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Haploid plant regeneration from anther cultures of three north american cultivars of strawberry (Fragaria x ananassa Duch.)

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Summary. A study was conducted to maxmuze plant regeneration frequencies from cultured anthers of' Chandler', 'Honeoye', and 'Redchief strawberries (Fragaria x ananassa Duch.). A comparison of auxins (IAA, NAA), cytokinins (BA, BPA, KIN) and carbohydrates (sucrose, glucose, maltose) in MS medium showed that the highest shoot regeneration across cultivars (8%) occurred when using a medium containing 2 mgtl IAA, I mgtl BA, and 0.2 M glucose. A comparison ofMS, NN, and HI inorganic medium (a new formulation based on the anther culture literature) solidified with either agar or gellan gum and containing IAA, BA, and glucose, showed the highest shoot regeneration across cultivars (19%) when using HI and gellan gum. Lastly, media containing Fe-EDTAyielded more shoots than media containing Fe-Metalosate, and anthers cultured on Fe-EDT A media in darkness for 30d followed by 30d in white light produced more shoots (16% average regeneration) than those cultured on Fe-EDTA media under white or yellow light (16h photoperiod) for the initial 30d (0.3% and 5% respectively). Plants were acclimated ex vitro where they flowered and set :fruit. Chromosome counts of root tip cells confirmed that haploid plants were obtained from all three cultivars.

Abbreviations: IAA: indoleacetic acid, NAA: naphthaleneacetic acid, BA: 6-benzylarninopurine; BP A: N-benzyl-9-(2-tetrahydropyranyl)-adenine, KIN: 6-furfurylaminopurine; MS: Murashige & Skoog (I 962); NN: Nitsch & Nitsch (1969)

Introduction

Cultivated strawberry **(**Fragaria x ananassa Duch.) is a highly heterozygous, octoploid species (2n = 8x = 56). Traditional breeding efforts to improve strawberry quality and yield are labor intensive, costly, and time-consuming, since many generations of crossing and selection are routinely required for cultivar development. Reducing the ploidy level of breeding material via androgenesis may accelerate plant improvement efforts by more direct exposure of genetic traits at the haploid level, by phenotypic expression of gametes for assessment of pollen-donor potentials, and, together with chromosome redoubling techniques, by the production of highly homozygous lines for fwther use as parental lines.

Several laboratories have attempted to regenerate haploid plants via anther culture of a number of Fragaria x ananassa cultivars (Fowler eta/. 1971; Hennerty eta/. 1987; Laneri & Damiano 1981: Quarta eta/. 1991; Rosati eta/. 1975; Sayegh & Hennerty 1989; Svensson & Johansson 1994). In these investigations, however, haploid plants were not obtained. Successful regeneration of haploids from anther culture has been reported for only four cultivars, produced and cultivated in Europe (Niemirowicz-Szczytt & Zakrzewska 1981; Niemirowicz-Szczytt et a/. 1983). Haploid plants have also been produced by crossing F. x ananassa with Potentilla species where the Potenti/la chromosomes have been eliminated after hybridization (Niernirowicz-Szczytt 1987). In one of these studies (Niemirowicz-Szczytt & Zakrzewska 1981), a combination of IAA,

BA, and 2,4-dichlorophenoxyacetic acid (2,4-D) was favorable to haploid plant regeneration, with an overall haploid regeneration frequency of approximately 2.5%. Unfortunately, use of this combination of growth regulators did not result in the production of either callus or shoots in preliminary experiments in our laboratory using several North American cultivars. This is not surprising, given the very broad genetic base from which modem, North American cultivars have been developed (Sjulin and Dale 1987). Therefore, a series of experiments was designed to investigate the cultural influences on callus and shoot regeneration from anther cultures of several North American cultivars.

