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In vitro attempts to overcome the cross-incompatibility between *Vaccinium corymbosum* L. and *V. elliottii* Chapm. *

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Summary. Seed was readily obtained from *V. corymbosum* zygotes using embryo rescue techniques, even when embryos were cultured at proembryonic stages. Best in vitro seed development was obtained when ovules were cultured attached to placental tissues. Successful fruit and seed development in culture occurred only when the fruit was cut longitudinally or when the basal portion of the fruit was removed previous to plating. Addition of various vitamins, amino acids, and growth regulators to the nutrient medium did not increase seed production. Attempts to rescue hybrid embryos from *V. croymbosum* (tetraploid) × *V. elliottii* (diploid) crosses by in ovulo and in ovary culture gave a few presumably hybrid seed, but at a rate no greater than when normal crossing procedures are used.

Key words: Interspecific hybridization – Embryo rescue – Ovule culture – Ovary culture

Introduction

Vaccinium elliottii, a wild diploid blueberry species, has potential for use in developing cultivars adapted to the southeastern United States because of its early ripening, tolerance to dry upland soils, disease resistance, and good fruit flavor and aroma (Lyrene and Sherman 1977). Hybrids between this species and the cultivated tetraploid blueberry, *V. corymbosum*, are extremely difficult to obtain. It has been found that, in crosses between these two species, a strong postfertilization barrier prevents development of hybrid seed and that embryo abortion usually occurs before the zygote starts dividing (Munoz 1984). A relatively large number of hybrids are needed in an interspecific breeding program to adequately sample the genetic variability in the species involved and to avoid inbreeding depression in advanced generations. Since embryo and ovule culture have not previously been studied in *Vaccinium*, a series of experiments was conducted to evaluate some embryo rescue techniques that can be used when abortion occurs at proembryonic stages of development.

Materials and methods

During the flowering seasons of 1982 and 1983, pollinations were made using several cultivars and selections from the University of Florida blueberry breeding program. The 12 tetraploid clones used are considered to be *V. corymbosum* although other species are included in their genetic background (Lyrene and Sherman 1983). Diploid *V. elliottii* plants were selected from native populations in Silverhill, Alabama (Baldwin County) and LaCrosse, Forida (Alachua County). Pollen of *V. elliottii* was also collected from 12 randomly selected wild plants from LaCrosse and three plants from Baldwin, Florida (Nassau County). These two pollen mixtures were assigned the numbers FL 82-226 and FL 82-227, respectively.

Both intra- and interspecific reciprocal crosses were made in the greenhouse using potted plants. Flowers were emasculated before anthesis, and pollen was applied to the stigmas using the fingernail technique (Galletta 1975).

In ovulo embryo culture

Flowers used in these experiments were surface sterilized by immersing them for 15 min in 100 ml of a 1% sodium hypochlorite solution (ca. 20% Clorox) to which one drop of poly-

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Table 1. Modified Murashige and Skoog medium used for in ovulo and in ovary embryo culture of *Vaccinium corymbosum* and *V. elliottii* [a]

Constituent	Concentration (mg/l)
$Ca(NO_3)_2 \cdot 4\,H_2O$	684.0
NH_4NO_3	400.0
KNO_3	190.0
$MgSO_4 \cdot 7\,H_2O$	370.0
KH_2PO_4	370.0
Na_2EDTA	74.4
$FeSO_4 \cdot 7\,H_2O$	55.8
$MnSO_4 \cdot H_2O$	22.3
$ZnSO_4 \cdot 7\,H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2\,H_2O$	0.25
$CuSO_4 \cdot 5\,H_2O$	0.025
$CoCl_2 \cdot 6\,H_2O$	0.025
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxin · HCl	0.5
Thiamine · HCl	0.1
Glycine	2.0
Casein hydrolysate	500.0
Sucrose	30,000.0
Agar	4,000.0

[a] pH adjusted to 5.7 with HCl before autoclaving at 103 kPa (121 °C) for 20 min

oxyethlene sorbitan monolaurate (Tween 20) had been added. They were then rinsed five times in sterile, distilled water. Ovules were dissected out of the ovary under a laminar flow hood with the aid of a dissecting microscope. Ovules were more easily removed undamaged if the walls of the ovaries were removed first and the ovules inside each locule were removed attached to the placenta. Extracted ovules were plated on Petri dishes containing 5 ml of a highly modified Murashige and Skoog (1962) medium (Table 1).

