo, so triploid plants can be produced (Pan et al., 1998; Xu et al., 2005; Yamashita et al., 1993, 1998; Sun et al., 2011). In grape embryo rescue, most of the reports were about the researches in which the seedless or early ripening cultivars were used as a female parent, and the researches of cross between subgenus. There are few works on embryo rescue from interspecific hybridization between diploid and tetraploid grape species. Wakana et al. (2003) and Motosugi et al. (2003) studied the formation and developments of hybrid seeds from cross between diploid and tetraploid, and then obtained triploid progenies through embryo rescue. Yang et al. (2007) and Li et al. (1998) studied the effects of inoculation time and medium on embryo rescue, and also obtained triploid progenies. These previous studies imply that embryo rescue technique is an effective method to obtain the triploid grape plants. However, this technique remains to be

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**Abbreviations: IBA,** Indole-3-butyric acid; **$GA_3$,** gibberellic acid; **BA,** benzyladenine; **DPS,** data processing system; **MS,** Murashige and Skoog; **NN,** Nitsch and Nitsch's; **$B_5$,** Gambor~s.

#These authors contributed equally to this work.

**Table 1.** Cross combinations.

| Combination | Female parent | | Male parent | | Inoculating time |
|---|---|---|---|---|---|
| | Variety | Ploidy | Variety | Ploidy | (days after blossom)/d |
| Jumeigui×XinghuaNo.1 | Jumeigui | 4x | XinghuaNo.1 | 2x | 55、60、65、70、75 |
| Jumeigui×Hongqitezao | Jumeigui | 4x | Hongqitezao | 2x | 55、60、65、70、75 |
| Kyoho×Muscat Hamburg | Kyoho | 4x | Muscat Hamburg | 2x | 60、70、80、90、100 |
| Red globle×Kyoho | Red globle | 2x | Kyoho | 4x | 70、80、90、100、110 |
| 87-1×Kyoho | 87-1 | 2x | Kyoho | 4x | 50、55、60、65、70 |

improved. The low emergence rate is an important problem demanding solution, and the effect factors throughout the rescue are still to be investigated in detail.

In this study, medium type, inoculation time and phytohormone concentration were optimized. The embryo rescue technique from crosses between diploid and tetraploid grape was preliminarily established, so as to increase the emergence rate and apply to further triploid grape breeding.

## MATERIALS AND METHODS

### Plant materials

Plant materials were supplied from the vineyard of Shenyang Agricultural University. There were totally five cross combinations (Table 1). Emasculation was done 3 to 5 days before blooming, and 30-40 clusters were pollinated for each combination.

### Embryo rescue

Trails were carried out in cell engineering laboratory, College of Horticulture, Shenyang Agricultural University from 2004 to 2008. Ovules of different development stages were sampled. After disinfected, ovules were inoculated into the growth medium with 3 bottles for each treatment, and 20-25 ovules for each bottle. The growth medium was mixed with 6% sucrose, 0.6% agar and 0.1% active carbon. 10 weeks later, ovules were transversely cut and the mouth was also cut, then ovules were transferred into the emergence medium (1/2 Murashige and Skoog (MS)+2% sucrose+0.6% agar+0.1% active carbon+benzyladenine (BA) 0.5 mg/L+ indole-3-butyric acid (IBA) 1.5 mg/L+ gibberellic acid ($GA_3$) 0.5 mg/L). Emergence rate was investigated after 2 months. The germinated ovules were transferred into seedling medium within 10 days to induce seedlings. The culture conditions were 25±1°C, 2000lx, 12-14 h lighting per day.

$L_{25}(5^5)$ orthogonal experiment design was applied in ovule growth medium experiment. Factors and levels were as follows. A, medium ① MS② B5③ ER④ modified Nitsch⑤ White; B, inoculation time (Table 1); C, IBA concentration, ①0.5 mg/L②1.0 mg/L③1.5 mg/L④2.0 mg/L⑤2.5 mg/L; D: 6-BA concentration, ①0.1 mg/L②0.3 mg/L③0.5 mg/L④0.7 mg/L⑤0.9 mg/L; E: $GA_3$ concentration, ①0.1 mg/L②0.3 mg/L③0.5 mg/L④0.7 mg/L⑤0.9mg/L. All plant growth regulators were added into media before sterilization.

