

Enhanced Strawberry Seed Germination through in Vitro Culture of Cut Achenes

A. Raymond Miller¹, Joseph C. Scheereus, and Patricia S. Erb

Department of Horticulture, The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, OH 44691

Craig K. Chandler

Agricultural Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 13138 Lewis-Gallagher Road, Dover, FL 33527

Additional index words. *Fragaria x ananassa*, tissue culture, hormones

Abstract. A tissue culture protocol was developed that increased the germination percentage and decreased the lag time to germination for strawberry (*Fragaria x ananassa* Duch.) achenes. This technique involved cutting surface-sterilized achenes across the embryo axis then placing the shoot apex/radicle-containing sections on semisolid Murashige and Skoog medium lacking hormones. Cut achenes began germinating 5 days after culture and achieved maximum germination (97% to 100%) in less than 2 weeks, compared to whole achenes, which began to germinate 7 to 10 days after sowing and required more than 7 weeks for maximum germination (<50%). Enhanced germination of cut achenes was a general phenomenon since achenes from 231 hybrid crosses responded similarly. Following placement on culture medium, cut achenes could be stored up to 8 weeks at 4C then removed to 27C, where germination and seedling development occurred at percentages and rates comparable to freshly cut achenes. Achenes did not require stratification before cutting to exhibit increased germination. Nearly 100% of the achenes from freshly harvested red-ripe, pink and white strawberries germinated after cutting and culture, although cut achenes from white and pink berries germinated more slowly than those from red-ripe berries. Achenes from green berries, whether whole or cut, did not germinate. This method of "embryo rescue" could be used to generate more seedlings from poorly germinating hybrid crosses, would considerably decrease the time from sowing to seedling production compared to traditional means, and would produce seedlings of uniform age for subsequent field evaluation.

Strawberry achenes often exhibit variable and/or low germination percentages. Further, emergence of seedlings may be staggered, occurring from 2 to 12 weeks after sowing (Henry, 1935; Iyer et al., 1979; Scott and Draper, 1967; Wilson et al., 1973). For a strawberry breeding program, these aspects of strawberry seedling production result in the loss of potentially valuable genotypes and evaluation of plants of different ages. Hence, the development of a technique that would consistently result in a high germination percentage occurring over a short period for numerous genotypes would be of great value.

Several treatments have been reported to affect strawberry achene germination, including acid scarification (Scott and Ink, 1948), cold treatment (Bringhurst and Voth, 1957; Thompson, 1969), light exposure (Nakamura, 1972; Scott and Draper, 1967), gibberellic acid (Nakamura, 1972; Thompson, 1969), and ethephon (Iyer et al., 1979; Wilson et al., 1973). To some extent, all of these treatments were successful. However, strawberry genotypes exhibited variable responses in maximum germination percentage, and germination still occurred over an extended period.

Recently, we reported (Miller and Chandler, 1990) that an excised shoot apex/radicle-containing portion of strawberry achenes germinated and developed into a normal seedling. In a subsequent preliminary study (Erb et al., 1989), we found that nearly 100% of the cut achenes tested germinated in less than

1 month. A similar treatment has been reported to significantly improve the germination of *Rubus* seeds (Ke et al., 1985). The present paper reports the results of four experiments that compared the temporal and developmental characteristics for the germination of cut achenes (shoot apex/radicle-containing explants) with that of whole achenes subjected to chemical and/or physical treatments previously reported to stimulate germination. In all experiments, whole (intact), untreated achenes were used as controls.

Materials and Methods

Plant material. Strawberry achenes for this study were from open-pollinated 'Catskill', a hybrid cross of 'Chandler' × 'Totem', and crosses of other selected cultivars used as parents in a breeding program. All achenes, except those from 'Catskill', were dried to 10% to 14% water content. After drying, 'Chandler' × 'Totem' achenes were stored at 4C for at least 4 months before experimentation, whereas breeding program seed lots were cold-stored for variable periods.