To study the effect of time of excision, ovules from both intra- and interspecific crosses were extracted from each of the five locules and placed in culture attached or detached from placental tissues. Ovules were placed in culture every 7 days, starting about 15 days after pollination (DAP) and continuing for 4 weeks. Cultures were kept in the dark, at 25 °C for about 90 days. Ovules that developed into seed were then transfered to 0.6% water-agar and placed under indirect sunlight to induce germination.

Various additives to the basic nutrient medium were evaluated. Coconut milk (5%), five different sucrose levels (15–20–30–40–60 mg/l), three different vitamin levels, (1x–2x–3x normal concentration of myo-inositol, nicotinic acid, pyridoxin, and thiamine) were tested. For the evaluation of growth regulators, a factorial experiment was conducted in which three levels (0–2–4 mg/l) of 2-isopentyl adenine (2iP) and three levels (0.0–0.5–1.0 mg/l) of 2,4 dicholorophenoxy-acetic acid (2,4-D) were tested with or without adding 0.2 mg/l of succinic acid-2,2-dimethylhydrazide (Alar). Further testing of growth regulators included addition of 0.006 mg/l of 4-amino-3,5,6-trichloropicolinic acid (Picloram), 0.25 mg/l of ABA, and 1 mg/l of the antiauxin 7-azaindole.

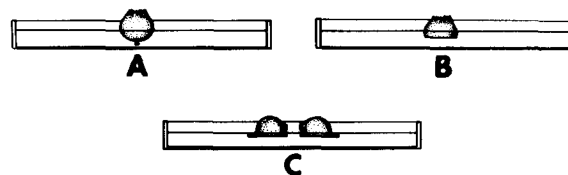

Fig. 1. Methods tested to plate young fruits for in-ovary embryo culture (see "Materials and methods")

To study the effect of auxins applied previous to the culture of the embryos, 1% naphthalene acetamide in lanolin paste was applied to the external part of the ovary at pollination (Darrow 1956). Following this treatment ovules were extracted and cultured 20 and 27 DAP as described above.

In ovary embryo culture

Surface sterilization and culture conditions were the same as for in ovulo embryo culture. Ovaries were placed in culture between 11 and 24 DAP. The following media components were tested: sucrose (15–30–60–120 g/l), alanine (0.00–0.09–0.45–0.89 g/l), glutamine (0.00–0.15–0.75–1.45 g/l), proline (0.58 g/l), and a combination of alanine, glutamine, and proline at 0.45, 0.73, and 0.58 g/l, respectively. Growth regulators tested included picloram (0.006 mg/l), 2iP (5 mg/l), and gibberelic acid (1 mg/l).

To study the effect of the position of the ovaries in the culture medium, the following treatments were tested: a) two thirds of the whole young fruit was inserted into the solid nutrient medium (Fig. 1A); b) the basal ⅓ of the fruit was cut, and the remainder of the fruit was inserted ½ way into the nutrient medium with its calyx upwards (Fig. 1B); and, c) the fruit was cut longitudinally, and both halves were placed with the cut surface in direct contact with the nutrient medium (Fig. 1C).

Results and discussion

In ovulo embryo culture

A series of preliminary experiments demonstrated that ovules cultured detached from the placenta turned brown and died during the first 10 days in culture. Culture of ovules attached to placental tissues resulted in various degrees of success depending on the stage of embryo development at the time ovules were placed in culture. Table 2 shows the effect of time of excision on seed development after intraspecific crosses. As many as 8.9% of the cultured ovules of *V. elliottii* developed into mature seeds, even if they were excised as early as 15 DAP, at a time when the zygote had just started to divide (Munoz 1984). As embryos advanced in maturity the percentage capable of developing in culture increased up to 29 DAP, when embryos had reached the cotyledonary stage. From then on, the percent of seed that attained maturity in culture did not increase further.

V. corymbosum ovules showed a similar trend in culture, but fewer ovules completed development when

Table 2. In ovulo culture of embryos from intraspecific crosses in *Vaccinium* as affected by the stage of embryo development at the time of excision

Days after pollination	Ovules placed in culture	Ovules that developed into seeds		Seed germination	
	No.	No.	%	No.	%
V. elliottii					
15	225	20	8.9	1	5.0
22	150	29	19.3	2	6.9
29	150	39	26.0	3	7.7
36	225	143	63.5	7	4.9
43	225	131	58.2	13	9.9
50	150	96	64.0	7	7.3
V. corymbosum					
14	250	6	2.4	0	0.0
21	250	3	1.2	0	0.0
28	375	35	9.3	11	31.4
35	125	97	77.6	43	44.3
42	125	61	48.8	21	34.4
49	125	69	55.2	33	47.8

