In Vitro Culture of Ovules and Embryos from Seedless Grapes (*Vitis vinifera* L.)

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Ovules of stenospermocarpic seedless grapes were cultured under different conditions. The number of embryos was not significantly increased when ovules of Muscat Seedless were cultured on a medium supplemented with the plant growth regulators indole-3-acetic acid and gibberellic acid compared to the basal medium. No correlation was found between the ovule size of Sultanina and the presence of embryos. Comparable percentages of embryos were found in small, medium and large ovules following weekly culture from four to 10 weeks after bloom. Varying results were obtained when ovules of four open-pollinated seedless cultivars were cultured on two basal media with or without activated charcoal. In all four cultivars the highest number of embryos was found at the later culture date. With three cultivars the best results were obtained when ovules were cultured on the medium of Bouquet & Davis (1989), supplemented with activated charcoal. Isolated embryos of Muscat Seedless and Sunred Seedless were cultured under various conditions and it was found that the absence of growth regulators resulted in the highest number of plantlets with normal leaves.

Breeding new seedless table grape cultivars has a high priority world-wide due to consumer preference. Conventional breeding techniques limit the use of seedless cultivars to pollen parent only, resulting in a low percentage of seedless progeny. Development of embryo culture techniques, however, makes it possible to generate plants from the embryos of seedless plants and thus from seedless x seedless crosses. A much higher frequency of seedlessness is found in the progeny of such crosses (Spiegel-Roy, Sahar & Baron, 1990).

In stenospermocarpic cultivars a high number of the ovules possess normal embryo sacs, fertilisation occurs and embryos may develop (Pearson, 1932; Barrit, 1970). Embryo development, however, is often arrested and normal seeds do not develop. By applying *in vitro* techniques these embryos can be rescued and allowed to develop into plants. Embryo culture has been successfully applied by a number of researchers, using various media and techniques (either liquid or solid, supplemented with or without plant growth regulators) (Cain, Emershad & Tarailo, 1983; Emershad & Ramming, 1984; Gray, Fisher & Mortensen, 1987; Barlass, Ramming & Davis, 1988).

Embryos of stenospermocarpic grapes are able to germinate directly from the ovule when cultured on an artificial medium (Spiegel-Roy *et al.*, 1985). However, a certain percentage of embryos fail to germinate while embedded in the ovule. To ensure the highest frequency of germination, a number of authors found it necessary to excise embryos after a period in culture (Cain *et al.*, 1983; Emershad & Ramming, 1984; Gray *et al.*, 1987; Barlass *et al.*, 1988). Various techniques are applied to stimulate the growth of isolated embryos and their development into plants. Embryos obtained from cultured ovules appeared to be dormant, but cold stratification of the isolated embryos at 4°C for six weeks failed to promote germination, while the addition of N^6-Benzyladenine to the medium stimulated rapid germination (Gray *et al.*, 1990). Bouquet & Davis (1989), however, found that BA was detrimental to plant development and that gibberellic acid was ineffective in breaking dormancy.

The aim of this study was to investigate different factors which may affect the success of *in vitro* culture of grape embryos.The effect of plant growth regulators, developmental stage and ovule size on embryo growth was investigated. The effect of different culture conditions on the growth of isolated embryos was also studied.

MATERIALS AND METHODS

Ovule culture: A standard procedure as described by Spiegel-Roy *et al.*, (1985) with appropriate modifications was followed. Berries were collected in the vineyard at different stages after pollination, rinsed under running tap water (20 min), surface-sterilised for 15 min in 1% NaOCl and rinsed twice in sterile water. Ovules were aseptically removed and cultured on the basal medium of Nitsch & Nitsch (1969) (BMN), modified by increasing the $FeSO_4.7H_2O$ concentration 10X, or on the basal medium of Bouquet & Davis (1989) (BMB). Sigma agar was used. Ovules were cultured in petri dishes (90 mm x 10 mm) under standard conditions of 26 ± 2°C and 16 h cool-white fluorescent (G.E.C. 65 W) illumination. In some of the experiments (see below) 3-indoleacetic acid (IAA), gibberellic acid (GA_3), N^6-Benzyladenine (BA) and indole-3 butyric acid (IBA) were added to the media. Media were sterilised at 121°C for 15 min and the plant growth regulator solutions were filter-sterilised (0,2 µm Millipore filters)

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and added after autoclaving.