Achene culture and germination. In Expt. 1 (Table 1), 'Chandler' × 'Totem' achenes to be cut were surface-sterilized with 10% Clorox (0.5% final NaOCl) for 10 min, then rinsed (4×) in sterile, double-distilled (SDD) water. Cut achenes were prepared under sterile conditions in a laminar flow hood (Baker, Sanford, Maine) by cutting the sterilized achenes transversely with a scalpel (Miller and Chandler, 1990). Cut achenes were then placed in plastic-capped baby food jars containing 50 ml of MS (Murashige and Skoog, 1962) medium solidified with 0.2% (w/v) Phytagel (Sigma Chemical Co., St. Louis) and supplemented with 3% (w/v) sucrose. Comparative treatments with intact achenes were prepared under sterile conditions as follows: 1) Surface-sterilized whole achenes were placed on MS medium as described above [i.e., without hormones (control)] or on MS medium containing various hormone combinations. N-(phenyl-

Received for publication 11 Sept. 1990. Accepted for publication 12 Nov. 1991. Journal Article no. 249-90. Salaries and research support provided in part by state and federal funds appropriated to the Ohio Agricultural Research and Development Center and a Seed Grant to A.R. M. from The Ohio State Univ. We thank Greg Breneman, Jennifer Kyler, Susan Buckingham, and Jamie Chapman for their excellent technical assistance, and Bonnie Beck for typing the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement solely* to indicate this fact.

¹To whom reprint requests should be addressed.

Table 1. Total germination percentage and time to maximum germination of whole, acid-scarified, mechanically scarified, and cut 'Chandler' x 'Totem' strawberry achenes.

Achene treatment	Germination medium	Germination (%)	Time for maximum germination (months)
<i>Experiment 1</i>			
Whole			
Untreated	Soilless mix	27 abc ^a	3
	Moistened filter paper	39 bcd	3
	MS medium/no hormones	32 abcd	3
	MS medium/5 μ M BA + 5 μ M NAA	47 cd ^y	2.5
	MS medium/5 μ M BA	57 d ^y	2.5
	MS medium/5 μ M NAA	33 abcd ^y	2.5
	MS medium/1 μ M GA ₃	53 d ^y	2
	MS medium/5000 ppm ethrel	37 abcd ^x	3
Acid-scarified	Moistened filter paper	13 a	2.5
	MS medium/no hormones	21 ab	3
Cut	MS medium/no hormones	97 e	0.5
<i>Experiment 2</i>			
Whole			
Untreated	Moistened filter paper	10 a	2
Machine-scarified	Moistened filter paper	20 a	2
Hand-scarified	Moistened filter paper	27 a	2
Punctured			
hilum	Moistened filter paper	63 b	1.25
Cut	Moistened filter paper	100 c	0.25

^aValues represent the mean where n = 6 replicates, Mean separation within an experiment by Duncan's multiple range test ($t = 0.05$).

^ySeedlings emerged from achenes, but then formed callus.

^xSeedlings emerged from achenes, but became chlorotic and were stunted.

methyl)- 1H-purin-6-amine (BA) and 1-naphthaleneacetic acid (NAA) were added to MS medium before being autoclave, whereas gibberellic acid (GA₃) and 2-chloroethylphosphonic acid (ethrel) (adjusted to pH 5.8 with 2 N NaOH) were filter-sterilized, then added to the cooled, autoclave medium. 2) Surface-sterilized whole achenes were germinated in 100 x 15-mm petri dishes containing two layers of sterile Whatman #1 filter paper moistened with 5 ml SDD water or on the surface of moistened soilless mix containing equal parts of vermiculite and perlite (v/v) held in plastic-wrap-covered pots. 3) Dry, whole achenes were acid-scarified in cold, concentrated HCl for 15 min, then rinsed (4x) in SDD water. Scarified, sterile achenes were cultured on MS medium (no hormones) or germinated on moistened filter paper as described above.

