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Seedless grape breeding for disease resistance by using embryo rescue

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Summary

An efficient system of seedless grape breeding for disease resistance through embryo rescue was developed by using an interspecific hybrid 'Beichun' of *V. vinifera* × *V. amurensis* as the pollen donor. Genotype and medium were confirmed to play important roles in this system, when a combined culture phase of solid plus liquid was used. 'Emerald Seedless' showed the highest percentage of plant development (19.6 %) in Emershad and Ramming (1994) medium (ER) among the females, suggesting it is more sensitive to ovule culture. To further improve the breeding efficiency, different amino acids were tested by using ovules from 'Emerald Seedless' × 'Beichun'. The addition of asparagine, glycine, arginine and glutamine (2.0 mmol·l^{-1} respectively) yielded a higher plant development rate than the basal medium. The best result was obtained from asparagine supplemented medium, with 55.0 % ovules generated plants. The field performance to downy mildew [*Plasmopara viticola* (Berk. and Curtis) Berl. and de Toni] and anthracnose [*Elsinoë ampelina* (de Bary) Shear] of the parents and progenies was also evaluated. Disease resistance in F_1 generation demonstrates continuous variation, with some resistant progenies, accounted for 5.7 % from offsprings, beyond the range observed in the parents. No correlation was observed between the resistance to the two pathogens in this research.

K e y w o r d s : grapevine breeding, disease resistance, embryo rescue, germplasm, seedless grape.

Introduction

Seedless grapes were classified into two types, stenospermic and parthenocarpic. In the first type, fertilization occurred but embryos aborted during their earlier development (Stout 1936). Many stenospermic cultivars have large bunches and good fruit qualities, preferred for table grapes or raisin production around the world. In recent years, the demand for diversity of stenospermic varieties is increasing, promoting the breeding programs in this crop. Earlier in 1982, Ramming and Emershad (1982) first reported stenospermic grapes could generate plants *via* ovule culture. It enhanced the application of this *in vitro* method, embryo rescue, in seedless grape breeding. Presently, the conventional strategies adopting seeded cultivars as female parents have been replaced by using stenospermic grapes as females directly. Compared to the old method, a higher seedless proportion can be obtained and fewer generations are needed with the new strategies (Ramming *et al.* 1990). Hence, it has become a routine approach for new cultivars in seedless grapes, comprised by crossing, ovule culture, embryo excise and plant formation etc. (Burger *et al.* 2003, Kebeli *et al.* 2003).

Most European seedless cultivars (*Vitis vinifera*) are highly susceptible to fungi diseases which is a worldwide problem for all breeders. Each year, expensive chemical sprayings for disease control can seriously contaminate the environment. Therefore, breeding for new resistant seedless cultivars has been a common purpose around the world. Moreover, the tool of embryo rescue seems to be much reliable, without any potential dangers to human health and the environment. It is reported that Goldy *et al.* (1988) attempted to introgress disease resistance of *Vitis rotundifolia* as pollen parent into seedless *Vitis vinifera* as female parent through field cross and embryo rescue and that Ramming *et al.* (2000) investigated and demonstrated embryo rescue. In this case, a stenospermic hybrid 'C41-5' of *V. vinifera* × *V. rotundifolia* was obtained from Goldy *et al.*'s cross combinations and embryo rescue. Therefore, it is very difficult to obtain hybrids between *V. vinifera* × *V. rotundifolia.*

The reason is chromosome inconsistence between the two subgenera (*V. vinifera*, 2n = 38; *V. rotundifolia*, 2n = 40) and often leads to cross incompatibility. China is one of the origin centers of *Vitis* species, with many disease resistant resources (He *et al.* 1991). But most of wild species and clones often have small clusters and berries, low sugar and high acidity as fruit quality characteristics, which are more easily transferred when serving as females (He *et al.* 1981, Luo and He 1999). Among the Chinese wild *Vitis* germplasms, *Vitis amurensis* was earlier evaluated for its disease and cold resistance (He *et al.* 1981, Li *et al.* 1983, Song *et al.* 2005). Through interspecific crossing, a later-maturing hybrid of 'Beichun', derived from 'Muscat Hamburg' (*V. vinifera*) × 'Heilongjiang' (*V. amurensis*), was released by Peking Botanical Garden in China (Li *et al.* 1983). Compared to its male parent, many economic traits were largely enhanced in this F_1 hybrid. The most valuable, 'Beichun' generally exhibits significantly higher level of resistance to both downy mildew and anthracnose than its wild parent (Li *et al.* 1983, Liu *et al.* 2002, 2004), apart from good resistance to chilling, moisture and other environmental stresses (Li *et al.* 1983; Wang 1989). These characteristics resulted in wide adaptability to various culture

