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Rapid In Vitro Multiplication of Non-Runnering *Fragaria vesca* Genotypes from Seedling Shoot Axillary Bud Explants

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Abstract: *Fragaria vesca* L. has become a model species for genomic studies relevant to important crop plant species in the Rosaceae family, but generating large numbers of plants from non-runner-producing genotypes is slow. To develop a protocol for the rapid generation of plants, leaf explants were compared to single axillary bud shoot explants, both from in vitro-grown *Fragaria vesca* seedlings, as sources of shoots for new plant production in response to benzyladenine (BA) or thidiazuron (TDZ) combined with indolebutyric acid (IBA) on Murashige and Skoog's Basal Salt (MS) medium. BA at 2.0 and 4.0 mg L^{-1} and TDZ at 1.5 mg L^{-1} promoted the greatest number of shoots produced per shoot explant. There were no IBA effects or IBA interactions with BA or TDZ. Significant interactions between BA and IBA, but not TDZ and IBA, occurred in leaf explant callus formation and % explants with callus at 6 and 9 weeks of culture and on shoots per leaf explant at 9 weeks. TDZ treatments produced uniformly high levels of callus but low numbers of shoots. The treatment generating the most shoot production was BA at 4.0 mg L^{-1} plus IBA at 0.50 mg L^{-1}. After 9 weeks of culture, leaf explants of the non-runner-producing genotype Baron Solemacher had generated 4.6 shoots per explant with the best treatment, while axillary bud explants had generated 30.8 shoots with the best treatment. Thus, in vitro culture of shoot axillary bud explants can generate high numbers of clonal shoots from a single seedling plant in vitro.

Keywords: in vitro multiplication; alpine strawberry; TDZ; BA; IBA; non-runnering; shoot explant

1. Introduction

Fragaria vesca is a self-pollinating diploid species that has become a model species for the commercial strawberry (*F. X ananassa* Duch.) and other members of the Rosaceae family because it has a small genome (240 Mb), a short generation time, and an available full genome sequence [1–3]. In order to facilitate research in ameliorating the qualitative (i.e., flavonoid biosynthesis and other polyphenols) and quantitative characters of the plant with *F. vesca* [4], a large number of replicate clonal plants are required for individual experiments. Most *Fragaria* species produce clonal plants on stolons (commonly called runners) that develop from axillary buds [5], but there are some important non-runnering genotypes within *F. vesca* [6], notably the *F. vesca semperflorens*, that flower constantly, with progeny that are primarily seed-derived and do not produce any runners (stolon, vegetative self-propagating unit).

Studies of in vitro micropropagation of *F. vesca* have successfully regenerated shoots from leaf and petiole explants using combinations of benzyladenine (BA) and indolebutryic acid (IBA) following

transformation by *Agrobacterium* [7–11]. *F. vesca* leaf explants placed abaxial side up regenerated shoots more rapidly than those placed adaxial side up after an *Agrobacterium* transformation treatment [11]. No difference was observed in the regeneration response of leaf explants for *F. vesca* versus *F. vesca semperflorens*, runnering versus non-runnering phenotypes, respectively, although differences in petiole responses did differ as more shoots were produced by the latter form at comparable levels of BA plus IBA [10]. However, genotypic variation in post-transformation shoot regeneration was noticed in *F. vesca* genotypes [11]. Thidiazuron (TDZ) was shown to replace BA and promote callus and shoot initiation from *F. vesca* leaf explants [12,13], although a *F. vesca* × *F. vesca semperflorens* interspecific hybrid produced fewer shoots than *F. vesca* in response to TDZ [14], suggesting some genotype sensitivity to TDZ. TDZ may also reduce subsequent shoot elongation [2]. Within the majority of these studies, it was not clear how the transformation and subsequent selection protocols may have determined shoot regeneration potential separately from the inherent capacity for such potential within each genotype. Regeneration of plants after callus formation, as is common in transformation studies, leads to much greater somaclonal variation among progeny than after meristem micropropagation [15], and this lack of uniformity is a problem for subsequent genetic studies.