Experiment 2 (Table 1) compared germination of cut 'Chandler' x 'Totem' achenes to that of whole achenes after physical scarification treatments or to that demonstrated by a control (unscarified). Scarification treatments were as follows: 1) Hand scarification was accomplished by abrading individual achenes (4x) between facing sheets of sandpaper (3MK 413Q Wet or Dry Tri-m-ite A wt. W2). 2) Achenes were scarified mechanically for 60 min at 35 kPa (supplied by compressed air) in a Hoffman seed scarifier (Hoffman Manufacturing, Albany, Ore.) equipped with 3M sandpaper (Crystal Bay Crocus Cloth NT1). 3) Scarification was also achieved by puncturing achenes through the hilum with a no. 20 gauge syringe needle (i.e., boring into the achene to breach the endocarp without cutting it and without damaging the shoot apex/radicle-containing portion of the em-

bryo). As in Expt. 1, cut and control seed lots were prepared by surface-sterilizing whole achenes in sodium hypochlorite before treatment (cut achenes only) and subsequent placement on the germination medium. In contrast, achenes subjected to scarification procedures were surface-sterilized after treatments were performed. All achenes in Expt. 2 were germinated using the moistened filter paper technique described above.

The effect of fruit maturity at harvest on cut achene germination was examined in Expt. 3 (Table 2). Open-pollinated 'Catskill' achenes were isolated from fresh receptacles at the green, white, pink, and red-ripe stages of development. Immediately after isolation (i.e., without drying or cold treatments), achenes were surface-sterilized, cut, and then cultured on hormone-free MS medium as described for Expt. 1. Corresponding whole achenes serving as controls were comparably surface-sterilized and cultured.

A fourth experiment was designed to determine whether cut achenes could be stored before germination. 'Chandler' x 'Totem' achenes were surface-sterilized, treated, and then placed on hormone-free MS medium as above (Table 3). Jars containing cut achenes were stored (4C, dark) for 0, 3, 7, 14, 28, or 56 days before their removal and placement under standard germination conditions.

For all experiments, achenes were germinated at 27 \pm 1C under a 12-h photoperiod supplied by General Electric F40-W warm-white fluorescent lamps (fluence of 100 μ mol \cdot m⁻²·s⁻¹ photosynthetically active radiation).

Each experiment was repeated at least twice with at least three replicate jars, pots, or petri dishes per experiment and a minimum of five achenes per replicate. All treatments were scored daily for seedling germination as evidenced by protrusion of the root and shoot.

Table 2. Effect of fruit maturity on the germination percentage of cut and whole, nonstratified achenes from 'Catskill' strawberries.

Weeks after sowing	Fruit maturity					
	Seed of white		Seed of pink		Seed of red-ripe	
	Whole	Cut	Whole	Cut	Whole	Cut
<i>Germination (%)</i>						
1	0 \pm 0 ^x	17 \pm 3	---	---	0 \pm 0	73 \pm 22
2	---	---	0 \pm 0	0 \pm 0	0 \pm 0	83 \pm 17
3	0 \pm 0	67 \pm 7	0 \pm 0	97 \pm 3	0 \pm 0	90 \pm 10
4	0 \pm 0	77 \pm 9	3 \pm 3	97 \pm 3	0 \pm 0	90 \pm 10
7	13 \pm 9	83 \pm 7	30 \pm 6	97 \pm 3	23 \pm 12	100 \pm 0

^aValues represent the mean \pm SE (n = three replicates, each containing 10 achenes).

^xNot determined.

Table 3. Effect of storage of cut achenes at 4C on subsequent germination of achenes from 'Chandler' x 'Totem' strawberries.

Duration of storage at 4C (days)	Germination (%)	Time to maximum germination (weeks)
0	94 \pm 3 ^x	2
3	92 \pm 4	3
7	90 \pm 4	3
14	70 \pm 4	2
28	64 \pm 9	3
56	72 \pm 7	3

^aValues represent the mean \pm SE (n = 10 replicates, each with 10 seeds).

Results and Discussion

In our initial experiment, whole 'Chandler' × 'Totem' achenes sowed on soilless mix, moistened filter paper, or MS medium lacking hormones exhibited < 40% germination (Table 1, Expts. 1 and 2). Scarification of whole achenes with acid or treatment with 5 μ M BA + 5 μ M NAA, 5 μ M NAA alone, or 5000 ppm etrel did not enhance germination. Germination of whole achenes increased slightly (up to 57% total) in the presence of 5 μ M BA or 1 μ M GA₃, but these seedlings became stunted or formed callus (Table 1, Expt. 1). In our next experiment, we attempted to affect germination of achenes by mechanically scarifying the endocarp (Table 1, Expt. 2). As shown, neither machine nor hand scarification significantly enhanced germination, compared to untreated achenes. Both of these treatments removed substantial portions of the endocarp, as observed by dark-field microscopy (data not shown), and further scarification resulted in severe damage to the embryo.