Table 3. In ovulo culture of embryos from interspecific crosses in *Vaccinium* as affected by the stage of embryo development at the time of excision

Days after pollination	Ovules placed in culture	Ovules that developed into seeds		Seed germination	
	No.	No.	%	No.	%
V. elliottii (female) × *V. corymbosum* (male)					
15	225	3	1.3	0	0.0
22	150	2	1.3	0	0.0
29	150	3	2.0	0	0.0
V. corymbosum (female) × *V. elliottii* (male)					
14	250	1	0.4	0	0.0
21	250	0	0.0	–	–
28	375	0	0.0	–	–
35	375	0	0.0	–	–
42	375	10	2.7	2	20.0
49	375	3	0.8	3	100.0

cultured at proembryonic stages, i.e. before 29 DAP. As in the case of *V. elliottii,* once the cotyledonary stage of embryo development was attained, there was no further increase in the percent of ovules capable of developing in culture.

Germination of the cultured seed was low, particularly in *V. elliottii.* This may be explained, at least in part, by the fact that warm-season germination of blueberry seed is normally difficult.

Table 3 shows similar data for interspecific crosses. Less than 2% of the cultured ovules developed into seed when *V. elliottii* was the female parent, and none germinated. When *V. corymbosum* was the female parent, no ovules developed if excised before 35 DAP. Only ovules placed in culture at late stages of embryo development resulted in complete seed development in culture and in subsequent seed germination.

In ovules from interspecific crosses placed in culture, only a little cell division occurred, and growth was mainly due to rapid increase in cell size, particularly at the internal layers of the integument. Ovules usually showed rapid cell enlargement during their first 2 weeks in culture, but showed no further sign of growth or development from then on, even if transplanted into fresh medium. Since the epidermal cells of the ovule did not grow or divide, enlargement of the internal cells of the integument resulted in the rupture and cracking of the epidermis. In some cases, callus was formed from the internal cell layers of the integument, but in only one case was differentiation of this callus observed. Placental tissues also showed some growth, and often callus was formed, particularly at the point where the placenta was attached to the ovary. Placental tissues remained green during most of the culture period, and the callus that occasionally formed showed no signs of differentiation.

Anatomical observations of cultured embryos from intraspecific crosses showed that development in vitro was similar to that in vivo. In cultured ovules from interspecific crosses, the endosperm started to divide normally but disintegrated shortly after being placed in culture. The zygote, however, did not abort immediately following endosperm degeneration and instead remained alive but undivided for more than 30 days (Fig. 2).

Application to the ovaries of 1% naphthalene acetamide in lanolin paste immediately after interspecific pollinations resulted in partial parthenocarpic fruit development but no seed development. Naphthalene acetamide delayed fruit abscission and stimulated growth of the ovary walls at the sites where the paste was applied, but had no effect on ovule development. More than 2000 ovules of *V. corymbosum* were placed in culture 25 DAP following auxin treatment, but none developed into normal seed.

Growth regulators added in various combinations to the nutrient medium gave various responses, but none increased the amount of hybrid embryos obtained. Addition of 2iP resulted in the production of large masses of callus from placental tissues. Ovules showed little growth and they were almost entirely covered by callus formed in all areas of the placenta.

