Proceeding Fifth Plant Breeding Conference May 27, 2007 (Giza)

Egypt. J. Plant Breed. 11(1): 335-349 (2007) Special Issue

EMBRYO RESCUE IN TWO SEEDLESS GRAPE CULTIVARS

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ABSTRACT

This investigation was executed on two seedless grape cultivars; Beauty and Thompson Seedless during 2004 &2005 seasons, at the tissue culture lab of Fruit Section, Faculty of Agriculture, Assiut University to establish a protocol for embryo rescue as a step to introduce genes of the new introduced colored seedless grape cultivars into the white Banaty (Thompson Seedless) most adapted and accepted cultivar in Egypt.

Results indicated that the optimal time for embryo isolation was during the 7th and 8th week beyond fruit set. The best media to rescue the aborted embryos were MS, WP and B5. There was a positive correlation between the average length of the embryo and the percentage of success.

Key Words: ***Embryo rescue, Seedless grapes, Embryo culture, Embryo abortion***

INTRODUCTION

Grapes are the largest and oldest fruit crops in the World. They ranked second in Egypt after citrus. The total area devoted for grapes is 152,488 feddans producing about 1,073,815 tons with an average of 8.02 tons/feddan. Until the nearest time, most of vineyards in Egypt were planted with Thompson Seedless and Red Roomy cultivars in addition to a few areas planted with some other local cultivars. During the last two decades, many new grape cultivars like Beauty Seedless, Red Globe and Flame Seedless have been introduced to Egypt. Thompson Seedless grape cultivar is still the major common cultivar in Egypt where it is adapted to local environmental conditions in addition to its widely acceptance to consumers. The new introduced colored seedless cultivars have a good appearance but with less acceptable taste. Therefore, it would be of great importance to introduce genes responsible for color and flavor of the new cultivars into Thompson Seedless cultivar. Hybridization of seedless x seedless will result in a seedless hybrid that needs a tool such as embryo rescue to germinate the aborted embryo. Many of the seedless grapes are stenospermocarpic in which fertilization occurs but embryo development stops at an early stage and then aborts. In Black Corinth, according to Pearson (1932), there is no ovule development beyond the time of bloom. Parthenocarpy, accordingly, was attributed to defective embryo sac formation with some or all of the nuclei degenerating. There are heterotrophic and autotrophic stages in embryo development. The embryo in the heterotrophic stage of development is smaller (termed as proembryo) then in the autotrophic stage and usually requires presence of growth regulators to allow for its proper development in the autotrophic stage. Development of an embryo does not depend on exogenous sources of growth regulators (Raghavan and Srivastava, 1982). The inheritance of stenospermic seedlessness was studied in F_1 populations

obtained from seeded X seedless crosses. Two distinct types of stenospermic male parents were used: male parents were obtained either from the progeny of seeded X seedless crosses (or naturally occurring seedless mutations such as Thompson Seedless and Black Monukka) or from *in ovulo* embryo cultures of seedless X seedless hybridizations. The historic development of *in vitro* plant cell and tissue culture has undoubtedly been a major factor in the advancement of our knowledge of cell biology, physiology, biochemistry (Bhojwani and Razdan, 1996), and more recently, molecular biology (Raghavan, 1997). Plant biotechnology utilizes a range of *in vitro* techniques to manipulate plant germplasm, including clonal multiplication, generation of novel variants and the production of genetically modified plants through somatic hybridization and genetic transformation (Vasil and Thorpe 1994).

The use of embryo culture is important in many classical breeding programmes and has been used to rescue immature embryos from early ripening fruits (El-Agamy and Sherman 1982 and Ramning and Emershad. 1990). Therefore, the objectives of this study were to: a) rescue embryos of Thompson and Beauty Seedless grape cultivars, b) define the optimal age and size to isolate the embryo as well as the best media for planting under *in vitro* conditions and c) determine the anatomical stage of embryo development of the two seedless cultivars to illustrate the embryo abortion and rescue ages.

MATERIALS AND METHODS

This study was conducted at Fruit Crops orchard and tissue culture laboratory, Assiut University during 2004 and 2005 seasons.

Explant Preparation:

Fruits of Beauty seedless and Thompson seedless grape were collected three weeks after fruit set for eight weeks from the middle portion of the clusters. Fruits were divided as follows:

(1) 50% were cleaned and sterilized as follows:

(a) Washing under running water for ten minutes.