Specifically, the objective of the present study was to successfully regenerate shoots and haploid plants from three current, commercial, North American strawberry cultivars in sufficient quantities to be useful in a strawberry breeding program. Further, we wished to examine and quantify the effects of several cultural factors (plant growth regulators, carbohydrates, inorganic basal media, culture medium gelling agent, iron chelate, and light exposure and quality) on shoot regeneration and haploid plant production across all three cultivars examined in order to develop a procedure with the potential to be applicable to a range of North American cultivars. These factors were examined in three separate experiments, for the purpose of manageability, and sequenced in terms of their expeCted influence, from highest to lowest, after an examination of previously published tissue culture media developed for a range of plant genera, including Fragaria.

Materials and Methods

In all experiments, three cultivars were examined ('Chandler', a California variety, and 'Honeoye' and 'Redchief, two varieties developed for the eastern United States). All treatments utilized 10 ml semisolid medium (see individual experiments) in 15 x 60 mm petri plates, 15 anthers **(1** mm length, uninucleate stage) per plate, and five plates per treatment (75 anthers per treatment). Floral buds were surface-sterilized by a 30 sec. dip in 70% ethanol, followed by inunersion in a 5.25% sodium hypochlorite solution for 20 min. and 3 rinses in sterile, distilled water. Contamination rates were very low (1-2%, data not shown). All media contained MS vitamins (Murashige & Skoog 1962) and were prepared to obtain a post-autoclave pH of5.8 (Owen et aL 1991).

Experiment 1: Two auxins (2 mglliAA or 0.2 mgll NAA), three cytokinins (BA, BPA, or KIN at 1 mgll), and three carbohydrates (0.1 M sucrose, 0.1 M maltose, or 0.2 M glucose) were examined in corrected MS medium (Owen & Miller 1992) solidified with 0.6% Phytagar (Gibco, Grand Island, NY). BPA is structurally similar to BAP, and has recently become commercially available. Glucose was used at 0.2 M to give equivalent moles of available monosaccharide; however, osmotic potentials would be different. A total of 4050 anthers were cultured. Callus formation after 30d dark culture (21-24C) and shoot formation after an additional30d light exposure (16h photoperiod) were recorded.

Experiment 2: Three inorganic medium formulations [MS; Nitsch & Nitsch 1969 (NN); and H1] and two gelling agents (0.6% Phytagar or 0.15% Gelrite (Carolina Biological)]

were examined in media containing the growth regulators and carbohydrate shown to produce the highest overall shoot regeneration frequencies from experiment 1 (2 mglliAA, **1** mgll BA, and 0.2 M glucose). HI inorganic medium was formulated after a comprehensive examination of basal media reported for successful androgenesis from a broad range of plant species (fable 1). Successful anther culture techniques have used a number of different of medium formulations. Thus, H1 medium was formulated to take into account several of these differences (see discussion). A total of 1350 anthers were cultured. Callus formation after 30d dark culture (21-24C) and shoot formation after an additional 30d light culture (16h photoperiod) were recorded.

Table 1. Composition of H1 Medium

	M.W.	mg/l	Molarity
NH_4NO_3	80.05	400	5mM
KNO_3	101.10	2022	20mM
KH_2PO_4	136.09	272	2mM
$MgSO_4 \cdot 7H_2O$	246.38	246	1mM
$CaCl_2 \cdot 2H_2O$	146.99	294	2mM
$FeSO_4 \cdot 7H_2O$	277.91	27.8	100µM
Na_2-EDTA$\cdot 2H_2O$	372.25	37.2	100µM
H_3BO_3	61.84	6.2	100µM
$CoCl_2 \cdot 6H_2O$	237.85	0.024	100nM
$CuSO_4 \cdot 5H_2O$	249.61	0.025	100nM
$MnSO_4 \cdot 4H_2O$	223.00	22.3	100µM
$Na_2MoO_4 \cdot 2H_2O$	241.92	0.24	1µM
KI	166.02	0.17	1µM
$ZnNa_2$-EDTA$\cdot 4H_2O$	471.63	9.4	20µM