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conditions in the north, south and center of China during the 1980s (WANG 1989). On the other hand, the fruit quality of 'Beichun' is not as good as some of the mainly cultivated varieties (LI *et al.* 1983). This limits the direct utilization of 'Beichun' as female in breeding for table grapes because of its small clusters and berries, low sugar and also big seed set. So we selected 'Beichun' as male pollen and several better-quality stenospermic grapes as female parents, in order to generate novel disease resistant seedless germplasm combined with improved fruit quality. In this program, the technique of embryo rescue was employed as a tool.

In this field, genotype and medium have been found playing crucial roles for the success of this technique (GOLDY and AMBORN 1987, EMERSHAD *et al.* 1989, GRIBAUDO *et al.* 1993, PONCE *et al.* 2000). Based on the earlier findings, the objective of this research was to develop an efficient system for disease resistance in seedless grape breeding. Specific objectives were to screen suitable genotypes for embryo rescue, to improve the efficiency by medium adaptation, and to identify the hybrid responses to fungi diseases subsequently.

Materials and Methods

Genotype screening: To determine the optimum genotype for embryo rescue breeding, 8 stenospermic cultivars served as females for crossing with 'Beichun' (Tab. 1). Plant materials are all grown in the nursery of grape germplasm of Northwest A & F University, located in Yangling, Shaanxi province of China. Crossing was made during May of 2004-2005. Hybrid fruits were harvested 3 d before véraison, surface sterilized in 70 % ethanol for 1 min, followed by 0.1 % $HgCl_2$ for 8 min, and rinsed 3 times with sterilized water before dissecting aseptically. Ovules were cultured in Erlenmeyer flasks containing EMERSHAD and RAMMING (1994) medium (ER). After 2 months, embryos were excised from the ovules under a stereomicroscope. The recovered embryos were recorded and then removed to woody plant medium (LLOYD and MCCOWN 1981) supplemented with 1.0 μM 6-benzyladenine and 0.15 % activated charcoal to germinate (Figure, b). Within 3 months, the plants developed from germinated embryos were recorded and propagated, using shoot cuttings cultured in MURASHIGE and SKOOG (1962) medium (MS) containing 0.5 μM indole-3-butyric acid. After 30-40 d, the well-rooted clones were transplanted into pots containing a mixture (perlite: peat: soil = 3:1:1 v/v) after rinsing the adhering medium. These pots were covered with plastic cups to maintain initial humidity (Figure, d), and then the cups were uncovered gradually for plant hardening. The hardened plants were moved to a green house