(b) Under the laminar flow hood, fruits were sterilized by soaking in 2% sodium hypochlorite solution for 15 minutes.

(c) Sterilized fruits were washed in sterilized distilled water for 2 rinses.

(d) Embryos from seedless grape fruits were isolated in sterilized conditions and divided into three replicates and vertically replaced in Murashige & Skoog,1962, Gamborg,1968, Woody Plant,1982), Nitsche & Nitsche, 1969) and White ,1963 media(Vasil and Thrope, 1994) with organic supplements for aseptic culture establishment.

(2) 40% divided in 10 replications for fruits measurements "fruit length, fruit diameter" using vernier caliper and embryo length using the eye piece micro meter lens.

(3) 10% were fixed in FAA solution for anatomical study. Fruits of each cultivar were picked weekly for ten weeks and fixed in FAA solution .The fruits were transferred from FAA and were dehydrated in a graded series of ethyl alcohol and cleaned in xylol. Then they were embedded in paraffin wax and sections were stained with safranine and light green and mounted by DPX (Sass, 1951).

Media preparation

Five known media were used in this study. These media were Murashige & Skoog, 1962, Gamborg, 1968, Woody Plant, 1982), Nitsche & Nitsche, 1969) and White, 1963 media (Vasil and Thrope, 1994). Media salts were dissolved in water and supplements were added as follow: agar was added as 6.8-7.0 g/l using hot plate with stirrer, organic compounds 1/2 ml BA / L, 5 ml GA/L and 0.02 mg/l IBA, sugar as 30 g sucrose/L, myo-inositol as 0.1 g/L, vitamins 5ml/L of Murashige& Skoog vitamin in stock solution and pH was adjusted before autoclaving to 5.6-5.8. Media were poured in 200 ml jar as 25 ml of medium/jar then jar were autoclaved at 1.5 kg/cm^2 pressure and 120°C temperature for 20 minutes. Incubation of cultured jars was made in culture room at temperature of 22 ±2 °C under cool florescent light 1500 Lux for 16 hours /day. Normal incubation period was proceeded as 6-8 weeks /culture.

The following measurements were recorded a) fruit dimensions, b) embryo length, c) number of survived embryos (remain green) and d) number of germinated embryos. Data of embryo length represent the whole seed components but exclusively the embryo since degradation occurs in seedless grapes used cultivars. Statistical analysis was undertaken.

C- Statistical correlation was made to a) embryo length, b) percentage of survived Embryos (remain green) and c) percentage of germinated embryos.The aforementioned experimental designs were made after (Steel& Torrie, 1980). Means were separated according to least significant difference LSD at 5% level (Snedecor & Cochran, 1990).

RESULTS AND DISCUSSION

Results of this manuscript will be presented as follow: (1) effect of media and embryo age on embryo, survival and germination percentages (2) the relationship between fruit length, diameter and embryo length and (3) the relationship between embryo length, survival percentage and germination percentage.

Effect of media and embryo age on embryo survival and germination percentages

a) Survival percentage of Thompson Seedless cultivar

Data presented in Fig.(1) showed that there were no survived embryos in samples taken 3 and 4 weeks after fruit set(premature stage)

for any type of used media. While in the 5th week slight survival occurred in MS and WP media and reached its maximum at the 7th week. Slight decrement occurred during the 8th week followed by a dramatic decrement then reached its minimum survival percentage at the 9th and 10th weeks on MS and WP media respectively. On the other hand, there was no survival recorded at weeks 3,4,5 and 6 for embryos cultured on WH medium. Beyond the 7th week and two weeks later, the embryo survival percentages were less than 50%. As for NN and B5 media there were no survived embryos in samples taken during 3,4 and 5 weeks after fruit set. Then, there were survived embryos in samples taken after these embryo ages and reached their maximum at the 7th and 8th weeks for B5 and NN medium, respectively and reached nil at the 10th and 9th weeks for the same media during the two studied seasons. The maximum survival percentages were 100, 95 and 80% during the 7th week after fruit set for WP ,B5 and MS medium respectively, while embryo survival was 65 and 45 % for NN and WH medium at the 8th and 9th weeks respectively. Therefore, the best medium for embryo culture was WP. Gradual and significant increase occurred from the 5th week till the 7th week, followed by slight and insignificant decrease at the 8th week after fruit set (embryo age) then sharp and significant decrease two weeks later, also B5 medium had significant increase for embryo survival percentage from 6th to 7th week (45 to 95 %).After this time, the percentage of embryo survival gradually and significantly decreased till the 9th week of culture. Data of the current study confirmed the results of previous study made by Pommer *et al* 1995 who suggested that the best time for culturing for grape embryo rescue was 6 and 10 weeks post- bloom. At these dates, the largest number of embryos, germinated embryos and transplantable plants were obtained.