A further consideration with all of the treatments in Expts. 1 and 2, except cutting, was that it took from 2 to 3 months for maximum germination to occur. Therefore, although the above treatments have been reported to increase germination of seeds from other plant species (Hartmann and Kester, 1959) and achenes from certain strawberry genotypes (Bringhurst and Voth, 1957; Iyer et al., 1979; Nakamura, 1972; Scott and Draper, 1967; Scott and Ink, 1948; Thompson, 1969; Wilson et al., 1973), they apparently have little effect on others, including 'Chandler' × 'Totem'.

Two treatments that did significantly enhance germination and yielded normal seedlings were puncturing of the endocarp near the hilum region and cutting achenes across the embryo axis. Puncturing did increase germination, relative to untreated, whole achenes, but it still took 5 weeks to achieve maximum germination. Further, the total germination percentage for punctured achenes was less than that for cut achenes (Table 1, Expt. 2).

Cutting achenes and then placing the shoot apex/radicle-containing portion on MS medium lacking hormones significantly increased total germination of 'Chandler' × 'Totem' achenes to a level higher than that observed with any other treatment (> 97%; Table 1, Expts. 1 and 2). Further, maximum germination occurred in 2 weeks or less. During a detailed time course examination, we found that most achenes germinated (> 70%) within the first week after cutting and sowing the achenes (Fig. 1). Embryo swelling was seen after 3 days, and germinated seedlings were clearly evident after only 5 days (Fig. 1, inset). Germination of cut achenes began to plateau after 7 days. For comparison, germination of whole achenes, regardless of acid scarification, was not observed until after at least 7 days, began to plateau after 6 to 7 weeks, and did not reach completion until 13 weeks (Fig. 1). Ke et al. (1985) reported that nicking *Rubus* seeds increased germination and decreased the time to maximum germination, relative to whole seeds. Thus, this technique may be applicable to many rosaceous crops with a hard endocarp surrounding the embryo. The utility of the cut achene technique is further demonstrated by its success with numerous genotypes. To date, we have germinated more than 97% of more than 15,000 achenes from 231 divergent strawberry crosses (data not shown).

Factors other than embryo germination are involved in seedling survivability, since not all of the cut achenes that germinated produced mature field-grown plants. Only 56% of the cut achenes from all hybrid crosses survived in the field. The remainder (44%) were lost at previous handling steps. The largest

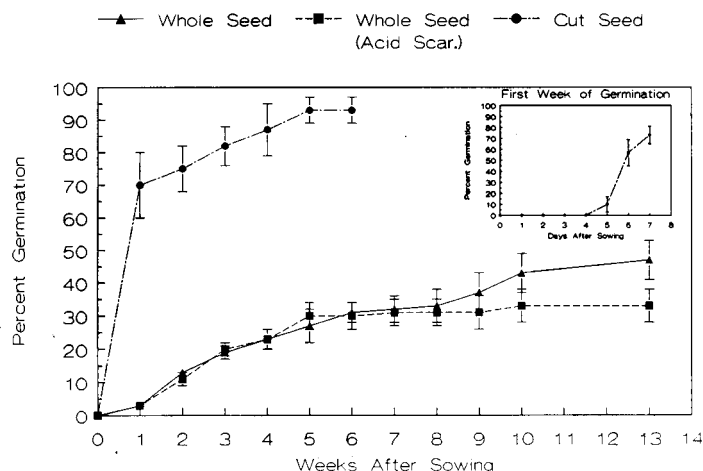


Fig. 1. Germination time course of whole, whole (acid-scarified), and cut 'Chandler' × 'Totem' achenes cultured on MS medium lacking hormones. Inset shows germination of cut achenes during the first week after cutting and culture. Bars indicate SE.