The effect of GA_3 and IAA: Muscat Seedless ovules were cultured on BMN or BMN supplemented with 1 x 10^{-5} M IAA and 1 x 10^{-6} M GA_3. Ovules were collected at five, seven, nine and eleven weeks after bloom and cultured as described above. After 12 ± 1 weeks in culture the percentage of germinated ovules was determined for each treatment. The Spiegel-Roy *et al.*, (1985) technique was adapted by excising the remaining (ungerminated) ovules and culturing the isolated embryos on BMN in darkness at 26 ± 2°C. Germinated embryos were transferred to a growth chamber and cultured under standard conditions. The resulting plants were micropropagated and acclimatised in a greenhouse.

The effect of cultivar, basal medium and addition of activated charcoal: Ovules of open-pollinated Muscat Seedless, Flame Seedless, Festival Seedless and a selection, 72-413, were cultured at six and eight weeks after bloom. Mean ovule masses at six weeks after bloom were 6,25 mg; 3,30 mg; 6,22 mg and 5,84 mg respectively, while it was 10.36 mg; 4,48 mg; 7,25 mg and 6,56 mg at eight weeks after bloom. Two basal media (BMN and BMB) with and without activated charcoal (2 g/L) were included in the experiment. The standard procedure described above was followed and isolated embryos were cultured on BMB.

The effect of ovule size: Sultanina ovules were collected weekly from four to 10 weeks after bloom and visually grouped into three classes according to size (small, medium and large). Size criteria varied according to culture date (weeks after bloom). One hundred ovules of each size class were cultured on BMN for 12 ± 1 weeks after which their embryos were excised. Isolated embryos were cultured on BMN.

All the experiments described above were arranged in a factorial design and replicated in either two or three blocks. An experimental unit consisted of five petri dishes with 10 ovules per petri dish. The number of embryos germinating from intact ovules was counted and afterwards the ungerminated ovules were dissected to determine the number containing embryos. The total embryo percentage could thus be calculated. The number of ovules per unit differed (attributed to contamination) and a working logit transformation was thus applied. The transformed values were then analysed. In order to compare treatment means Student's t-LSD test was applied at 5% significance. Treatments were developmental stage (i.e. time measured as weeks after bloom), different media (including the addition of plant growth regulators and activated charcoal) and ovule size (small, medium and large).

Development of excised embryos: During 1991 isolated embryos of Muscat Seedless were submitted to different treatments to stimulate growth and further development into normal seedlings. Treatments were as follows: (1) BMN + 16h illumination at 26 ± 2°C (control), (2) BMN + darkness at 4°C, (3) BMN + darkness at 26 ± 2°C; (4) BMN + BA + darkness at 26 ± 2°C, (5) BMN + GA_3 + IAA + darkness at 26 ± 2°C, (6) BMN + GA_3 + darkness at 26 ± 2°C.

Based on the results of the previous year, the following media were included in a similar experiment with Sunred Seedless and isolated embryos were submitted to the following treatments (1992): (1) BMN + darkness at 26 ± 2°C (control), (2) BMB + darkness at 26 ± 2°C, (3) BMB + BA + darkness at 26 ± 2°C, (4) BMB + GA_3 +IAA + darkness at 26 ± 2°C,(5) BMB + GA_3 + darkness at 26 ± 2°C.

Plant growth regulator concentrations were 1 x 10^{-5} M IAA, 1 x 10^{-6} M GA_3 and 1 x 10^{-6} M BA, respectively. Treatments included the addition of plant growth regulators to the medium and different illumination periods and were replicated in three blocks during 1991. An experimental unit consisted of five petri dishes with five embryos per petri dish (25 embryos per block). During 1992 treatments were replicated in five blocks and an experimental unit consisted of six petri dishes with 5 embryos per dish (30 embryos per block). After three weeks, when petri dishes were transferred to standard illumination and temperature conditions, observations were made by counting the number of germinated embryos. Data on the number of embryos which eventually developed into plantlets with normal leaves were recorded two months later. Data were subjected to a working logit transformation and analysis of variance was performed as described above.