Concerning the effect of embryo culture time, it is clear that the 7th week was the milestone in producing the highest percentage for WP, MS and B5 respectively. While the 8th and 9th weeks obtained the highest values for NN and WH respectively.The effectiveness of embryo age was in, line with those reported by Liu *et al* 2003. The most vigorous growth was observed for ovules cultured at 30 and 43 DAF, but more embryos were recovered from ovules cultured at 60 and 70 DAF. Ovule growth and embryo production *in vitro* were improved in Bouquet and Davis (BD) and Nitsche and Nitsche (NN) media.

Regarding the interaction between medium and embryo culture time, it is clear that WP medium at the 7th and 8th week embryo age induced the highest survival percentage (100 and 95 %) followed by B5 medium at the 7th week embryo age (95%).While the survival percentage

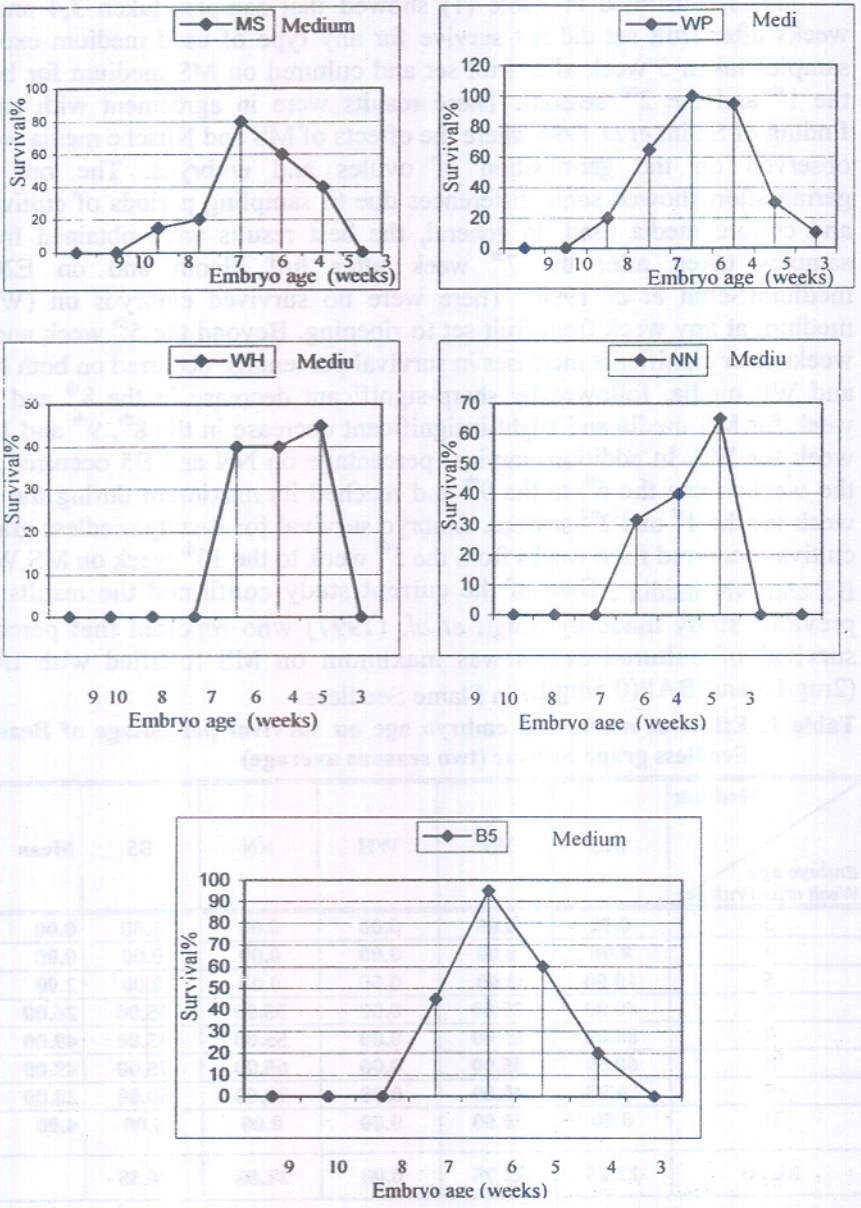


Fig 1. Survival Percentage of Thompson Seedless embryo on different media.