Even though *F. vesca* may be self-pollinated, seed-derived populations of genotypes that do not produce stolons exhibited some level of variability [2], necessitating lengthy periods of controlled intraspecific pollination of each genotype of interest to create reasonably uniform homozygous populations for subsequent research. Even without transformation, reliable and rapid in vitro propagation techniques for substantially and rapidly increasing the number of clonal plants from non-runnering genotypes for physiological and molecular studies are desirable. To date, there have been no protocols for shoot regeneration from shoot explants (i.e., a shoot with an axillary meristem) of non-runnering *F. vesca* genotypes. Thus, the present study was performed to establish such a protocol by (1) comparing the rates of new shoot production from seedling shoot axillary bud explants versus leaf explants, (2) determining the effective concentrations of BA, TDZ, and IBA for in vitro shoot regeneration from both leaf and shoot explants of *Fragaria vesca*, (3) determining if genotype has an effect on the responses, and (4) assessing if adaxial versus abaxial placement affects leaf explant response.

2. Materials and Methods

2.1. Plant Material

Seeds of four *F. vesca* genotypes, the non-runnering Baron Solemacher (*F. vesca semperflorens)*, rarely runnering Pineapple Crush, and runnering types Ivory and Yellow Wonder, were collected from several self-pollinating, individual plants of each genotype grown in a greenhouse at the University of Kentucky, Lexington, KY, USA, washed under tap water, and air-dried.

2.2. Seed Germination and Culture

Seeds were dipped into 30% Clorox bleach (*v/v;* 2.5% sodium hypochlorite) plus 10% sodium dodecyl sulfate (SDS) (*v/v*) solution for 20 min. Seed was then rinsed with sterilized water 3 times under a laminar flow hood. After surface sterilization, 20 seeds were placed in sterile 20 mL Petri dishes containing 4.4 g L^{-1} Murashige and Skoog's Basal Salt (MS) (Sigma® M5524) [16], 30 g L^{-1} sucrose, and 7 g L^{-1} Bacto agar (BD-Difco®) for germination. The medium was prepared by adjusting the pH to 5.7 prior to autoclaving at 121 °C and 105 kPa for 70 min. After 14 days of germination, 3 seedlings with little growth were transferred to each of the 50 mL jars containing the same medium to allow for better growth. Seed germinated in two to three weeks.

2.3. Explant Culture

Leaf lamina (36 mm^2) and shoot (6–8 mm) explants were excised from 5 week-old sterile, in vitro Baron Solemacher seedlings in order to regenerate shoots. The shoot explant consisted of a piece of the main stem, a petiole base, and an axillary meristem at the petiole base. To assess the effect of different

combinations and concentrations of plant growth regulators (PGRs) on shoot regeneration, two excised shoot explants were placed in each sterile 20 mL Petri plate containing MS Basal Salt with the following treatments: benzyladenine (BA) at 2 or 4 mg L^{-1} or thidiazuron (TDZ) at 1 or 1.5 mg L^{-1}, each with indole-3-butyric acid (IBA) at 0.125, 0.25, or 0.50 mg L^{-1}. Thus, there were 12 treatment combinations (BA+IBA or TDZ+IBA) in total and each was replicated 4 times. Possible genotypic variation was compared using four shoot explants from each of the 4 genotypes on 2 mg L^{-1} BA versus 1.5 mg L^{-1} TDZ, each combined with 0.25 mg L^{-1} IBA. There were 4 replicates of each genotype by treatment combination. In a third experiment, leaf explants were placed adaxial side up versus abaxial side up using the set of BA+IBA or TDZ+IBA treatments described above. With leaf explant placement as an additional treatment, there were 24 total treatments, each replicated 4 times. The Petri dishes were held in a laboratory at 22 °C temperature under fluorescent lighting with an 18 h daylength.

After 6 and 9 weeks of culture, callus production by each explant was rated as: 0 = no callus, 1 = low quantity of callus, 2 = medium quantity of callus, or 3 = high quantity of callus. After 10 weeks, regenerated shoots were transferred to Petri plates containing half-strength MS medium without growth regulator for rooting. The mean value of each set consisting of two explants in each Petri plate was considered as a replication. All the experiments were conducted in a completely randomized design (CRD).