Micropropagation and acclimatisation: Although normal healthy plants developed from the cultured embryos, abnormal growth of the cotyledons was frequently observed. When morphologically normal stems and leaves developed from embryos with abnormal cotyledons, these shoots were removed and rooted on medium containing 6,2 x 10^{-6} M IBA. In all experiments, when leaves developed, plantlets were transferred from petri dishes to BMN in 100ml glass jars. Plantlets were micropropagated by one-node microcuttings on medium supplemented with 4 x 10^{-6} M BA to encourage shoot proliferation. Resulting shoots were rooted on medium supplemented with IBA. Rooted plants were removed from the jars, the roots washed under running water, dipped in a 1% Benlate solution and planted in an unsterilised mixture of commercial "speeding" mix (Kompel) and vermiculite (1:1) in polystyrene seedling trays. Each tray was covered with a plastic bag and transferred to a greenhouse at 26 ± 2°C and 16 h illumination. After 10 days the plastic was gradually ruptured., followed by complete removal after 13 days.

RESULTS AND DISCUSSION

Polyembryos and secondary embryos obtained from one ovule were regarded as one embryo to interpret results.

The effect of GA_3 and 1AA: Only a very small number of embryos were able to germinate from intact Muscat Seedless ovules and for each culture date no significant differences were found for ovules cultured on media with and

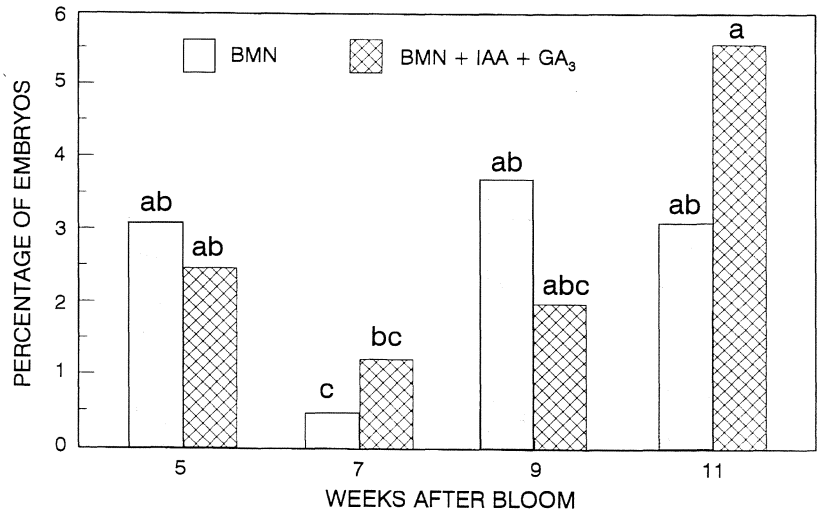

FIGURE 1(a)

The effect of plant growth regulators on the percentage of embryos germinating from intact Muscat Seedless ovules (BMN: Basal medium of Nitsch & Nitsch; GA_3: Gibberellic acid; IAA: 3-Indoleacetic acid). Percentages identified by the same symbols do not differ significantly ($p \leq 0,05$).

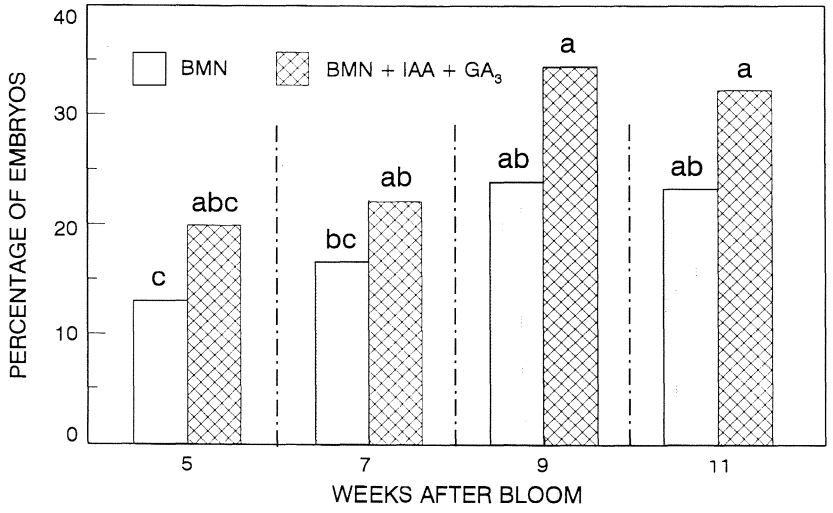

FIGURE 1(b)

The effect of plant growth regulators on the percentage of embryos germinating from intact Muscat Seedless ovules as percentage of the total number of embryos (BMN: Basal medium of Nitsch & Nitsch; GA_3: Gibberellic acid; IAA: 3-Indoleacetic acid). Percentages identified by the same symbols do not differ significantly ($p \leq 0,05$).