Experiment 3: Three light/dark treatments during the initial 30d callus formation period [16h photoperiod white light (130 µmol m-2 sec1 light intensity), 16h photoperiod under a yellow #2208 filter (Almac Plastics, Inc., Akron Ohio)(Stasinopoulos & Hangarter 1990), or dark culture] and two iron chelates [100 µM Fe-EDT A or Fe-Metalosate (Albion Laboratories, Clearfield Utah)] were examined in the medium shown to produce the highest overall shoot regeneration frequencies across cultivars from experiment 2 (2 mg/I IAA, 1 mg'I BA, 0.2 M glucose, H1 inorganic medium, and 0.15% Gelrite). A total of 1350 anthers were cultured. Callus formation after 30d and shoot formation after an additional 30d light exposure (16h photoperiod) were recorded. Regenerated shoots were rooted in the same medium devoid of growth regulators for 8-12 weeks, transplanted into coarse sand in 10 em pots, and acclimated ex vitro. Chromosome counts from root tips of the 34 plants which were successfully acclimated were made according to Owen & Miller (1993).

Results and Discussion

The best combination of auxin, cytokinin, and carbohydrate for callus formation and shoot production across all three cultivars (53% and 8%, respectively) was 2 mg/1 IAA, I mg/1 BA, and 0.2 M glucose (Table 2). This combination is very similar to that used previously in strawberry micropropagation media (Boxus 197 4), and confirms its utility for androgenesis as well as organogenesis. Kinetin was found to be virtually ineffective for stimulating shoot formation at the levels tested in this study. BP A was shown to be only moderately effective. Of the three carbohydrates tested, glucose was moderately effective for callus proliferation (46%) and superior for shoot regeneration (2.9%) across the three cultivars and five growth regulators examined. This may be due to the higher osmotic potential of the medium containing 0.2 M glucose than media containing 0.1 M disaccharide. Cultivar differences were observed, particularly for carbohydrate source; however, the combination of glucose/IAA/BA resulted in shoot formation for all three cultivars examined, and thus it was used in the second experiment.

The influence of inorganic basal media and medium gelling agents (experiment 2) on callus formation and shoot regeneration from cultured anthers are listed in Table 3. Across all cultivars and gelling agent treatments, HI inorganic medium was similar to MS and NN inorganic medium formulations for callus formation (61% vs. 59% and 61%, respectively), but superior for plant regeneration (17% vs. 6% and 9%, respectively), illustrating that a medium may influence cell division and organ regeneration events differently. Notable differences between HI and either MS or NN include a significant reduction of ammonium nitrate (400 mg/1 vs. 1650 mg/1 and 720 mg/1, respectively), an increase of potassium phosphate (272 mg/1 vs. 170 mg/1 and 68 mg/1, respectively), and an increase of potassium nitrate (2022 mg/1 vs. 1900 mg/1 and 950 mg/1, respectively). Anthers cultured on either Phytagar or Gelrite regenerated shoots; however, the combination of HI and Gelrite produced a greater percentage of shoots across all three cultivars (19%) than HI and Phytagar (15%). Therefore, HI basal medium and Gelrite were used in the third experiment.

Iron in plant tissue culture media has been shown to catalyze the photo-oxidation ofEDTA to formaldehyde and is involved in light-induced IAA degradation (Hangarter & Stasinopoulos 1991). These types of photochemical changes in culture media can be prevented with a yellow long-pass filter (Stasinopoulos & Hangarter 1990). In the present study, we examined the influence of light and iron chelating agent on shoot regeneration frequencies, since the regeneration medium contained IAA. The combination of iron chelating agent and light/dark treatment (experiment 3) produced interesting results (Table 4). In this experiment, callus formation and shoot regeneration were reduced when anther cultures containing Fe-EDTA were exposed to yellow light during the first 30d and shoot regeneration was virtually eliminated when the cultures were exposed to white light. These results show that light is inhibitory to plant regeneration from strawberry anther cultures, presumably by some mechanism other than via EDT Amediated IAA degradation.