reached its maximum at 8th and 9th weeks for MS and NN (80 and 65%), respectively.

Survival percentage of Beauty Seedless cultivar

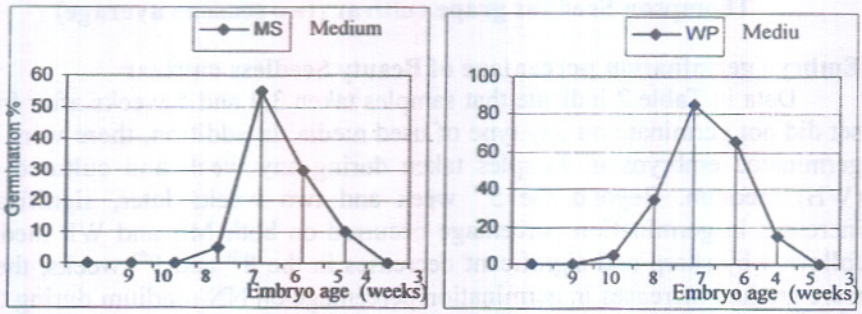
Data illustrated in Table (1) showed that samples taken 3,4 and 5 weeks after fruit set did not survive for any type of used medium except samples taken 5 week after fruit set and cultured on MS medium for both the 1st and the 2nd seasons. These results were in agreement with early finding of Selim *et al* 1996 where the effects of MS and Nitsche media were observed on the germination of ovules and embryos. The rate of germination showed some differences due to sampling periods of cultivars and culture media used. In general, the best results were obtained from samples taken after the 7th week after full bloom and on E20A medium(Selim *et al* 1996). There were no survived embryos on (WH) medium at any week from fruit set to ripening. Beyond the 5th week and 2 weeks later significant increases in survival percentage occurred on both MS and WP media, followed by sharp-significant decrease in the 8th and 9th week for MS media and slight-insignificant decrease in the 8th, 9th and 10th week for WP. In addition, survival percentage on NN and B5 occurred on the weeks from the 6th to the 9th and reached its maximum during the 8th week for the 1st and 2nd seasons. Embryo survival for Beauty seedless grape cultivar occurred for 6 weeks from the 5th week to the 10th week on MS,WP, B5 and NN media . Data of the current study confirmed the results of previous study made by Singh *et al.* (1997) who reported that percent survival of cultured ovules was maximum on MS fortified with IAA (2mg/L) and BAP(0.5mg/L) in Flame Seedless.

Table 1. Effect of media and embryo age on survival percentage of Beauty Seedless grape cultivar (two seasons average)

Medium / Embryo age Week after Fruit set	MS	WP	WH	NN	B5	Mean
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00
5	10.00	0.00	0.00	0.00	0.00	2.00
6	40.00	20.00	0.00	35.00	35.00	26.00
7	80.00	65.00	0.00	55.00	45.00	49.00
8	60.00	45.00	0.00	65.00	75.00	49.00
9	20.00	40.00	0.00	20.00	60.00	28.00
10	0.00	20.00	0.00	0.00	0.00	4.00
Mean	22.25	23.75	0.00	21.88	26.88	
LSD 0.05 of Medium 1.88		*LSD 0.05 of Embryo age 4.93*			*LSD 0.05 of Interaction*	