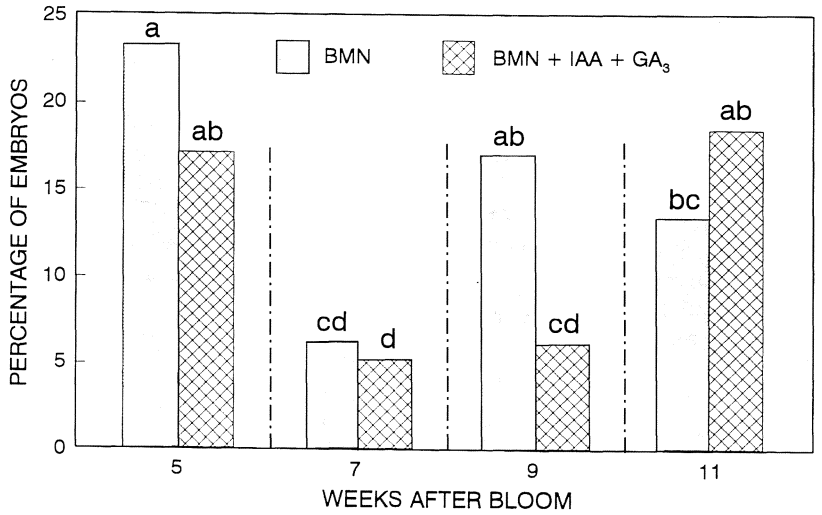

FIGURE 1(c)

The effect of plant growth regulators on the total percentage of embryos obtained from intact as well as dissected Muscat Seedless ovules (BMN: Basal medium of Nitsch & Nitsch; GA_3: Gibberellic acid; IAA: 3-Indoleacetic acid). Percentages identified by the same symbols do not differ significantly ($p \leq 0,05$).