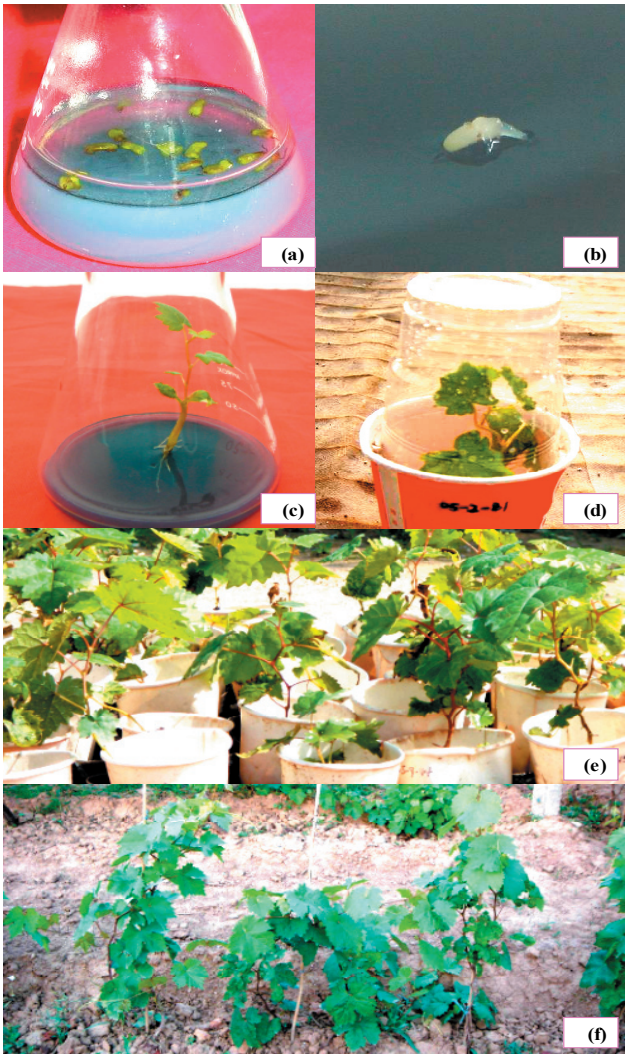

Figure: (**a**) Ovules cultured in solid plus liquid medium; (**b**) an excised embryo from ovules; (**c**) a plantlet with true leaves; (**d**) a transplanted clone under plastic cover; (**e**) plants in acclimatization; (**f**) progenies growing in field.

Table 1

Genotypes used in this study

Genotypes	Type of seeds	Species	Disease resistance	
			Downy mildew	Anthracnose
Emerald Seedless	Stenospermic	*Vitis vinifera*	Susceptible	Susceptible
Delight	Stenospermic	*Vitis vinifera*	Susceptible	Susceptible
Flame Seedless	Stenospermic	*Vitis vinifera*	Susceptible	Susceptible
Blush Seedless	Stenospermic	*Vitis vinifera*	Susceptible	Resistant/tolerant
Monukka	Stenospermic	*Vitis vinifera*	Susceptible	Resistant/tolerant
Thompson Seedless	Stenospermic	*Vitis vinifera*	Susceptible	Susceptible
Kishmish khishrau	Stenospermic	*Vitis vinifera*	Susceptible	Resistant/tolerant
Youngle	Stenospermic	*Vitis vinifera*	Susceptible	Susceptible
Beichun	Seeded	*V. vinifera* × *V. amurensis* hybrid	Resistant	Resistant

with natural daylight for acclimatization (Figure, e). After growing for 3-4 months, the surviving progenies were planted in the field in spring (Figure, f).

Medium adaptation: To enhance the breeding efficiency the addition of 8 amino acids were determined for ovules from 'Emerald Seedless' × 'Beichun'. The basal medium was a modified ER-medium with macro-nutrients as follows: $Ca(NO_3)_2 \cdot 4H_2O$, 235 mg·l^{-1}; KNO_3 660 mg·l^{-1}; KCl, 75 mg·l^{-1}; NH_4NO_3, 300 mg·l^{-1}; $MgSO_4 \cdot 7H_2O$, 1250 mg·l^{-1}; $NaH_2PO_4 \cdot 2H_2O$, 760 mg·l^{-1} (according to patent, 200610043024.0, P. R. China). Other components were all in accordance with ER (Emershad and Ramming 1994). Amino acids used included asparagine, glycine, arginine, glutamine, phenylalanine, serine, proline and methionine, in concentration of 2.0 mmol·l^{-1} supplemented to the basal medium, respectively.

Culture methodology and conditions: To improve the growth conditions, we used a solid plus liquid method for ovule culture in this study. In each medium, the nutrient components of the two layers were the same, except 0.6 % agar added into the solid phase. The two phases were made and autoclaved separately, and combined together under aseptic conditions. Each flask (50 ml) contained 10-15 ovules, half bathed in the liquid (Figure, a). All the materials were placed in a culture room at 25 ± 1 °C, maintaining 50 μmol·m^{-2}·s^{-1} illumination with a 14 h photoperiod. For each culture, medium was renewed monthly.

Disease resistance evaluation: To identify the responses to downy mildew and anthracnose, 35 accessions were randomly selected from progenies of 'Emerald Seedless' × 'Beichun', omitting any fungicide protections in the field. Each accession concludes 3 plants (replications) from the same shoot. The investigation was carried out during July 2006, when the field pathogens were fully epidemic. Under the same conditions, the disease resistance of both parents was evaluated as well. For each disease, 100 leaves were observed based on the necrotic lesions per plant. Each leaf was graded according to the standard displayed in Tab. 2 (Wang *et al.* 1995, 1998). The severity index of different clones was calculated as follows: severity index = Σ (number of leaves at each grade × grade number)/(number of total leaves × highest grade number). In this experiment, the disease resistance of each accession was measured by the severity index (averaged by the 3 plants evaluated) respectively.