Shoot number per shoot explant and the number of shoot explants producing new shoots were recorded after 6 and/or 9 weeks of culture. Shoot explants did not produce visible callus. With leaf explants, relative callus production and the number of new shoots per explant were recorded after 6 and 9 weeks of culture, and the % of explants producing callus was calculated. Analyses of variance (ANOVA) were performed (SigmaPlot 12.0, Systat Software, Inc., San Jose, CA, USA), and means were compared using the Student–Newman–Keuls method at $P < 0.05$. Results were expressed as least squares means ± standard error of the mean.

3. Results and Discussion

3.1. Shoot Axillary Bud Explants

BA at 2.0 and 4.0 mg L^{-1} and TDZ at 1.5 mg L^{-1} produced more shoots per axillary bud explant than with TDZ at 1 mg L^{-1} (Table 1). Preliminary work indicated that BA at 2 mg L^{-1} plus IBA at 0.125 mg L^{-1} had no effect compared to BA alone (data not shown), and IBA concentration up to 0.5 mg L^{-1} had no main effect and did not interact with BA or TDZ (data not shown). Thus, BA or TDZ alone were sufficient to generate new shoots from shoot explants. BA at 2 mg L^{-1} had a higher % of explants producing shoots than TDZ at 1 mg L^{-1}, with the remaining treatments at intermediate values (Figure 1).

Table 1. Effect of 6-benzylaminpurine (BA) and thidiazuron (TDZ) on shoot regeneration from shoot explants of *Fragaria vesca*. Data were collected 6 weeks after initiating the study.

BA or TDZ	Concentration (mg L^{-1})	Shoots per Explant	% Explants Producing Shoots
BA	BA 2.0	6.4 a [z]	100 a
BA	BA 4.0	7.3 a	86 ab
TDZ	TDZ 1.0	2.9 b	67 b
TDZ	TDZ 1.5	5.6 a	88 ab

[z] Mean separation by the Student–Newman–Keuls method at $P < 0.05$.

There was a genotype-by-treatment interaction on the number of shoots produced per shoot axillary bud explant (Table 2). With IBA at 0.25 mg L^{-1}, Baron Solemacher on TDZ at 1.5 mg L^{-1} produced more shoots per explant after 9 weeks of culture than those on BA at 2.0 mg L^{-1}. Baron Solemacher shoot explants on BA and TDZ produced equal numbers of shoots by 6 weeks of culture (Table 1), although the total numbers were considerably lower than those of the shoots shown in Table 2.

The longer 9-week culture period in the latter experiment led to more total shoot production and perhaps to the treatment difference, although the other cultivars in the latter experiment did not show the same BA versus TDZ difference observed with Baron Solemacher. It has been reported that the best shoot proliferation was obtained with 1 mg L^{-1} TDZ and 0.2 mg L^{-1} IBA from leaf explants of *F. vesca* cultivars (43.9% explants formed shoots) [14].

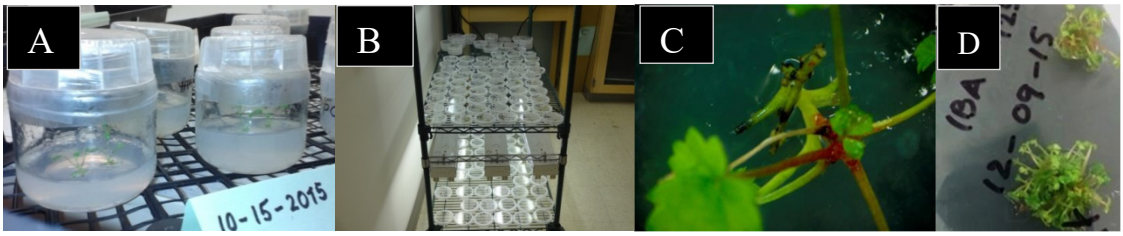

Figure 1. (**A**) Transfer of seedlings cv. Baron Solemacher to bottles with MS medium. (**B**) Setup of experiment. (**C**) Baron Solemacher single axillary bud shoot explants producing new shoots. (**D**) Baron Solemacher with single axillary bud shoot explants produced the maximum number of shoots at BA 2.00 (mg L^{-1}) + IBA 0.125 (mg L^{-1}).