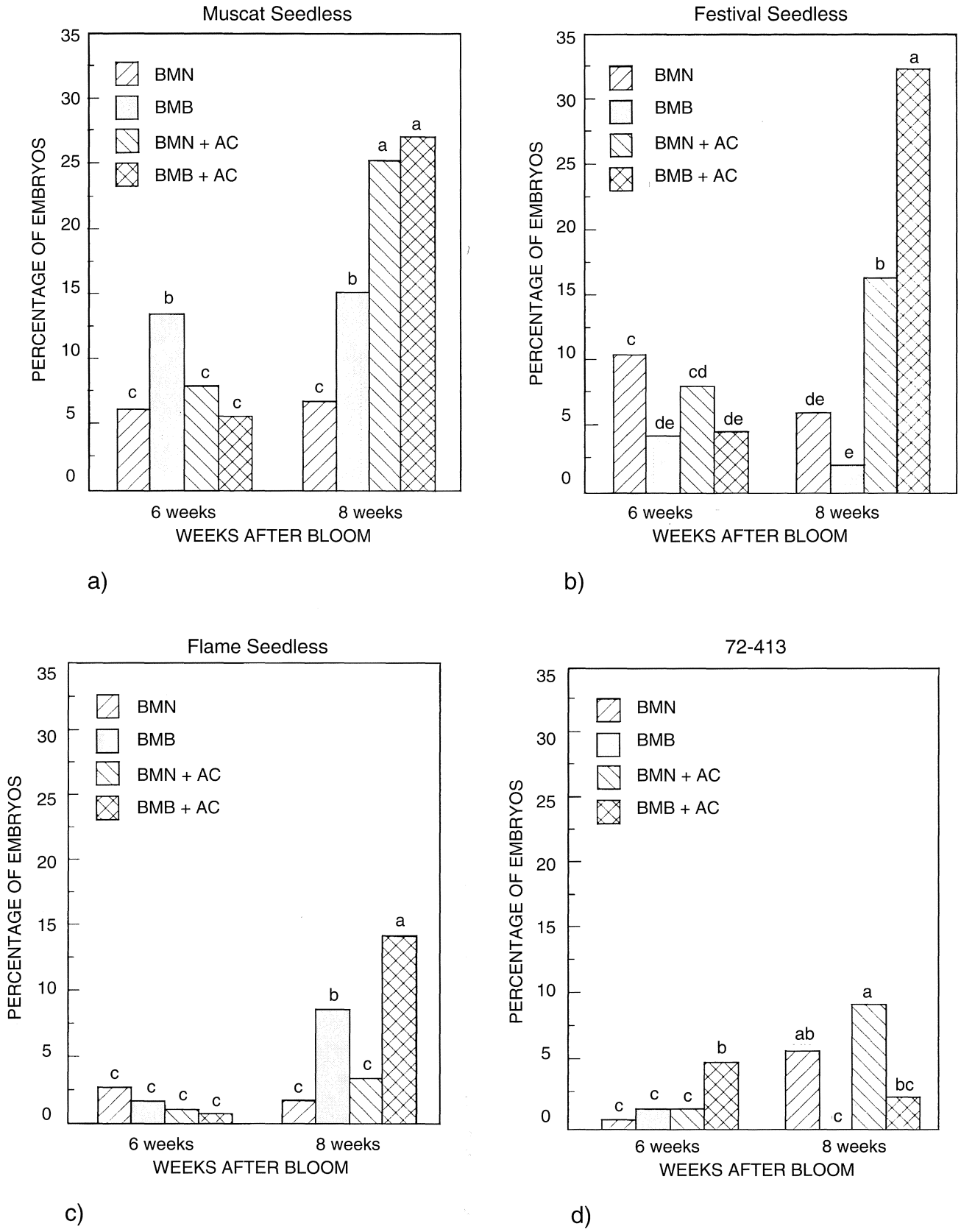

FIGURE 2

The effect of cultivar, medium and developmental stage of ovules on the percentage of embryos obtained (BMB: Basal medium of Bouquet & Davis; BMN: Basal medium of Nitsch & Nitsch; AC Activated charcoal). Percentages identified by the same symbols do not differ significantly ($p \leq 0,05$).