loss (25%) occurred at the in vitro culture step, and the majority of these (22%; 25% minus 3% not germinated) exhibited developmental abnormalities (no root development, no apical meristem, etc.). Seedling losses in the mist bench, greenhouse, and field were 14%, 4%, and 1%, respectively. The overall survival rate for seedlings from cut achenes (56%) was higher than the germination rate for whole achenes (10% to 39%, Table 1). Seedling losses in the mist bench, greenhouse, and field for whole achenes could be expected to be similar to seedling losses from cut achenes. Thus, cutting achenes offers a distinct advantage to increase final seedling counts. However, genotypic variation was evident, since seedling survivability ranged from 0% to 100%, depending on the hybrid source of the achenes.

On MS medium supplemented with sucrose, cut achenes underwent normal development into seedlings. When cut achenes were placed on moistened filter paper, the germinating seedlings died within 1 week after germination. Apparently, the germinating embryos required sucrose and/or other nutrients in the culture medium to replace the energy source normally found in the cotyledons, which were removed by the cutting process. Acclimatization to greenhouse conditions was necessary before seedlings could be transplanted to the field. The total time for the production of field-ready seedlings was 3.5 months.

Strawberry achenes normally require an extended cold treatment (up to 4 months) to achieve maximum germination, and only achenes from mature fruit are germinated (Bringhurst and Voth, 1957; Thompson, 1969). In an attempt to overcome these limitations of using whole achenes, we subjected achenes from freshly harvested immature and mature fruit to cutting and in vitro germination (i.e., the achenes were not dried or stratified). We found that our technique eliminated the need for this cold treatment, and achenes from immature strawberries germinated (Table 2) and produced normal seedlings. Untreated whole achenes from white, pink, and red-ripe fruit germinated poorly (13%, 30%, and 23%, respectively), and germination was not evident until at least 4 weeks after sowing. By contrast, cut achenes from white and red-ripe fruit germinated within 1 week, and cut achenes from pink fruit germinated within 3 weeks, with those from red-ripe fruit germinating more rapidly and at higher percentages than cut achenes from white and pink fruit. Germination, regardless of fruit maturity, was maximum by 7 weeks after sowing and > 80% (Table 2). Achenes from green

fruit, whether whole or cut, did not germinate (data not shown), probably due to immaturity of the embryo.

A drawback of cutting achenes to achieve maximum germination is that it is labor-intensive. In our laboratory, it took 6 weeks (≈ 225 person hours) to cut and culture 15,000 achenes, which results in the planting and evaluation of seedlings of different age. To overcome this age-related problem, we tested the effectiveness of cold storage to arrest germination and development of cut achenes. Cut achenes stored for 3 or 7 days at 4C then placed at 27C exhibited germination percentages similar to the cut unstored control (Table 3). Cold storage for longer periods (14, 28, or 56 days) decreased germination compared to the controls; however, the germination percentage for these achenes ($> 60\%$) was still higher than that observed for whole achenes ($< 40\%$; Table 1, Fig. 1). Further, germination of the cut, stored achenes occurred in the same time period as the unstored controls (Table 3), and seedlings from these stored achenes appeared normal and developed into healthy plants (data not shown). Hence, many cut achenes could be placed in cold storage, then these achenes could be moved to suitable germination temperatures to produce seedlings of uniform age for subsequent planting.

We do not know the means by which cutting affects strawberry achenes to allow more rapid germination and higher total germination percentages than with control achenes. From preliminary experiments, we know that the lack of germination by whole achenes is not due to embryo inviability, since whole achenes that have not germinated after 2 months on MS medium will germinate within 7 days after cutting and placement on fresh hormone-free MS medium (Miller, unpublished). Possible explanations for the cut achene effect are: 1) cutting may mimic mechanical scarification, which makes the tissue surrounding the embryo permeable to water and gases (Hartmann and Kester, 1959). However, treatments that scarified the endocarp tissue did not enhance germination of whole achenes (Table 1). Puncturing the endocarp to allow gas exchange and water uptake doubled the overall germination percentages, but did not achieve the percent germination of cut achenes (Table 1). Therefore, cutting must weaken additional barriers that are unaffected by normal mechanical scarification. 2) The hard strawberry endocarp simply acts as a physical barrier to expansion and germination of the embryo, and cutting is an effective means to separate the endocarp from the embryo. 3) Strawberry achenes may contain one or more endogenous germination inhibitors. After cutting the achenes, the inhibitor(s) would diffuse away from the embryo and germination would occur. The hypothesis that strawberry achenes contain potential inhibitors, such as cyanogenic glycosides found in many rosaceous plants (Corm, 1980) has not been tested.

