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Full Length Research Paper

In vitro embryo rescue culture of F1 progenies from crosses between tetraploid grape and *Vitis amurensis* Rupr.

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Using three tetraploid table grape varieties as the female parents and *Vitis amurensis* Rupr. as the male parent, the effects of inoculation time and medium state on embryo rescue were studied. The results showed that inoculation time had a great effect on ovule germination percentage. 60 to 70 days after pollination was the best inoculation time, which resulted in the highest ovule germination percentage. Among solid medium, liquid medium, semi-solid medium and liquid over solid double layer medium, the last medium had the highest germination percentage, and then the solid medium. Liquid and semi-solid media were not good.

Key words: Grapes, embryo rescue, inoculation time, medium.

INTRODUCTION

As an important method to obtain new grape cultivar with large berries, polyploidy breeding has been holding the interest of breeders for many years. The triploid grape has a commercial importance because of its seed-lessness, so triploid grape breeding breaks a new path to obtaining big berry and seedless grape cultivars. There exists a crossing barrier (ovule abortion at the early stage) between diploid and tetraploid grapes, so embryo rescue must be carried out to prevent the abortion, and finally to form a triploid plant (Yamashita et al., 1993; Xu, 1997). Utilization of embryo rescue technique on a cross between tetraploid and diploid table grape (*Vitis vinifera* L.) has already been reported, and triploid progeny had also been obtained (Yamashita et al., 1998; Pan et al., 1998; Wakana et al., 2003; Xu et al., 2005; Jiang et al., 2007; Yang et al., 2007; Sun et al., 2011). Embryo rescue technique was initially used in seedless grape breeding (Spiegel-Roy et al., 1985; Emershad et al., 1989; Ramming, 1990; Valdez and Ulanovsky, 1997), and then this technique had been modified gradually. However, there are few reports on embryo rescue technique based on a cross between tetraploid and diploid grape. Influencing factors, including inoculation time, medium and plant growth regulators, are still to be researched in detail.

Vitis amurensis Rupr. is the most hardy species among Vitis., and it is a fine parent of cold hardiness breeding for grape. In this study, we used tetraploid grape cultivars with high fruit quality crossed with diploid *V. amurensis* Rupr.. We used embryo rescue technique to prevent the abortion of hybrid embryo. The effects of inoculation time and medium state on embryo rescue were all studied. We look forward to obtaining new triploid grape germplasm that was cold hardy, and to laying the foundation for further breeding on large berry, seedlessness and cold hardiness.

MATERIALS AND METHODS

Plant materials

Materials were collected from the vineyard of Shenyang Agriculture University. Taking Shuangyou (*V. amurensis* Rupr.) as the male parent, three cross combinations of diploid and tetraploid were carried out, including Xiangyue (4x) × Shuangyou (2x), Kyoho (4x) × Shuangyou (2x) and Zuijinxiang (4x) × Shuangyou (2x). Emasculation was done 3 to 5 days before anthesis. The stigma was cleaned by water and then bagged after being slightly dried. Staminate flowers were picked during initial bloom stage and anthers were taken out and desiccated. Pollens were collected in

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Table 1. Effect of different inoculating dates on the germination rate of ovule.

Day after pollination	Xiangyue ×Shuangyou			Kyoho×Shuangyou			Zuijinxiang×Shuangyou		
	Ovule number	Germination number	Germination rate (%)	Ovule number	Germination number	Germination rate (%)	Ovule number	Germination number	Germination rate (%)
50	59	8	13.6c	73	6	8.2c	72	12	16.7c
60	52	13	25.0b	84	17	20.2b	81	20	24.7a
70	62	21	33.9a	68	29	42.7a	72	17	23.6a
80	61	16	26.2b	86	39	45.4a	81	17	21.0b

Different letters indicate significance at the 0.05 level.

clean bottles after the crack of anther, and were stored at 4°C. During the whole flowering phase, pollination was done twice when mucilage appeared on the stigma.