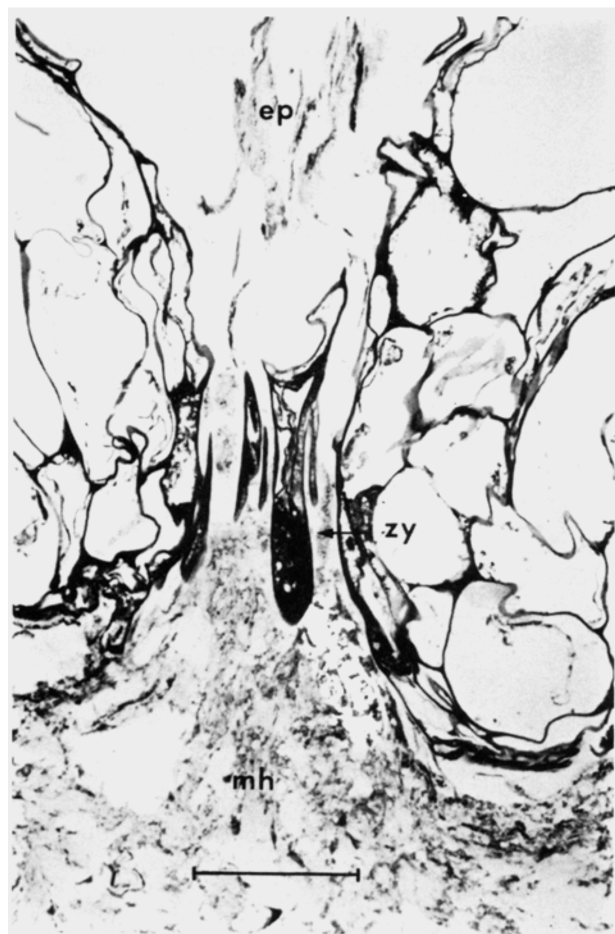

Fig. 2. Semi-thin section of a cultured embryo sac from interspecific crosses 30 days after plating, showing a large undivided zygote (*zy*) and the remnants of disintegrated cells from both the endosperm proper (*ep*) and the micropylar haustorium (*mh*). Horizontal bar 0.1 mm

After 90 days in culture, callus completely covered the undeveloped ovules. Increasing 2iP concentrations resulted in the growth of a more organized callus, particularly when 2,4-D was also present in the medium. Addition of 2,4-D was not essential for callus development, but 2,4-D added at 0.5 mg/l increased the amount of callus formed. Addition to the medium of Alar, a compound that has a reported antigibberellin action (Kochba et al. 1978), increased the final size attained by the ovules and inhibited callus growth from placental tissues, particularly when 2iP was also added. Addition of ABA to the medium had a striking effect. It completely suppressed the formation of placental callus and induced a rapid growth of the internal cells of the integument in such a way that the epidermis of the ovules was broken shortly after the ovules were plated.

Callus derived from ovules or placental tissues showed no signs of differentiation even after transfer to an auxin-free medium, except for the formation of a few roots. This root differentiation was not in response to a particular treatment, but rather occurred at random among the different treatments.

In ovary embryo culture

Position of the ovary in the culture medium had a great effect on fruit development in culture. Experiments with intraspecific crosses in *V. corymbosum* demonstrated that when whole, undisturbed young fruits were placed in culture (Fig. 1 A) they showed little growth and differentiation (Table 4), although they remained green and alive for up to 40–50 days. On the contrary, fruit that were cut longitudinally (Fig. 1 C) before plating or were plated with the basal portion of the ovary removed (Fig. 1 B) usually developed into normal looking fruit.

This striking difference in behavior might have resulted from an inability of the components of the nutrient medium to diffuse into the internal tissues of the uncut fruit. The epidermis of both fruit and leaves of blueberries possesses heavy wax depositions (Albrigo et al. 1980) which are probably not removed during surface sterilization and may constitute an important barrier to the diffusion of nutrients into the internal cells of the fruit. Cutting the fruit puts a large number of mesocarp cells in direct contact with the nutrient medium and also brings large portions of the vascular system in closer contact with it, thereby increasing the chances for rapid nutrient translocation to other parts of the fruit. An alternative explanation may be related to the wounding response induced by cutting the fruit. It is well established that tissue wounding induces various chemical changes in which several growth regulators are actively involved (Kahl 1978).

There was also an effect of the time at which ovaries were placed in culture. When fruit were cultured before the zygote started to divide, i.e. 10 DAP, they developed very few seed. When fruit were placed in culture 15 DAP, at a time when the embryos were past the globular stage of development, the number of viable seed that developed in culture was similar to the number of viable seed normally produced in vivo (Eck 1966).

An almost identical response in fruit and seed development in culture was observed when ovaries of *V. elliottii* were placed in culture (data not presented). Based on these observations, all experiments with interspecific crosses were made using ovaries from which the basal third was removed before plating.

Fruit from intraspecific crosses developed in culture in a way similar to that in nature. Even the normal changes associated with fruit ripening were observed after the fruits completed their development in culture (Table 4), which occurred about 40 days after plating.