Table 2

Standard of disease resistance evaluation in this study

Grades	Percentage of the lesions over the foliar area
0	0
1	0.1 ~ 5.0
2	5.1 ~ 15.0
3	15.1 ~ 30.0
4	30.1 ~ 45.0
5	45.1 ~ 65.0
6	65.1 ~ 85.0
7	85.1 ~ 100.0

Experimental design and data analysis: Three separate experiments were arranged in this research. For experiment 1, the factors were 8 different female genotypes to screen. For experiment 2, supplements of 8 different amino acids were factors to compare. And for the last experiment, to evaluate disease resistance in the progenies was the objective. All experiments were one factorial designed and repeated 3 times. Statistical analysis was conducted using SAS computing package (Cary N.C.). Data of percentage were subjected to arc sine transformation before comparison by Duncan's Multiple Range Test.

Results

Genotype: The responses of different females to embryo rescue are displayed in Tab. 3. Among the 8 genotypes, the production of hybrid plants was significantly different. 'Emerald Seedless' had the best plant development rate (19.6 %), followed by 'Delight' (13.3 %). This was significantly higher than that from other cultivars such as 'Flame Seedless', 'Blush Seedless', 'Monukka', 'Thompson Seedless', 'Kishmish khishrau' and 'Youngle', with percentages of plant development from 1.1 % to 6.6 % only. It is clear that 'Youngle' produced the poorest plants in this experiment, with only 1.8 % embryos recovered from ovules.

Media: The effects of different amino acids on embryo rescue were studied, and the results are presented in Tab. 4. In this experiment, the addition of asparagine, glycine, arginine and glutamine led to percentage of plant

Table 3

Responses of different female genotypes to embryo rescue

Female genotypes × Beichun	No. of ovules cultured	No. of embryos excised	No. of plants developed	Embryo recovery rate (%)	Plant development rate (%)
Emerald Seedless	352	94	69	26.7 a	19.6 a
Delight	600	116	80	19.3 b	13.3 b
Flame Seedless	180	30	12	16.5 b	6.6 c
Blush Seedless	630	43	33	6.8 d	5.2 c
Monukka	165	15	8	9.1 c	4.9 cd
Thompson Seedless	200	11	6	5.5 d	2.9 de
Kishmish khishrau	480	16	13	3.3 e	2.5 e
Youngle	175	3	2	1.8 f	1.1 f

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$.

development rate of 55.0 %, 45.0 %, 37.3 % and 35.8 % respectively, significantly higher than that of the basal medium (30.8 %). And the best result was obtained from the medium supplemented with asparagine, with the highest embryo recovery rate of 84.2 %. In contrast, some other amino acids such as serine, proline and methionine showed negative effects on the progeny production, with plant development rate of 26.7 %, 23.3 % and 20.8 % respectively. The lowest plant development rate was obtained from ovules cultured in methionine supplemented medium, although the embryo recovery rate of 38.1 % obtained. It also showed that phenylalanine supplemented medium produced 28.3 % plants from ovules, not significantly different to the basal medium.

Disease resistance: It seemed that the severity index of the diseases in the F_1 generation demonstrated continuous variation (Tab. 5). Within the parents, 'Emerald Seedless' was more susceptible to the pathogens, with severity index of 40.09 % and 30.29 % in downy mildew and anthracnose, respectively, much higher than that of 'Beichun' (12.46 % and 11.46 %, respectively). Two accessions with the average severity indexes of 9.1 % and 8.16 % demonstrated the higher resistance to both downy mildew and anthracnose than male parent. Eight accessions with the average severity index of 50.48 % and 2 accessions with the average severity index of 34.31 % showed higher susceptibility than female parent to downy mildew and anthracnose, respectively. The severity index of 25 accessions to downy mildew averaged 28.09 %, that of 31 accessions to anthracnose averaged 19.43 %. which indicated the resistance of intermediate types to the diseases between male and female parents. It suggested that accessions with low severity index can be used in the future selection. Based on the correlation analysis ($r = -0.02298$; $df = 34$), no significance was determined between the resistance to the two diseases.