Embryo rescue

Crossbreeding fruits were picked some certain days after pollination. Firstly, the fruits were washed with running water, then rinsed with 75% alcohol for 1 to 2 min and disinfected with 0.1% mercury for 10 to 20 min. Finally, the fruits were washed with sterilized distilled water for 5 times. Ovules were taken under sterile condition. Four or five bottles for each treatment. Fifteen or twenty ovules for each bottle. At first, ovules grew in growth culture medium. After ten weeks, ovules were cut transversely and then transferred to germination culture medium. Germination rates of ovule were calculated after three months. The seedlings from germinated ovules were transferred to seedling culture medium within ten days. The growth culture medium was 1/2MS + IBA 2.5 $mg \cdot L^{-1}$ + BA 0.5 $mg \cdot L^{-1}$ + GA_3 0.5 $mg \cdot L^{-1}$, supplemented with 0.1% active carbon, 0.6% agar and 6% sucrose. The germination culture medium was ½ MS + IBA 1.5 $mg \cdot L^{-1}$ + BA 0.5 $mg \cdot L^{-1}$ + GA_3 0.5 $mg \cdot L^{-1}$, supplemented with 0.1% active carbon, 0.6% agar and 2% sucrose. The seedling culture medium was B5 + BA 0.05 $mg \cdot L^{-1}$ + IBA 0.2 $mg \cdot L^{-1}$, supplemented with 0.1% active carbon, 0.6% agar and 2% sucrose. The culture conditions were 25±1°C, 2000Lx and 12 h light daily.

Effects of inoculation time on embryo rescue: ovules of each combination were taken 50, 60, 70 and 80 days after pollination, and then inoculated on solid growth culture medium. Ovule germination percentages of different treatments were compared. Effects of medium state on embryo rescue: in growth culture medium, the concentration of agar was 0 $g \cdot L^{-1}$ (liquid medium), 2 $g \cdot L^{-1}$ (semisolid medium) and 6 $g \cdot L^{-1}$ (solid medium), along with the liquid over solid culture as the fourth type (mean that: a layer of liquid medium of 3 to 4 mm thick added on the surface of the solid medium). Inoculation was done 63 days after pollination for Xiangyue (4x) × Shuangyou (2x), 65 days after pollination for Kyoho (4x) × Shuangyou (2x) and 65 days after pollination for Zuijinxiang (4x) × Shuangyou (2x). Ovule germination percentages of different medium types were compared.

RESULTS AND DISCUSSION

Table 1 showed the ovule germination percentages of three combinations on different inoculation time. It can be seen from the table that, the germination rates of the three combinations increased over time. An early inoculation led to the incomplete growth of ovule, which resulted in a low germination percentage. In Xiangyue × Shuangyou, the highest germination percentage was inoculation which happened 70 days after pollination. While when inoculation happened 80 days after pollination, the ovules aborted and the germination rate dropped. In Kyoho × Shuangyou, the highest germination percentage appeared when inoculation was done 80 days after pollination, but was not significantly different from that of 70 days after pollination. The proper inoculation time of Zuijinxiang × Shuangyou was 60 days after pollination, which created the highest germination rate.

Table 2 showed the effect of different medium state on embryo rescue. Higher ovule germination percentages were gained from solid culture medium and liquid over solid double layer medium, and the latter one is better. The highest germination percentages of Zuijinxiang × Shuangyou, Kyoho × Shuangyou and Xiangyue × Shuangyou reached up to 36.3, 37.7 and 26.6% respectively (Figure1). However, in liquid and semi-solid culture medium, the germination percentages were low, and it might have had bad aeration condition which prevented the growth of ovules. Referring to the inoculation time of embryo rescue on cross between diploid and tetraploid grape, Pan et al.(1998) reported that embryo rescue seedling was obtained when the inoculation took place 35 to 55 days after pollination. Xu et al. (2005) believed that the inoculation time was relative to the maturation stage of the female parent, meaning 6 to 9 weeks, 7 to 10 weeks and 9 to 12 weeks after pollination for early ripening, middle ripening and late ripening cultivars respectively. Guo et al. (2006) reported that 70 days after pollination was the proper inoculation time for late ripening cultivars.

Table 2. Effect of different medium on the germination rate of ovule.

Medium	Xiangyue ×Shuangyou			Kyoho×Shuangyou			Zuijinxiang×Shuangyou		
	Ovule number	Germination number	Germination rate (%)	Ovule number	Germination number	Germination rate (%)	Ovule number	Germination number	Germination rate (%)
Solid medium	79	25	31.7^{b}	85	31	36.5^{a}	78	19	24.4^{a}
Liquid medium	68	4	5.9^{d}	63	2	3.2^{c}	82	3	3.7^{c}
Semisolid medium	55	8	14.5^{c}	63	14	22.2^{b}	79	13	16.5^{b}
Liquid over solid culture	80	29	36.3^{a}	77	29	37.7^{a}	64	17	26.6^{a}

Different letters indicate significance at the 0.05 level.