Table 2. Effect of genotype and BA at 2.0 mg L^{-1} plus IBA at 0.25 mg L^{-1}, or TDZ at 1.5 mg L^{-1} plus IBA at 0.25 mg L^{-1}, on shoot regeneration from shoot explants of *Fragaria vesca*. Data were collected after 9 weeks of culture.

Genotype	BA or TDZ	Shoots per Explant
Baron Solemacher	BA	15.0 b [z]
Baron Solemacher	TDZ	30.8 a
Pineapple Crush	BA	22.4 ab
Pineapple Crush	TDZ	26.0 ab
Ivory	BA	27.0 a
Ivory	TDZ	26.9 ab
Yellow Wonder	BA	22.6 ab
Yellow Wonder	TDZ	29.6 a

[z] Mean separation by the Student–Newman–Keuls method at $P < 0.05$.

3.2. Leaf Explants

Starting at 4 weeks of culture, the cut edge of leaf explants exhibited the start of callus formation. From the callus, reddish-colored shoots and light-green leaves then developed. There were significant interactions between BA and IBA on the relative amount of callus formation and % explants with callus at 6 and 9 weeks of culture, and on shoots per leaf explant at 9 weeks (Figure 2, Table 3). Except for an interaction of TDZ at 1 mg L^{-1} with IBA on relative callus formation, there was no other TDZ–IBA interaction at 6 or 9 weeks. A high level of callus production did not always result in high shoot production, as the TDZ treatments produced uniformly high levels of callus but low numbers of shoots. The treatment generating the most shoot production was BA at 4.0 mg L^{-1} combined with IBA at 0.50 mg L^{-1}. Although IBA did not have an effect on shoot regeneration by shoot explants, as noted above, it did have an increasing effect on regeneration from leaf explants with BA. Only the treatment combination of BA at 2 and 4 mg L^{-1} and IBA at 0.125 mg L^{-1} did not produce any shoots through 9 weeks of culture.