Embryo germination percentage of Thompson Seedless cultivar

There were no germinated embryos in samples taken 3,4 and 5 weeks after fruit set for any type of used media except for (WP) medium where a slight percentage (5%) occurred for the age of 5 weeks after fruit set but significant increases occurred at 6 and 7 weeks after fruit set for (MS and WP) in the 1st and 2nd seasons followed by slight decreases during 8 and 9 weeks after fruit set (Figure 2) .On the other hand, for (WH) medium there were no germinated embryos before the 7th week and after the 9th week by percentage less than 40% for the 1st and 2nd seasons. While germination occurred beyond the 6th week for (B5 and NN) media followed by significant increase in the 7th and 8th week, however germination occurred on (B5) medium in the 9th week, while there was no germination for (NN) medium in the same week. On the other hand Ebadi *et al.(* 2002) suggested that embryo rescue and its germination were carried out successfully in five seedless grape cultivars; in most of cultivars culturing ovules at 20 days after flower opening in White Seedless and Red Seedless and 40 days after flowering . No germination occurred beyond the 9th week for any type of used media. The present results were accordant with those of Selim *et al.* (1996) who reported that embryo germination rate was rather high on all the tested media (MS, Nitsche, E20A) with the highest rate of (29.8%) obtained on E20A. Nearly all the germinated ovules on this medium developed into full plants (98.8%). The development into full plants from germination embryos on MS an NN media were 21.4 and 21.1 respectively. In addition Midani *et al (*2001) reported that the overall establishment was highest in the NN medium (68.5%). The interaction effect was most pronounced for Baharat Early cv and NN medium (69.8%), which was a par with Perlette and NN medium (76.2%).



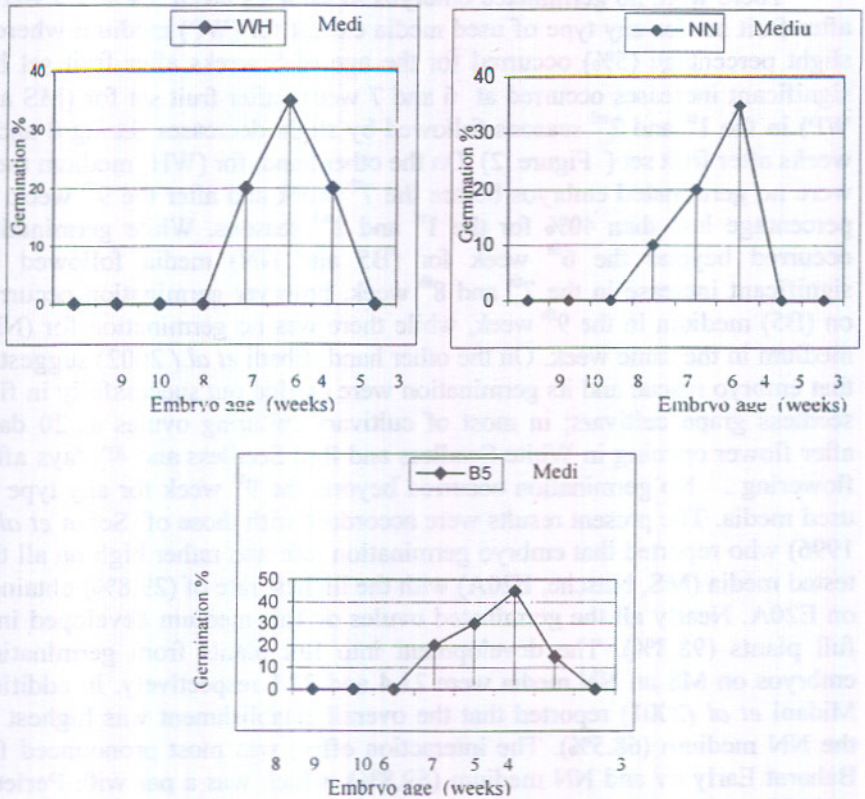


Fig. 2. Effect of media and embryo age on germination percentage of Thompson Seedless grape cultivar (two seasons average)

Embryo germination percentage of Beauty Seedless cultivar

Data in Table 2 indicate that samples taken 3,4 and 5 weeks after fruit set did not germinate on any type of used media. In addition, there were no germinated embryos in samples taken during any week and cultured on (WH) medium. Beyond the 5th week and two weeks later, significant increases in germination percentage occurred on both MS and WP media, followed by sharp and significant decreases in the 8th and 9th weeks, there were gradual increases in germination percentage on NN medium during the 6th, 7th and 8th weeks followed by a sharp and significant decrease in the 9th week. Data of the current study confirmed the results of previous study made by Selim *et al.* (1996) who suggested that, in Flame Seedless ovules, there was no germination on MS, 13,9% germination on Nitsche medium at the 7th week; 2.8% on E20A medium and 14.3% germination at the 6th and 7th weeks respectively, None of the ovules had alive embryos taken during the first two weeks (5 weeks age) .Flame Seedless embryos were found to