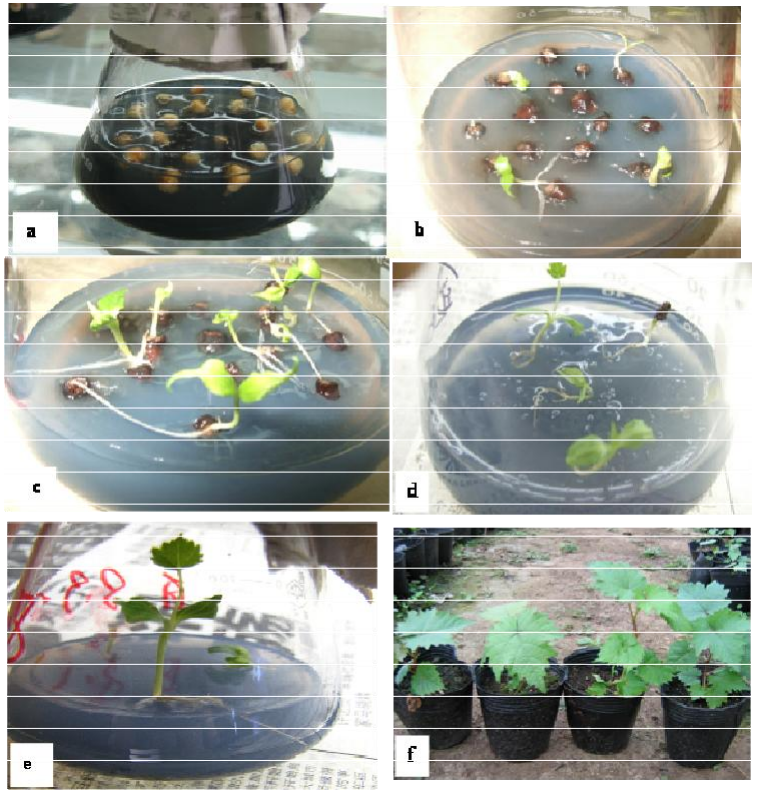

Figure 1. (a) Excised ovules; (b,c) germinated embryos; (d,e) plantlet with true leaves; (f) plants in acclimatization.

Yan et al. (2008) had a diploid female parent crossed with tetraploid, finding that the proper inoculation time was 40 to 50 days, 50 to 60 days and 60 to 80 days after pollination for early, middle and late ripening cultivars respectively.

In this study, we had three middle ripening tetraploid cultivars as the female parents crossed with diploid *V. amurensis* Rupr., the proper inoculation time was 60 to 70 days after pollination. We found that sampling too early could lead to defective developed ovules and a low germination percentage. As the time increased, the ovule germination percentage went up, but later some of the ovules inside the berries began to abort. So sampling too late could lead to a low number of well developed seeds. In medium research, previous studies mainly focused on the effect of medium type on the result of embryo rescue (Yang et al., 2007; Guo et al., 2006; Yan et al., 2008), while there were few reports about the effect of medium state on embryo rescue. Solid medium was the main medium used in previous studies (Yamashita et al., 1998; Pan et al., 1998; Wakana et al., 2003; Xu et al., 2005; Yang et al., 2007). we here compared the effect of solid medium, semi-solid medium, liquid medium and liquid over solid double layer medium on seed germination, and discovered that solid and double layer medium were better than liquid and semi-solid medium. The liquid over solid double layer medium produced the highest germination percentage. Because of the poor aeration condition of liquid and semi-solid medium, the ovules could not grow normally, and the germination percentage was low. The liquid over solid double layer medium was good, because it was beneficial to both nutrition absorption and aeration.

Conclusion

Hybrid ovules from cross Xiangyue× Shuangyou, Kyoho× Shuangyou and Zuijinxiang × Shuangyou were studied. The optimal inoculation time was 60-70 days after pollination, and the germination rates reached to 36.25, 37.65 and 26.56%, respectively. Among the four medium types, liquid over solid medium generated the highest germination rate.

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