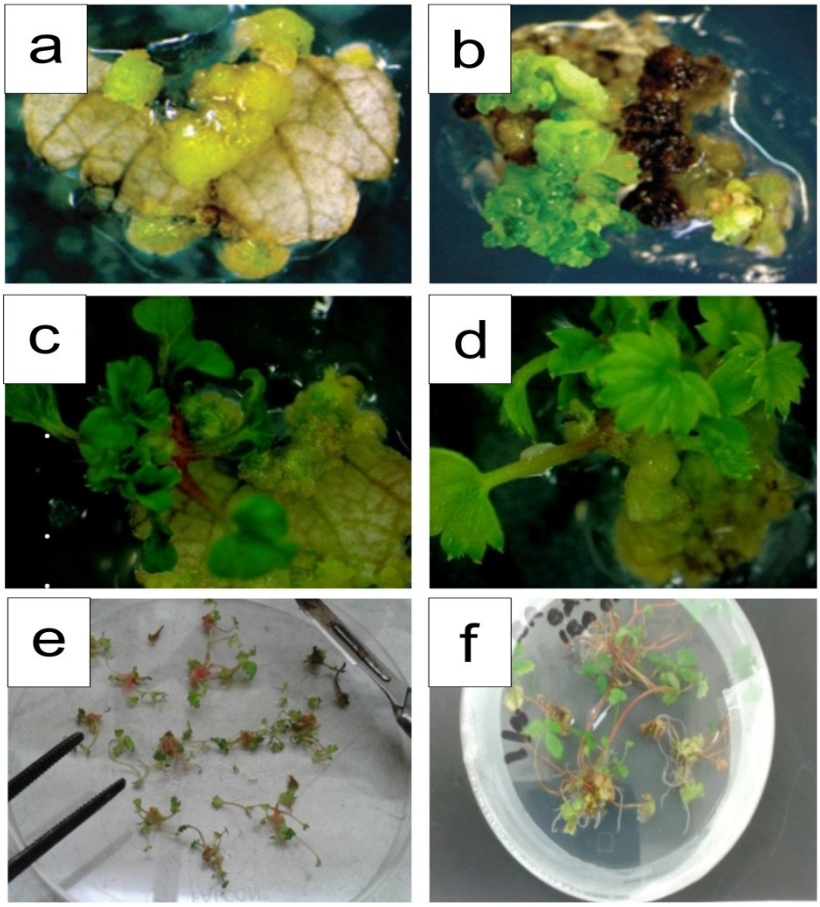

Figure 2. Shoot formation in 'Baron Solemacher' leaf discs treated with 6-benzylaminpurine (BA; 4.0 mg L^{-1}) plus indolebutyric acid (IBA; 0.5 mg L^{-1}). (**a**) Callus formation after six weeks in culture, (**b**) shoot formation after nine weeks, (**c**) abaxial side up with BA (4.0 mg L^{-1}) plus IBA (0.5 mg L^{-1}), (**d**) adaxial side up with BA (4.0 mg L^{-1}) plus IBA (0.5 mg L^{-1}), (**e**) plantlets, and (**f**) rooted plantlets.

Table 3. Effects of BA, TDZ, and IBA on relative callus formation [z], % explants with callus, and shoots per explant from seedling leaf explants of *Fragaria vesca* cv. Baron Solemacher.

PGR (mg L^{-1})			Six Weeks of Culture			Nine Weeks of Culture		
BA	**TDZ**	**IBA**	**Relative Callus Production [z]**	**Explants with Callus (%)**	**Shoots per Explant**	**Relative Callus Production**	**Explants with Callus (%)**	**Shoots per Explant**
2.0	0	0.125	0.25 c [y]	25 c	0 NS	0.33 bc	33 b	0 c
2.0	0	0.25	1.33 b	100 a	0.4	1.83 ab	100 a	0.85 b
2.0	0	0.50	1.42 b	83 ab	0	1.83 b	92 a	0.5 b
4.0	0	0.125	0 c	0 c	0	0 c	0 c	0 c
4.0	0	0.25	1.13 b	81ab	0.9	1.38 b	81 a	1.1 b
4.0	0	0.50	2.31 a	100 a	2.9	2.5 a	100 a	4.6 a
0	1.0	0.125	1.13 b	100 a	0	1.5 b	100 a	0.4 b
0	1.0	0.25	1.67 ab	100 a	0.4	2.08 ab	100 a	0.9 b
0	1.0	0.50	1.75 a	92 ab	0.4	1.92 ab	100 a	1.0 b
0	1.5	0.125	1.25 b	92 ab	0.2	1.5 b	92 a	1.4 b
0	1.5	0.25	0.83 bc	67 b	0	1 bc	75 a	0.4 b
0	1.5	0.50	1.50 b	100 a	0	1.94 ab	100 a	0.3 b

[z] Ratings were as follows: 1 = explant with low callus production; 2 = explant with medium callus formation; 3 = explant with high callus formation. [y] Mean separation within columns by the Student–Newman–Keuls method at $P < 0.05$. PGR: plant growth regulators.

F. vesca leaf explants cultured on MS media containing 1.5 mg L^{-1} TDZ + 0.5 mg L^{-1} IBA showed the best shoot production across a set of TDZ by IBA concentrations, but overall IBA had no effect, as noted in the present study when combined with TDZ, or even reduced shoot production as the concentration increased [13]. In another study, better shoot proliferation was recorded with TDZ plus IBA than without IBA in two *F. vesca* genotypes from leaf explants [14]. Neither of these cited studies compared TDZ to BA levels.

The placement of the leaf explants exerted a significant influence on explants with callus and % explants with callus, with leaf explants placed abaxial side up producing more callus, but not shoots, than those placed adaxial side up after 6 and 9 weeks of culture (Table 4, Figure 2). Placement did not interact with BA, TDZ, or IBA (data not shown). When young *F. vesca* leaf explants were placed abaxial side up on MS medium, shoot regeneration occurred in all the treatments with BA and IBA [8].

Table 4. Effect of leaf placement—adaxial or abaxial side up—on relative callus formation, % explants with callus, and shoots per explant from leaf explants of *Fragaria vesca* cv. Baron Solemacher.