### Chromosome number measurement rescued progenies

Chromosome number of root tip was counted to confirm the ploidy of the rescued progenies using the con-ventional squash method (Chang et al., 2003). Total of 50-60 root tip cells for every progeny in the metaphase of the mitosis were randomly selected to count the chromosome numbers per cell, and the percentage of specific chromosome numbers for every progeny was determined.

## RESULTS

### Effects of the different factors on ovule emergence rate in the orthogonal experiment

The results of the embryo rescue emergence are shown in Table 2. Data was analyzed by data processing system (DPS) data analyzing system. Germination of ovules in all combinations could be observed. However, variation of the germination rates was observed in the crossed combinations.

In the combination 87-1×Kyoho, the emergence rates varied from 0 to 39.13%. The highest rate was obtained from the treatment $A_5B_2C_1D_3E_4$, which is white medium, inoculation 55 days after blooming, IBA (0.5 mg/L), 6-BA (0.5 mg/L) and $GA_3$ (0.7 mg/L).

In the combination Jumeigui×XinghuaNo.1, the emergence rate was from 0-55.65%. The highest rate came from the treatment $A_2B_5C_1D_2E$, which is $B_5$ medium, inoculation 75 days after blooming, IBA (0.5 mg/L), 6-BA(0.3 mg/L) and $GA_3$ (0.7 mg/L).

In the combination Jumeigui× Hongqitezao, the emergence rate was from 0-71.43%. The highest rate came from the treatment $A_5B_3C_2D_4E_5$, which is white medium, inoculation 65 days after blooming, IBA (1.0 mg/L), 6-BA (0.7 mg/L) and $GA_3$ (0.9 mg/L).

In the combination Kyoho×Muscat Hamburg, the emergence rate was from 0-48.00%. The highest rate came from the treatment $A_5B_3C_2D_4E_5$, which is white medium, inoculation 80 days after blooming, IBA (1.0 mg/L), 6-BA (0.7 mg/L) and $GA_3$ (0.9 mg/L).

In the combination Red globle×Kyoho, the emergence rate was from 0-73.08%. The highest rate came from the treatment $A_5B_1C_5D_2E_3$, which is white medium, inoculation 70 days after blooming, IBA (2.5 mg/L), 6-BA (0.3 mg/L) and $GA_3$ (0.5 mg/L).

The range value of each cross combination is shown in Table 3. A larger range value may significantly influence the results, which means the factor is important. The order of range value was $R_B>R_E>R_A>R_C>R_D$, $R_B>R_D>R_C>R_A>R_E$, $R_B>R_C>R_A>R_E>R_D$, $R_B>R_A>R_D>R_C>R_E$ and $R_C>R_E>R_A>R_D>R_B$ for 87-1×Kyoho, Jumeigui×