Table 2. Influence of cultivar, carbohydrate source, and auxin/cytokinin treatment on anther culture response (n=75 for each treatment). All media contained MS inorganics and vitamins and were solidified with 0.6% Phytagar.

Treatment	Chandler		Honeoye		Redchief		average	
	% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots
Sucrose								
IAA + BA	65a*	19a	49abc	9a	57ab	0b	57ab	9a
IAA + BPA	45abcd	4bcde	53ab	1bc	56ab	4ab	51abcd	3abcde
IAA + KIN	43abcd	0e	29defg	0c	23e	0b	32fgh	0f
NAA + BA	32d	3cde	31efg	0c	23e	0b	29h	1def
NAA + BPA	64a	3cde	43abcde	1bc	35cde	0b	47abcd	1def
NAA + KIN	35cd	1de	25fg	0c	25e	0b	28gh	0ef
Maltose								
IAA + BA	61ab	11ab	43abcde	1bc	47abcd	4a	50abcd	5abc
IAA + BPA	49abcd	8abcd	39abcdef	0c	34bcde	0b	41cdef	3def
IAA + KIN	41bcd	0e	25fg	0c	23e	1ab	30gh	0ef
NAA + BA	63ab	8bcde	36bcdefg	0c	49abc	3ab	49abcd	4cdef
NAA + BPA	51abcd	7bcde	43abcdef	0c	51abc	5a	48abcd	4bcde
NAA + KIN	49abcd	0e	29efg	0c	51abc	0b	43bcdef	0f
Glucose								
IAA + BA	53abc	12abc	57a	9ab	49abc	3ab	53abc	8ab
IAA + BPA	48abcd	5abcde	31cdefg	3bc	53abc	4ab	44abcde	4abcd
IAA + KIN	63a	0e	35bcdefg	0c	24e	0b	41defg	0f
NAA + BA	56ab	4bcde	55ab	4abc	60a	0b	57a	3bcdef
NAA + BPA	49abcd	1de	48abcd	3bc	51abc	4ab	49abcd	3def
NAA + KIN	53abc	0e	23g	0c	29de	0b	35efgh	0f

* Values within columns followed by different letters are significantly different (P=0.05).

Table 3. Influence of cultivar, culture medium gelling agent, and basal medium on anther culture response (n=75 for each treatment). All media contained MS vitamins, 2 mg/l IAA, 1 mg/l BA, and 0.2 M glucose.

Treatment		Chandler		Honeoye		Redchief		average	
		% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots
Phytagar									
	MS	79a*	7ab	45ab	11bc	53ab	1b	59a	6c
	NN	72a	7a	53ab	13ab	48b	4ab	58a	8bc
	H1	81a	21a	48ab	15ab	55ab	9ab	61a	15ab
Gelrite									
	MS	72a	0b	48ab	3c	57ab	11ab	59a	5bc
	NN	84a	9a	57a	11bc	49b	7ab	63a	9abc
	H1	79a	13a	41b	28a	64a	16a	61a	19a

* Values within colums followed by different letters are significantly different (P=0.05).

Table 4. Influence of cultivar, iron chelating agent, and light/dark treatment on anther culture response (n=75 for each treatment). All media contained H1 basal medium, MS vitamins, 2 mg/l IAA, 1 mg/l BA, 0.2 M glucose, and 0.15% Gelrite.

Treatment		Chandler		Honeoye		Redchief		average	
		% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots	% forming callus	% forming shoots
Fe-EDTA									
	Light	43bc*	1b	8c	0b	4c	0b	18c	0c
	Yellow filter	61ab	4ab	40b	5b	35b	7ab	45b	5b
	Dark	77a	13a	65a	20a	68a	16a	70a	16a
Fe-Metalosate									
	Light	12c	0b	5c	0b	3c	0b	7c	0c
	Yellow filter	59ab	4b	60ab	7b	69a	7ab	63ab	6b
	Dark	79a	3b	52ab	5b	57ab	7ab	63ab	5b

* Values within columns followed by different letters are significantly different (P=0.05).