	Six Weeks of Culture			Nine Weeks of Culture		
Placement of Leaf Explant	**Relative Callus Production**	**Explants with Callus (%)**	**Shoots per Explant**	**Relative Callus Production**	**Explants with Callus (%)**	**Shoots per Explant**
Adaxial side up	1.03 b [z]	75 b	0.52	1.34 b	79	1.29
Abaxial side up	1.40 a	82 a	1.42	1.63 a	83	2.55

[z] Mean separation within columns by the Student–Newman–Keuls method at $P < 0.05$.

In vitro placing of regenerated shoots from all tissue sources, genotypes, and treatment conditions on half-strength MS medium without PGRs resulted in 100% rooting (data not shown) (Figure 2). A high level of root formation on MS basal medium in *F. vesca* has been noted [13]. All plants in these studies were successfully acclimatized to a greenhouse environment via a mist bed and culture in containers (Figure 3). There was no visible evidence of phenotypic variation within genotypes following one year of plant growth (data not shown).

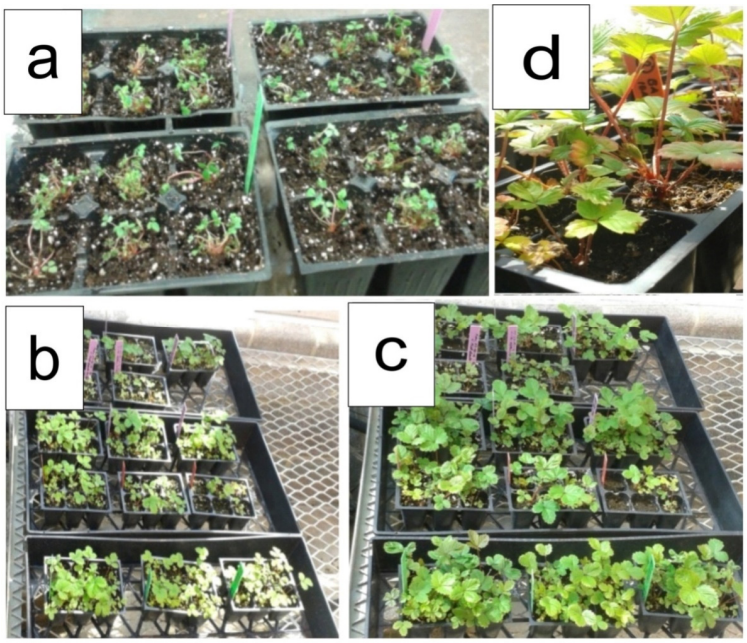

Figure 3. (**a**) Rooted plantlets placed in ProMix BX in a plastic pot in a mist chamber; (**b**) rooted plantlets in the greenhouse after 2 weeks in the mist chamber; (**c**) plants after being acclimatized in the greenhouse; (**d**) acclimatized plants with very good growth in the greenhouse.

4. Conclusions

An effective in vitro shoot regeneration protocol for *Fragaria vesca* was demonstrated from both shoot axillary bud and leaf explants. For shoot explants, BA at 2 and 4 mg L^{-1} and TDZ at 1.5 mg L^{-1} were the best treatments for shoot regeneration. However, there were no effects of IBA concentration on shoot regeneration. Only Baron Solemacher among the four *F. vesca* genotypes (including Pineapple Crush, Ivory, and Yellow Wonder) showed a difference between use of TDZ versus BA. From leaf explants, 4 mg L^{-1} BA plus 0.5 mg L^{-1} IBA resulted in the maximum callus production and number of shoots per explant, and the IBA effect increased from 0.125 to 0.5 mg L^{-1}. In contrast, TDZ promoted high callus production but resulted in reduced numbers of shoots, and IBA concentration had no effect on the production of callus and shoots. Leaf explants placed abaxial side up produced more callus but no more shoots than those placed adaxial side up. After 9 weeks of culture, Baron Solemacher leaf explants had generated 4.6 shoots per explant with the best treatment (Table 3), while shoot explants from Baron Solemacher had generated 30.8 shoots with the best treatment (Table 2). Thus, the protocol for using shoot axillary bud explants is a better alternative to leaf explants for generating high numbers of clonal shoots from a single seedling plant in vitro, avoiding callus production and with the possibility of a large homozygous population of plants for the non-runnering *F. vesca* genotypes.

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