germinate successfully. In general, except embryos taken at the 3rd week and placed on MS medium, embryo germination rates on MS, Nitsche and E20A were 50,79.2 and 97%, on MS and NN media, and 3rd, 4th and 6th weeks on E20A media. The number of viable embryos obtained from ovules on MS, Nitsche and E20A were 16.5,13.3 and 17.5 %and the germination percentages were 83.3,92.9 and 98.6, respectively.

Stable germination percentages occurred in the 6th and 7th week for B5 medium the end reached its maximum in the 8th week followed by slight decrease on the 9th week. There were no germinated embryos in samples taken 9 and 10 weeks after fruit set and cultured on any type of used media except WP. The maximum germination percentage found at the 7th week on MS and WP media (70 and 50%) respectively and in the 8th week for B5 medium 45%. Therefore, the optimal ages for embryo germination are 7 and 8 weeks after fruit set and the best media are MS, WP and B5, respectively(Plate 1). The effectiveness of embryo age was in, line with those of (Liu *et al* 2003) who reported that the most vigorous growth was observed for ovules cultured at 30 and 43 DAF, but more embryos were recovered from ovules cultured at 60 and 70 DAF. Ovule growth and embryo production *in vitro* were improved in Bouquet and Davis (BD) and Nitsche and Nitsche (NN) media.

Table 2. Effect of media and embryo age on germination percentage of 'Beauty Seedless grape cultivar as two seasons average.

Medium / *Embryo age Week after fruit set*	MS	WP	WH	NN	B5	Mean
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00
6	20.00	15.00	0.00	10.00	20.00	13.00
7	70.00	50.00	0.00	20.00	20.00	32.00
8	25.00	25.00	0.00	35.00	45.00	26.00
9	10.00	15.00	0.00	10.00	40.00	15.00
10	0.00	10.00	0.00	0.00	0.00	2.00
Mean	14.65	14.38	0.00	9.38	15.63	
LSD 0.05 of Medium 2.34		*LSD 0.05 of Embryo age 2.49*			*LSD 0.05 of Interaction 5.56*	