The cut achene technique we described offers several advantages over other methods used to germinate whole strawberry seeds: 1) Compared to germination of whole achenes in sphagnum, the time for production of field-ready plants from cut achenes is significantly reduced (6 to 8 months for whole achenes; 3.5 months for cut achenes), primarily because cutting of

achenes eliminates the need for cold treatment to achieve maximum germination. Also, the majority of cut achenes germinate within 1 week of sowing, whereas whole achenes may take 2 months. 2) Overall, a higher percentage of seedlings from cut achenes survive for field evaluation compared to whole achenes, probably due to higher germination of cut achenes, since losses of seedlings after germination of cut and whole achenes were similar. 3) The cut achene technique is effective for germinating all genotypes tested to date. Treatments intended to improve germination of whole achenes are genotype-dependent. 4) Cutting achenes can be used to rescue progeny from desirable hybrid crosses that exhibit poor or no germination. The technique also allows the evaluation of larger populations of plants from hybrid crosses that produce few achenes. 5) The cut achene technique enables one to plant and evaluate seedlings or plants of uniform age, since the majority of achenes germinate over a short period. If one has hundreds or thousands of achenes to germinate, achenes cut at various times can be stored on MS medium at 4C for up to 2 months, then all can be germinated at the same time by placing them at 27C.

Literature Cited

- Bringham, R.S. and V. Voth. 1957. Effect of stratification on strawberry seed germination. *Proc. Amer. Soc. Hort. Sci.* 70:144-149.
- Corm, E.E. 1980. Cyanogenic compounds. *Annu. Rev. Plant Physiol.* 31:433-451.
- Erb, P. S., A.R. Miller, J.C. Scheerens, and C.K. Chandler. 1989. Enhanced strawberry seed germination by in vitro culture of embryo axis sections. *HortScience* 24:71. (Abstr.)
- Hartmann, H.T. and D.E. Kester. 1959. *Plant propagation: Principles and practices*. Prentice-Hall, Englewood Cliffs, N.J. p. 124-125.
- Henry, E.M. 1935. The germination of strawberry seeds and the technique of handling the seedlings. *Proc. Amer. Soc. Hort. Sci.* 32:431-433.
- Iyer, C. P. A., E.K. Chacko, and M.D. Subramaniam. 1979. Ethrel for breaking dormancy of strawberry seeds. *Curr. Sci.* 39:271-272.
- Ke, S., R.M. Skirvin, K.D. McPheeters, A.G. Otterbacher, and G. Galletta. 1985. In vitro germination and growth of *Rubus* seeds and embryos. *HortScience* 20:1047-1049.
- Miller, A.R. and C.K. Chandler. 1990. Plant regeneration from excised cotyledons of mature strawberry achenes. *HortScience* 25:569-571.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Nakamura, S. 1972. Germination of strawberry seeds. *J. Jpn. Soc. Hort. Sci.* 41:367-375.
- Scott, D.H. and A.D. Draper. 1967. Light in relation to seed germination of blueberries, strawberries, and *Rubus*. *HortScience* 2: 107-108.
- Scott, D.H. and D.P. Ink. 1948. Germination of strawberry seed as affected by scarification treatments with sulfuric acid. *Proc. Amer. Soc. Hort. Sci.* 51:299-300.
- Thompson, P.A. 1969. The use of chilling and chemical treatments to promote rapid germination of strawberry achenes. *J. Hort. Sci.* 44:201-210.
- Wilson, D., A. Goodall, and J. Reeves. 1973. An improved technique for the germination of strawberry seeds. *Euphytica* 12:362-366.