**Table 2.** Statistic result of ovule germinating rate in different cross combinations (%).

| Number | Combination | 87-1×Kyoho | Jumeigui×Xinghua No.1 | Jumeigui×Hong qitezao | Kyoho× Muscat Hamburg | Red globle×Kyoho |
|---|---|---|---|---|---|---|
| 1 | $A_1B_1C_1D_1E_1$ | 6.12 | 0.00 | 21.43 | 0.00 | 20.41 |
| 2 | $A_1B_2C_2D_2E_2$ | 31.91 | 22.22 | 16.67 | 0.00 | 30.77 |
| 3 | $A_1B_3C_3D_3E_3$ | 24.00 | 16.13 | 0.00 | 18.18 | 28.13 |
| 4 | $A_1B_4C_4D_4E_4$ | 30.00 | 51.61 | 18.75 | 20.69 | 4.76 |
| 5 | $A_1B_5C_5D_5E_5$ | 8.89 | 30.43 | 8.7.0 | 0.00 | 21.74 |
| 6 | $A_2B_1C_2D_3E_4$ | 32.50 | 0.00 | 9.09 | 6.67 | 39.29 |
| 7 | $A_2B_2C_3D_4E_5$ | 0.00 | 24.44 | 5.56 | 0.00 | 21.31 |
| 8 | $A_2B_3C_4D_5E_1$ | 14.58 | 16.67 | 21.43 | 33.33 | 15.38 |
| 9 | $A_2B_4C_5D_1E_2$ | 6.98 | 35.19 | 0.00 | 35.71 | 19.23 |
| 10 | $A_2B_5C_1D_2E_3$ | 11.63 | 55.56 | 15.79 | 0.00 | 0.00 |
| 11 | $A_3B_1C_3D_4E_5$ | 9.52 | 0.00 | 16.22 | 12.50 | 37.74 |
| 12 | $A_3B_2C_4D_5E_1$ | 23.08 | 19.35 | 31.25 | 0.00 | 23.08 |
| 13 | $A_3B_3C_5D_1E_2$ | 28.57 | 22.73 | 7.69 | 18.18 | 15.38 |
| 14 | $A_3B_4C_1D_2E_3$ | 20.00 | 20.69 | 11.11 | 30.77 | 16.33 |
| 15 | $A_3B_5C_2D_3E_4$ | 4.35 | 37.78 | 13.64 | 0.00 | 8.33 |
| 16 | $A_4B_1C_4D_5E_2$ | 9.30 | 0.00 | 55.88 | 7.14 | 42.86 |
| 17 | $A_4B_2C_5D_1E_3$ | 14.81 | 26.83 | 11.11 | 38.46 | 50.00 |
| 18 | $A_4B_3C_1D_2E_4$ | 22.92 | 40.13 | 15.38 | 0.00 | 0.00 |
| 19 | $A_4B_4C_2D_3E_5$ | 4.44 | 30.77 | 15.79 | 35.29 | 12.24 |
| 20 | $A_4B_5C_3D_4E_1$ | 0.00 | 11.90 | 8.70 | 0.00 | 6.67 |
| 21 | $A_5B_1C_5D_2E_3$ | 6.98 | 0.00 | 13.16 | 15.38 | 73.08 |
| 22 | $A_5B_2C_1D_3E_4$ | 39.13 | 36.36 | 12.00 | 10.53 | 25.00 |
| 23 | $A_5B_3C_2D_4E_5$ | 18.37 | 25.00 | 71.43 | 48.00 | 29.41 |
| 24 | $A_5B_4C_3D_5E_1$ | 17.50 | 35.90 | 20.00 | 33.33 | 22.00 |
| 25 | $A_5B_5C_4D_1E_2$ | 10.29 | 12.50 | 19.23 | 10.00 | 3.57 |
| Average | | 15.83 | 22.88 | 17.60 | 14.96 | 22.66 |

For each cross combination, there were some treatments leading to higher emergence rates. The highest rate of the five cross combinations ranged from 39.13% to 73.08%, with an average emergence rate of 15.83%、22.88%、17.60%、14.96%、22.66%, respectively. Theoretically, the best treatment for each cross combination was $A_1B_2C_1D_3E_4$、$A_2B_4C_1D_4E_4$、$A_5B_3C_4D_1E_5$、$A_5B_4C_5D_3E_1$、$A_5B_1C_5D_3E_3$, respectively. However none of them appeared in the trail results. So the emergence rate of rescued ovules may be improved based on the results we got.

**Table 3**. R volume under analysis of ovule germinating rate in orthogonal test.

| Combination | 87-1× Kyoho | Jumeigui× XinghuaNo.1 | Jumeigui× Hongqitezao | Kyoho× Muscat Hamburg | Red globle× Kyoho |
|---|---|---|---|---|---|
| A | 9.89 | 6.26 | 16.79 | 15.67 | 11.57 |
| B | 14.75 | 34.83 | 10.05 | 29.15 | 34.61 |
| C | 9.75 | 12.87 | 21.17 | 13.28 | 23.53 |
| D | 9.41 | 15.56 | 16.45 | 13.60 | 7.70 |
| E | 14.72 | 4.408 | 19.29 | 9.81 | 9.08 |

XinghuaNo.1, Jumeigui× Hongqitezao, Kyoho×Muscat Hamburg and Red globle×Kyoho, respectively. It was indicated that the most important factor was inoculation time for most cross combinations. Hybrid embryo from cross between diploid and tetraploid would be aborted during the growing process, so inoculation time is very important. Other influencing factors, such as medium and hormone concentration, had different effects on embryo rescue due to the cultivar difference, which means that the sensibility of different genotypes to nutrient element

**Table 4.** Variation of ploidy in crossing progenies of diploid and tetraploid.

| Combination | Diploid (%) | Triploid (%) | Tetraploid (%) |
|---|---|---|---|
| Red globo (2x)×Kyoho (4x) | 39 (69.64) | 16 (28.57) | 1 (1.79) |
| 87-1 (2x)×Kyoho (4x) | 25 (83.33) | 4 (13.33) | 1 (3.33) |
| Jumeigui (4x)×Hongqitezao (2x) | 1 (25.00) | 2 (50.00) | 1 (25.00) |
| Jumeigui (4x)×XinghuaNo.1 (2x) | 9 (20.00) | 35 (77.78) | 1 (2.22) |
| Kyoho (4x)×Muscat Hamburg (2x) | 0 (0.00) | 3 (100) | 0 (0.00) |
| Total | 74 (53.62) | 60 (43.48) | 4 (2.90) |

and hormone is different.