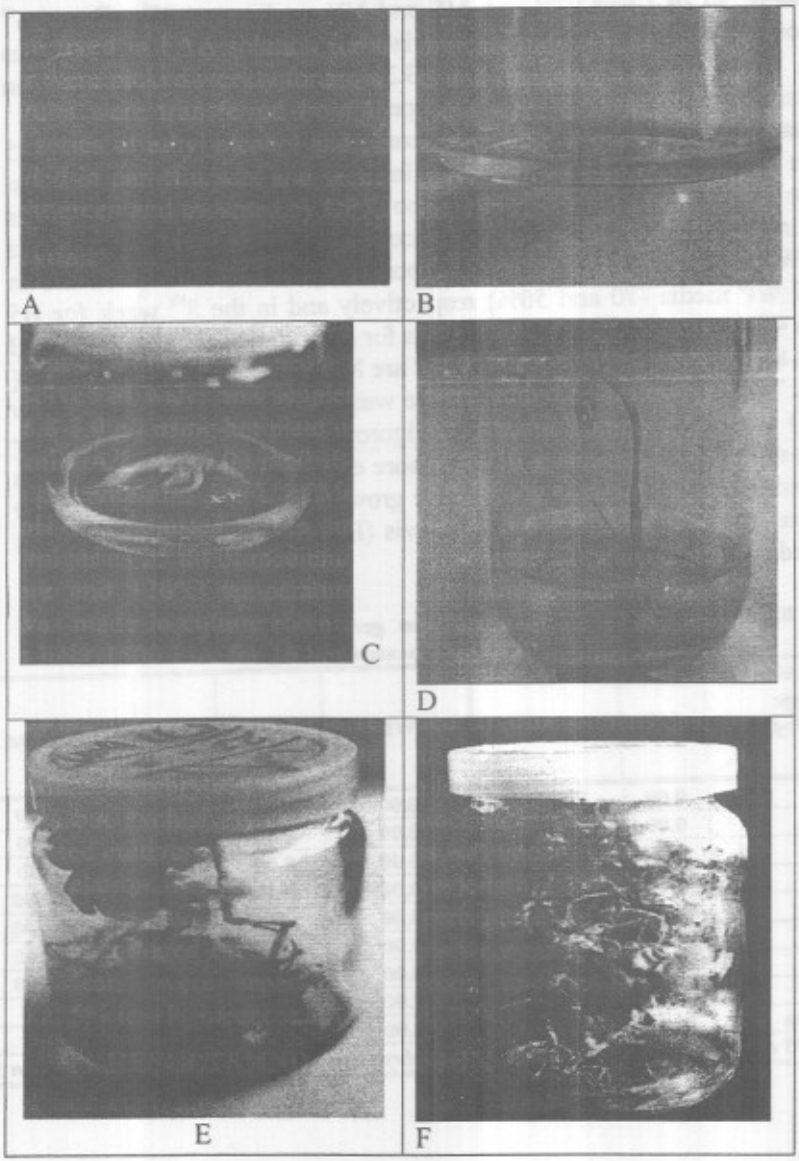


Plate 1. Embryo rescue in Thompson & Beauty seedless grape cultivars.

A: Embryo culture
B: Differentiation
C: Development
D: Plant Formation
E: Full Plant
F: Root Formation

The relationship between fruit length, diameter and embryo length of Thompson seedless grape cultivar.

Data of the two seasons average(Table 3) showed gradual and significant increase in fruit length till the 8th week after fruit set when fruits of Thompson Seedless reached (85.6%) from its maximum length, while changes in fruit length after the 8th week till the 10th week after fruit set were not significant. In addition, significant increases in fruit diameter occurred from week to week till the 8th week after fruit set and insignificant increase occurred from the 8th to the 9th week when fruits of Thompson Seedless reached (79.2 %) from its diameter at the 10th week. Slight increases occurred in fruits diameter from the 8th week till the 10th week. There were gradual insignificant increase in embryo length from the 3rd till the 8th week after fruit set where embryos of Thompson Seedless grape cultivar reached their maximum length, followed by a sharp and significant decrease in the 9th week (24.5%); slight and insignificant changes in embryo length in the 9th and 10th week.

There was a considerable relationship between embryo growth and fruit growth in length and diameter from the 4th to the 8th weeks respectively, where percentage of increases in embryo length reached (4.0,17.0,17.3,1.4,8.1 and 21.3) when increases in percentage of fruit length reached (14.5,16.1,5.5,16.4 and 23.8) and increases in percentages of fruit diameter reached (14.4, 16.0, 14.3, 21.2 and 14.3).

Table 3. The relationship between fruit length, fruit diameter and embryo length of Thompson seedless grape cultivar (two seasons average)

Properties / Week after fruit set	Fruit length (cm)	Fruit diameter (cm)	Embryo length (mm)
3	0.574	0.435	2.49
4	0.657	0.500	2.59
5	0.763	0.586	2.92
6	0.805	0.670	2.96
7	0.939	0.872	3.20
8	1.163	0.927	3.88
9	1.141	0.995	2.93
10	1.158	1.060	2.80
$LSD_{0.05}$	0.067	0.087	0.66

Data of Beauty Seedless cultivar (data not shown) had a similar trend as obtained in Thompson Seedless one.

The correlation between embryo length, survival percentage and germination percentage.

Thompson Seedless cultivar.

Table (4) illustrates correlation coefficients of survived embryos and germinated embryos on media means (two seasons average). Data indicated positive significant correlation coefficients (r^2) between survival and germination starting from the 3rd week after (FS) to the 10th week after (FS) in Thompson Seedless grape cultivar. Embryo length was also found to be positively associated with either survival and germination.

Table 4. The relationship between embryo length, survival percentage and germination percentage of Thompson Seedless grape cultivar (two seasons average).

	Survival (%)	Germination (%)	Embryo length (mm)
Survival (%)	1.00		
Germination (%)	0.984**	1.00	
Embryo Length(mm)	0.865**	0.904**	1.00

Beauty Seedless cultivar

Data in Table (5) illustrate correlation coefficients of survived embryos and germinated embryos on media means during two seasons average. Data indicated positive significant correlation coefficients (r^2) between survival and germination starting from the 3rd week after (FS) to the 10th week after (FS) in Beauty Seedless grape cultivar. Embryo length was also found to be positively associated with both survival and germination.