Anthers cultufed on a mediwn containing Iron Metalosate (an amino acid chelate formulation developed for field application). behaved similarly to anthers cultured on a medium containing Fe-EDTA for both callus formation and shoot regeneration when cultures were exposed to white or yellow light, but produced fewer shoots than anthers cultured on a medium containing Fe-EDTA when cultures were incubated in darkness for the initial30d. Additionally, anthers cultured on a medium containing Iron Metalosate and incubated in darkness showed a plant regeneration frequency similar to anthers incubated under yellow light. These results demonstrate that Fe-Metalosate is less effective for shoot regeneration than Fe-EDTA at equal concentrations when cultures are incubated in darkness, but unlike Fe-EDTA it is not inhibitory ii1 the presence of yellow light. Additional experiments would be needed to determine if other concentrations of Fe-Metalosate would be effective in increasing plant regeneration frequencies to equal or surpass the frequencies observed with Fe-EDTA for cultures incubated in darkness.

Table 5. Chromosome counts of regenerated plants

Genotype designation	Mean	SD	Genotype designation	Mean	SD
C3	53.4	3.2	C24	52.0	7.7
C4	49.2	7.5	C25	32.6	3.3
C5*	28.4	2.4	C26	27.4	2.1
C6	52.8	2.4	C27	50.8	6.4
C7*	27.2	1.9	H2*	28.0	2.6
C8	55.6	1.0	H9*	26.0	0.9
C9	55.2	1.2	H10	55.2	2.5
C10	53.6	3.4	H13	52.6	3.4
C11*	28.4	2.1	H14	54.4	1.5
C13	55.8	2.6	H15*	28.0	1.4
C14*	27.2	1.5	H16	53.8	2.2
C15	55.6	2.4	R3	52.6	3.9
C16	54.8	2.5	R4*	29.0	1.7
C18	54.8	1.9	R6	50.0	2.8
C19	54.2	2.9	R9*	27.4	2.0
C21	54.6	3.8	R11	53.8	3.2
C23*	27.8	2.5	R13	53.6	3.5

C= Chandler, H= Honeoye, R= Redchief, *= haploid range, n= 10

Chromosome counts of regenerated plants are listed in Table 5. Of the 34 plants which survived acclimation to greenhouse conditions, II exhibited chromosome nwnbers within the haploid range (n = 4x = 28), giving an overall haploid regeneration frequency of0.8% for the fmal experiment. This frequency is lower than the frequency reported by Niemirowicz-Szczytt and Zakrzewska (1981) for European cultivars, but it is within an acceptable range for generating haploids for use in a breeding program. It was not determined whether the 23 plants having chromosome numbers within the diploid range

were regenerants from somatic cells, or from gametic cells that had doubled during in vitro culture, producing ditetrahaploids. The acclimated plants were grown in a greenhouse and have since flowered and set fruit. Thus, fertility was maintained at the tetrahaploid level, suggesting their potential, after chromosome doubling, for use as homozygous parental material for a traditional plant breeding program. By the anther culture method described in this paper (HI medium, 2 mg/IIAA, I mg/1 BA, 0.2M glc, 0.15% Gelrite, 30d darkness, 30d light incubation), haploid plants can be produced at frequencies of 0.4-1% for the three North American cultivars examined. These frequencies are sufficient to allow plant breeders the opportunity to better describe the genetic compliment of desirable cultivars and also use haploid plants in much the same way as inbred lines are used in traditional plant breeding programs. Further studies would be needed to confirm gametic ploidy levels of regenerants (for possible use in interploidy crosses), to determine fertilization mechanisms (male and/or female fertility), and to quantify phenotypic characteristics of the regenerants, including strawberry quality attributes.

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