### Chromosome numbers of progenies from different cross combinations

Chromosome numbers were measured in 138 randomly sampled seedlings. Diploid, triploid and tetraploid cells were recognized. There were 74 diploid, 60 triploid and 4 tetraploid cells, accounting for 53.62, 43.48 and 2.90%, of the sampled seedlings, respectively. In the combinations whose female parent was diploid, 64 (74.42%) out of 86 identified seedlings were diploid, 20 (23.26%) triploid and 2 (2.32%) tetraploid. In the combinations whose female parent was tetraploid and male parent was diploid, 10 (19.32%) out of 52 identified seedlings were diploid, 40 (76.92) triploid and 2 (3.85%) tetraploid (Figure 1; Table 4).

## DISCUSSION

### Effects of inoculation time and medium on embryo rescue

Embryo rescue from cross between diploid and tetraploid grape is affected by many factors. Hybrid embryo would abort during its growing process (Xu et al., 2005; Yamashita et al., 1993), so the proper inoculation time must be the key factor that affect embryo rescue. Previous studies had showed different results on the choice of best inoculation time. Pan et al. (1998) found that 35 to 50 days after blooming was the proper embryo inoculation time for cross between diploid seedless grape (female) and tetraploid grape. Yang et al. (2007) also found that 35 to 45 days after blooming was the best inoculation time. Zhao et al. (2005) believed the highest emergence rate would reveal when inoculation happened before the fruits were nearly to be ripe. Xu et al. (2005) reported that embryo rescue sampling time could be determined by the mature stage of the female parent, being 6-9 weeks after pollination for early maturing cultivars, 7-10 weeks after pollination for medium maturing cultivars and 9-12 weeks after pollination for late maturing cultivars. Based on the study using five different cross combinations, it was suggested that the inoculation time is determined mostly dependent on the female parent. The proper inoculation time was 65-80d after pollination for most cultivars, but about 55 days after pollination for extreme early maturing cultivars as a female parent. Excessive early inoculation time will lead to an imperfect development of the embryo, while excessive late inoculation time will resulted in the abortion of hybrid embryo or physiological dormancy.

Proper medium can provide suitable nutrient for young embryo. It's also an important factor of embryo rescue. In embryo rescue from cross between diploid and tetraploid grape, MS, Gambor~s ($B_5$), Nitsch and Nitsch's (NN) and 1/2MS medium has been used in previous studies (Motosugi et al., 2003; Pan et al., 1998; Xu et al., 2005). Yan et al. (2004) compared MS, $B_5$ and NN medium, and found MS the best. Yang et al. (2007) also believed NN medium was the best. Based on this study, we consider that the proper medium for embryo rescue from cross between diploid and tetraploid grape was White medium with a low mineral salt concentration. The widely used NN medium in seedless grape embryo rescue was not suitable for this study. It is found in research that relatively higher phytohormone concentration is beneficial to the development of young embryo. The cultured ovules were *in vitro*, so the young embryo can only continue growing under high concentrations of exogenous plant growth regulators. We carried out 3-step embryo rescue to finish embryo development, germination and into seedling process, and we also investigated inoculation time for different cultivars, medium type and phytohormone concentration. In further study, other growth regulators and nutrients are to be researched. What's more, the effect of growth regulators' interaction on ovule development is to be researched.