Table 5. The relationship between, embryo length, survival percentage and germination percentage of Beauty Seedless grape cultivar (two seasons average)

	Survival (%)	Germination (%)	Embryo length (mm)
Survival (%)	1.00		
Germination (%)	0.989**	1.00	
Embryo Length(mm)	0.742**	0.710**	1.00

The main objective of the current study was to establish a protocol for embryo rescue of Thompson Seedless and Beauty Seedless grape cultivars as a step for further work to introduce genes of the new introduced colored grape cultivars such Beauty Seedless, Flame Seedless and other's into the white Banaty (Thompson Seedless) most adapted and accepted cultivar in Egypt. For this purpose, fruit and embryo development of the two cultivars

under the study were monitored starting from the 3rd week after fruit set till the 10th week. Fruit and embryo dimensions were also determined in association with embryo survival and germination on different proposed media.

Similar studies were utilized to set a proposed protocol for embryo rescue and obtaining complete plantlets of either cultivar. Embryo age at which it can be rescued was investigated on different cultivars at different environments. For instance, Midani *et al* (2002) found that the ovule age had a significant effect on culture establishment. The maximum (65.9%) and minimum (56.9%) culture establishment was observed at 28 and 16 days of ovule age after pollination. Nearly similar results were obtained by Ebadi *et al* (2002).

On the other hand, most studies indicated that embryo age of 6 to 10 weeks after fruit set was; the best culturing time for grape embryo rescue (Pommer *et al* 1995). At these dates the largest number of embryos, germinated embryos and transplantable plants were obtained. In addition to Liu *et al.* (2003) who stated that the most vigorous growth was observed for ovules cultured at 30 and 43 DAF, but more embryos were recovered from ovules cultured at 60 and 70 DAF and Selim *et al* (1996) who determined the 7th week to rescue embryos in Turkey. Such findings support the present study results where 7th and 8th weeks after fruit set were the best ages to secure Thompson Seedless and Beauty Seedless grown under Assiut environmental conditions.

Several media were suggested to secure grapes embryos at different degrees of success; Murashige & Skoog (1962), Gamborg (1968), Woody Plant (Wisconsin) (1982), Nitsche & Nitsche (1969) and White (1963) . In the current study Murashige & Skoog (1962), Gamborg (1968), Woody Plant (Wisconsin) (1982) were found to produce the best results in embryo rescue of Thompson Seedless and Beauty Seedless grape cultivars. These support early studies by Selim *et al* (1996), Singh *et al* (1997), Kuden *et al* (1999), Chen Xangbo *et al* (2000), Liu *et al* (2003) and Midani *et al* (2001).

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إنقاذ الأجنة في صنفين من الأعناب اللابذرية

سمير زكي العجمي ، حسن عبد القوي عبد الجليل ، مخلص أحمد السيسي

قسم البساتين ـ كلية الزراعة ـ جامعة أسيوط

أجري هذا البحث علي صنفي العنب اللابذري تومسون سيدلس (البناتي الأبيض) وبيوتي سيدلس خلال موسمي 2004 ، 2005 بمعمل زراعة أنسجة بفرع الفاكه بكلية الزراعة جامعة أسيوط بهدف عمل بروتوكول لإنقاذ الجنين كوسيلة لإدخال جينات الأصناف الملونة اللابذرية المستوردة حديثا إلي صنف العنب البناتي الأبيض (توممسون سيدلس) الأكثر زراعة وقبولا في مصر.

أظهرت النتائج أن أنسب موعد لعمر الجنين لإنقاذه قبل ضموره هو بين الأسبوعين السابع و الثامن من عقد الحبات تحت ظروف التجربة بأسيوط. و قد كانت البيئات الغذائية: موراشيجي و سكوج ، بيئة النباتات الخشبية(وودي) و بيئة جامبوج (ب 5)هي أفضل البيئات لإنقاذ الأجنة قبل ضمورها (إجهاضها). كما كان هناك إرتباط إيجابي بين متوسط طول الجنين و نسبة النجاح المئوية

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المجله المصريه لتربية النبات ١١(١): ٣٣٥ـ٣٤٩ (عدد خاص)