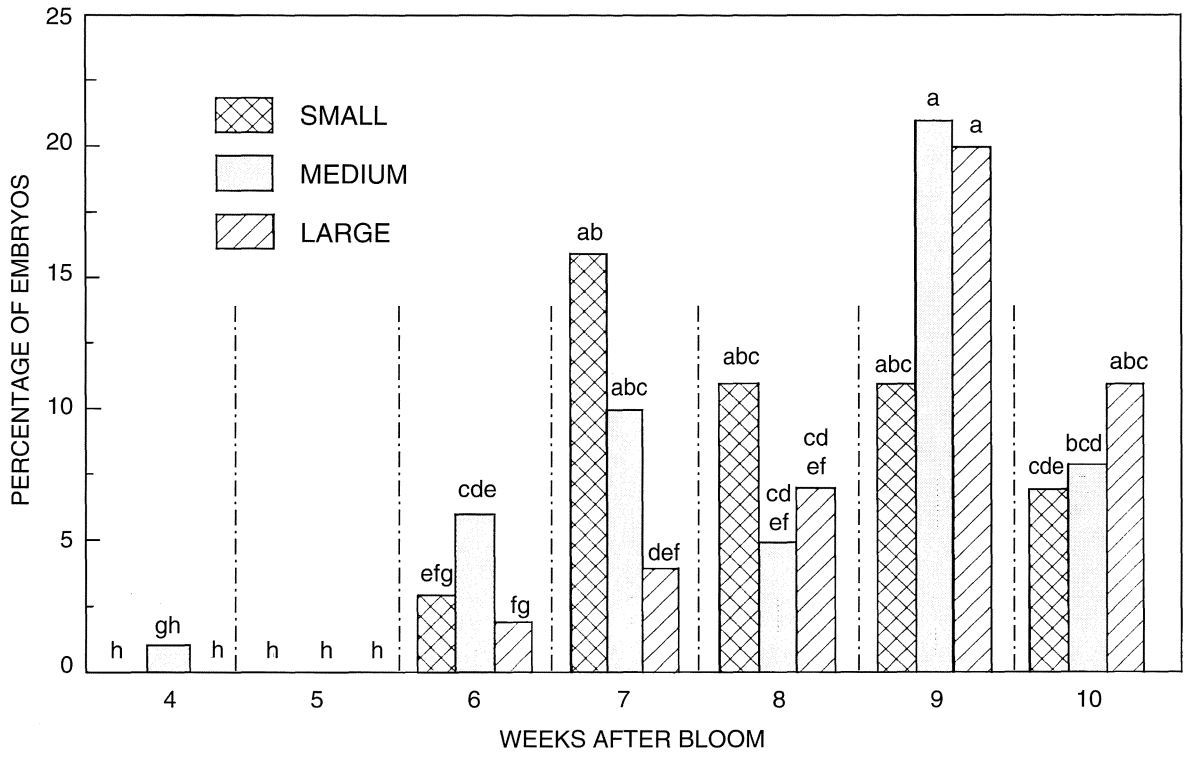

FIGURE 3

The effect of ovule size on the percentage of Sultanina embryos obtained. Ovules were cultured on BMN (BMN: Basal medium of Nitsch & Nitsch). Percentages identified by the same symbols do not differ significantly ($p \leq 0,05$).

without plant growth regulators (Fig. 1a). Preliminary results were Sultanina ovules cultured with the inclusion of different concentrations and combinations of BA, GA_3 and IAA indicated that these growth regulators were not essential for embryo recovery (Burger, 1992). When considering the number of embryos that germinated from intact ovules as percentage of the total number of embryos, a higher number germinated when cultured on medium supplemented with growth regulators. These differences, however, were not significant (Fig. 1b). However, despite the indication of plant growth regulators promoting germination from intact ovules, it was indicated that a higher total number of embryos (as percentage of ovules) is found when ovules are cultured on medium without plant growth regulators (Fig. 1c). For each culture date significant differences in the number of embryos were found only for the ovules cultured nine weeks after bloom, in which case the higher embryo percentage was found in the ovules cultured on the basal medium (Fig. 1c). In order to obtain the most plants, the ovules need to be dissected and the isolated embryos cultured. This observation confirms that of a number of authors who found it necessary to dissect the embryos from the ovules after a certain culture period (Barlass *et al.*, 1988; Cain *et al.*, 1983; Gray *et al.*, 1987; Emershad *et al.*, 1989; Gray *et al.*, 1990; Ramming *et al.*, 1990).

The effect of cultivar, basal medium and addition of activated charcoal: Significantly more embryos were obtained with Muscat Seedless ovules when cultured eight weeks after bloom on medium supplemented with activated charcoal irrespective of basal medium (Fig. 2a). The same trend was observed for Festival Seedless (Fig. 2b). However, in this instance the results for the two basal media supplemented with activated charcoal differed significantly. The highest number of embryos was obtained from ovules cultured on BMB (Fig. 2b). For both Flame Seedless and 72-413 the highest numbers of embryos were obtained from ovules cultured on media supplemented with activated charcoal (Fig. 2c,2d). It seems that activated charcoal has no beneficial effect at six weeks after bloom. These results are in accordance with those of Bouquet & Davis (1989), who also found that the addition of activated charcoal increased the percentage of embryos, irrespective of culture date. Although not statistically confirmed, it was observed that the cultivars with the highest embryo percentages had ovules with higher mean masses. Cain *et al.*, (1983), Goldy & Amborn (1987) and Spiegel-Roy *et al.*, (1990) related differences in embryo percentages to the ovule size of different cultivars. More embryos were obtained from cultivars with large ovules than from those with small ovules. The highest number of embryos for all four cultivars was obtained at eight weeks after bloom.

The effect of ovule size: No correlation was found between ovule size and the presence of embryos in Sultanina ovules. Contrary to expectation, a relatively high percentage of small ovules yielded embryos and the highest percentage of ovules that contained embryos at seven and eight weeks after bloom was classed as small ovules (Fig. 3).

TABLE 1

The effect of different treatments on embryo growth and plant development *in vitro* (Muscat Seedless, 1991).