### Nuclear ploidy in rescued progenies

The ultimate goal of cross between diploid and tetraploid grape is the obtainment of triploid, so as to open up a new way of seedless grape breeding. Many previous works had obtained triploid progenies, but the ratio of progenies with different ploidy and the difference of cross combination were seldom mentioned. We determined the root tip chromosome number in the hybrid progenies, which proved that the triploid germplasms had been obtained. It was also found that a higher average per-

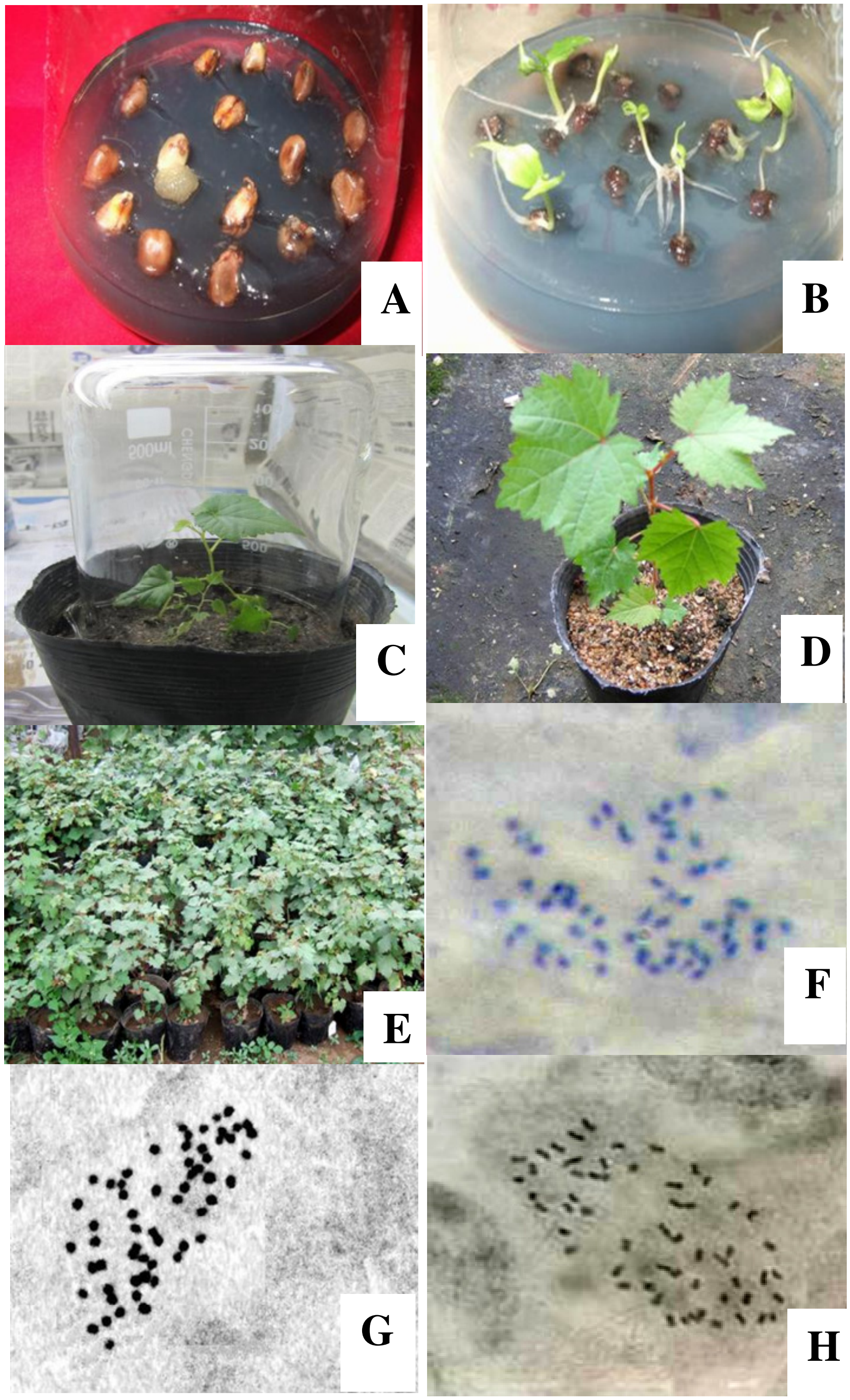


**Figure 1.** A. Embryo development; B. Embryo germination; C,D,E. Plants transplanted successfully; F, G, H. Chromosome number of triploid root tip (2n = 3x = 57)

centage of triploid progenies had been obtained from the cross combination in which the female parent was tetraploid, compared with the cross combination in which the female parent was diploid. After acclimation, the rescued seedlings have been transplanted into the field. Observation in the laboratory and from the field showed that most of the triploid plants had dark green leaves and vigorous growth habit.

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