Tables 5 and 6 show the effect on seed production of the time at which the ovaries were placed in culture following interspecific pollinations. When *V. corymbosum* was the female parent (Table 5), 8.4% of the

Table 4. Fruit development in culture after intraspecific pollinations in *Vaccinium corymbosum*

Days after pollination	Increase in fruit diameter[a] (mm)			Mature seed per fruit (no.)			Fruit ripening in culture		
	Whole[b]	Cross[c]	Long.[d]	Whole	Cross	Long.	Whole	Cross	Long.
10	0.9	5.6	5.8	0.0	1.9	2.3	no	no	yes
15	0.8	5.4	5.2	3.2	19.5	11.5	no	yes	yes

[a] Increase in fruit diameter from the time of plating to fruit maturity
[b] Whole: the whole fruit was plated
[c] Cross: the basal third of the fruit was cut before plating
[d] Long.: the fruit was longitudinally cut in halves before plating

Table 5. Seed development after crossing various clones and selections of *Vaccinium corymbosum* (female) and *V. elliottii* (male) as affected by the time at which ovaries were placed in culture

Clones or selections crossed	Days after pollination	Ovaries plated	Ovaries with seed		Seed/ ovary
		No.	No.	%	No.
V. corymbosum × *V. elliottii*					
FL 5-12 × FL 82-226	11	18	1	5.6	1.0
FL 81-166 × FL 82-226	13	22	0	0.0	–
FL 79-25 × FL 82-226	13	20	0	0.0	–
Sharpblue × FL 82-226	14	26	5	19.2	2.0
NC-1688 × FL 82-226	15	26	0	0.0	–
Avonblue × FL 82-226	18	36	6	16.7	1.7
FL 64-76 × FL 82-226	18	5	1	20.0	1.0
Flordablue × FL 82-226	18	20	3	15.0	1.7
FL 79-26 × FL 82-226	19	12	0	0.0	–
FL 64-76 × FL 82-226	24	6	0	0.0	–
Total	11–24	191	16	8.4	1.7

Table 6. Seed development after crossing various clones and selections of *Vaccinium elliottii* (female) and *V. corymbosum* (male) as affected by the time at which ovaries were placed in culture

Clones or selections crossed	Days after pollination	Ovaries plated	Ovaries with seed		Seed/ ovary
		No.	No.	%	No.
V. elliotti × *V. corymbosum*					
FL 81-198 × FL 82-229[a]	12	24	1	4.2	1.0
FL 81-198 × FL 82-229	14	48	2	4.2	1.0
FL 82-178 × FL 82-229	14	22	3	13.6	1.0
FL 82-145 × FL 82-229	15	37	3	13.6	1.0
FL 82-147 × FL 82-229	17	39	0	0.0	–
FL 82-178 × FL 82-229	19	26	1	3.8	1.0
FL 80-69 × FL 82-229	21	36	2	5.6	1.0
FL 80-69 × FL 82-229	24	10	0	0.0	–
Total	12–24	242	12	5.0	1.0

[a] FL 82-229 is a pollen mixture of 'Sharpblue', 'Avonblue', 'Flordablue', NC 1688, and FL 64-76

plated ovaries developed an average of 1.7 seed, most of which were produced when ovaries were placed in culture between 14 and 18 DAP. Non-hybrid embryos are past the globular stage of embryo development 14 DAP (Stushnoff and Palser 1969).

When *V. elliottii* was the female parent (Table 6), 5.0% of the plated ovaries developed seed, averaging one seed per fruit. The time at which the ovaries were placed in culture seemed to be less critical than when *V. corymbosum* was the female parent.

Since none of the compounds tested in the in ovulo experiments enhanced the ability of the hybrid embryos to grow in culture, a different group of chemicals was tested for the in ovary experiments. Although casein hydrolysate, one of the components of the nutrient medium used, contains all the essential amino acids, several experiments were conducted incorporating additional amounts of some of the amino acids that have been claimed to be essential. Addition of alanine, glutamine, proline, or a combination of all three did not increase the number of hybrid seed obtained, nor did they affect the developmental pattern of the cultured ovaries. The various sucrose concentrations tested did not influence the development of hybrid seed.

As in the case of in ovulo embryo culture, addition of growth regulators to the nutrient medium did not augment the ability of hybrid seed to develop in culture. A tendency to differentiate callus from ovary walls and from the sepals was observed when either 2iP and/or picloram were present in the nutrient medium. Little callus formation was observed when no growth regulators were added to the nutrient medium. Addition of GA resulted in complete parthenocarpic fruit development, but had no effect on seed development.

Results presented here indicate that both in ovulo and in ovary embryo culture are feasible after intraspecific *Vaccinium* pollinations, even at proembryonic stages of seed development. The failure to obtain viable hybrid embryos after interspecific crosses indicates that most hybrid zygotes show an intrinsic developmental failure that can not be overcome by culturing the embryos in a simple culture medium. It has been shown that when *V. corymbosum* is the female parent some hybrid zygotes will abort later during seed development (Munoz 1984). Hybrid zygotes that are capable of reaching the cotyledonary stage of development, are probably the ones that successfully developed into a normal seed in culture.

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