Treatment[y)]	Number of embryos	Number of germinating embryos p≤0,05	Number of plants with normal leaves p≤0,05
1	64	55[a]	35 (54,7%) [a]
2	62	39[b]	23 (37,1%) [ab]
3	65	43[b]	26 (40,0%) [ab]
4	65	53[ab]	19 (29,2%) [b]
5	68	46[b]	29 (42,6%) [ab]
6	64	55[a]	33 (51,6%) [ab]

[y)] Treatments applied to isolated embryos: (1) BMN + 16 h illumination at 26 ± 2°C (Control)., (2) BMN + darkness at 4 °C., (3) BMN + darkness at 26 ± 2 °C., (4) BMN + BA + darkness at 26 ± 2 °C., (5) BMN + GA_3 + IAA + darkness at 26 ± 2 °C., (6) BMN + GA_3 + darkness at 26 ± 2 °C.

Embryos cultured in darkness were transferred to conditions of 16 h illumination at 26 ± 2 °C after 3 weeks. Germinating embryos were counted at this stage and the data for plants with normal leaves were recorded two months afterwards.

Values in columns designated by the same symbol do not differ sigfnificantly. Values in brackets represent the percentage of embryos that developed into plants.

TABLE 2

The effect of different treatments on embryo growth and plant development *in vitro* (Sunred Seedless, 1992).

Medium	Number of embryos	Number of germinating embryos	Number of plants with normal leaves
BMN	140	107[a]	83 (59,3%) [a]
BMB	132	98[ab]	80 (60,7%) [a]
BMB + BA	140	92[bc]	59 (42,1%) [b]
BMB + GA_3	138	86[c]	51 (36,9%) [c]
BMB + GA_3 + IAA	132	85[bc]	20 (15,0%) [d]

Embryos were cultured in darkness for 3 weeks. before transference to 16 h illumination at 26 ± 2 °C Germinating embryos were counted at this stage and the data for plants with normal leaves were recorded two months afterwards.

Values between brackets showt the percentage of embryos which developed to plants with normal leaves..

Values in columns designated by the same symbol do not differ sigfnificantly (p≤0,05).

These results are in contrast to those of Bouquet & Davis (1989), who observed a correlation between the size of the ovules and viable embryos within a given cultivar. Embryo percentages in small ovules decreased when the time span increased, while the highest percentage of medium and large ovules containing embryos was found at nine weeks after bloom. A gradual increase in percentage of embryos was found in ovules that were cultured at later developmental stages (up to nine weeks) than at four and five weeks after bloom and it was concluded that Sultanina ovules should be cultured at nine weeks after bloom and only medium and large ovules should be cultured (Fig. 3).

Development of excised embryos: The addition of BA stimulated further development of embryos and germination, but not the development of plants with morphologically normal leaves. The number of embryos (excised from Muscat Seedless ovules) that eventually produced leaves were significantly higher when embryos wer subjected to a 16 h illumination period (control), compared to those obtained when embryos were cultured on medium containing BA (Table 1). During the following year the highest percentage of plants with normal leaves was obtained when Sunred Seedless embryos were cultured on the two basal media. The results for the two basal media did not differ significantly, but the number of normal plants was significantly higher compared to the treatments with plant growth regulators included in the media (Table 2). Gray *et al.*, (1990) found that the addition of 1 x 10^{-6} M BA to solid Murashige & Skoog (MS) medium stimulated rapid growth of excised embryos. However, abnormalities were recorded in these embryos. In the present study it was found that although BA stimulated embryo germination, embryos and plantlets developed abnormalities, as was also reported by Bouquet & Davis (1989). The results of the present study regarding the inefficiency of GA_3 to break dormancy are in accordance with those of Emershad & Ramming (1984) and Bouquet & Davis (1989).

CONCLUSIONS

Plants were obtained by culturing the ovules and embryos of open-pollinated seedless cultivars and it was clear that a number of factors contributed to the number of plants that eventually developed. Cultivar and ovule age at culturing greatly influenced the number of embryos rescued and it was clear that ovules should not be cultured earlier than six weeks after bloom. Depending on culture date, the addition of activated charcoal to the medium resulted in higher numbers of embryos. It may be concluded that illumination has no effect in the initial development of isolated embryos and that these embryos should be cultured on medium without growth regulators. The practice of culturing intact ovules, followed by excision of the ovules and culturing of isolated embryos on medium without plant growth regulators, ensures the preservation of the widest spectrum of genetic diversity from a specific cross. This procedure is now routinely followed in the table grape breeding programme of Nietvoorbij and the first plants obtained from seedless x seeedless crosses fruited during 1994.

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