Discussion

From this study, breeding efficiency influenced by genotype of the female was clearly demonstrated (experiment 1). The highest plant production obtained from 'Emerald Seedless', indicating this cultivar being more suitable for embryo rescue. This superiority could be controlled by the specific genotype, like some other cultivars in earlier findings (GOLDY and AMBORN 1987, PONCE *et al.* 2000). Among the medium supplements, asparagine gave the best result, followed by glycine, arginine and glutamine (experiment 2). The beneficial effects of these amino acids could be explained by their preferential utilization during embryo development. In stenospermic grapes, the exact reason for embryo abortion is unclear, although *in vitro* culture has been applied in breeding. A fact that the breakdown of endosperm prior to embryos was observed *in vivo*, inferring that a nutrient deficiency occurred during embryo development (STOUT 1936, RAGHAVAN 1966). Nutrient analysis revealed high levels of amino acids in the endosperm tissue (SMITH 1973). This enhanced the application of these compounds in the medium (EMERSHAD

Table 4

Responses of the ovules from Emerald Seedless×Beichun to different amino acids

Medium supplents	No. of ovules cultured	No. of embryos excised	No. of plants developed	Embryo recovery rate (%)	Plant development rate (%)
Asparagine	120	101	66	84.2 a	55.0 a
Glycine	120	82	54	68.3 b	45.0 b
Arginine	150	70	56	46.7 cd	37.3 c
Glutamine	100	49	36	49.2 c	35.8 c
Phenylalanine	120	56	34	46.7 cd	28.3 de
Serine	150	78	40	52.0 c	26.7 ef
Proline	120	44	28	36.7 e	23.3 fg
Methionine	100	38	21	38.1 de	20.8 g
Basal medium	120	47	37	39.2 de	30.8 d

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$.

Table 5

The severity index (%) of the disease resistance of the accessions evaluated

Disease	Emerald Seedless (♀)	Beichun (♂)	Mid-value of the parents	F_1 average	F_1 distribution			Correlation coefficient
					Higher than female	Between female and male	Lower than male	
Downy mildew	40.09	12.46	26.28	32.12 (35)	50.48 (8)	28.09 (25)	9.1 (2)	-0.02298^{NS}
Anthracnose	30.29	11.46	20.88	19.63 (35)	34.31 (2)	19.43 (31)	8.16 (2)	

^{NS} shows no significance determined between the two diseases at $p \leq 0.05$.
Figures in the parantheses indicate the total number of accessions.

and Ramming 1984, Emershad *et al.* 1989). Emershad *et al.* (1989) also stated ovules response to amino acids differently, supporting our results. In this program, embryo rescue has proved to be an efficient tool for obtaining progenies successfully (Figure, f). Furthermore, the resistance to downy mildew and anthracnose of the F_1 generation shows continuous variation, in correspondence with the theory of quantitative traits (experiment 3). The observed progenies (5.7 %) with lower severity index than 'Beichun' showed better resistance as well, inferring their potential value in the future. In addition, no correlation was found between the diseases, implying the resistance to the two pathogens rely on independent genetic factors.

With regard to the cross of 'Emerald Seedless' × 'Beichun', it is also clear that experiment 2 showed a better result than experiment 1. The basal medium produced 30.8 % plants from ovules (Tab. 4), evidently higher than ER medium (Tab. 3), indicating that the embryos benefit from the modifications. During the process of the *in vitro* culture, we also observed most embryos on woody plant medium enlarged and germinated soon, with differentiated cotyledons and taproot and followed by true leave development (Figure, c). But in some cases, embryos formed plantlets abnormal in morphology, some of them could convert to normal plants via subculture.

In fact, breeding for disease resistant stenospermic grapes is a long-term task. We have to point out this study is only a preliminary report. For other factors, such as embryo age, medium components such as mineral nutrients and plant growth regulators could also influence the breeding efficiency with complex interactions. In our laboratory, further experiments on various parameters are in progress. With the increasing number of progenies, the identification of different agronomic characteristics should be performed. However, from the results obtained, this research can clearly indicate the feasibility and perspective of our protocol. It is hoped to select some desirable accessions combined with disease resistance and seedlessness in the near future.

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