AGRICULTURAL RESEARCH FOUNDATION FINAL REPORT
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Title: Validating the model medium formulations for a wider range of blueberry cultivars

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Project background and justification

Micropropagation is important for a wide range of nursery crops. However, because individual cultivars differ in their cultural requirements, developing growth media for specific and unique cultivars is often difficult. Micropropagation can be also difficult for some elite clonal stocks and is not well developed for newer cultivars or for some species that are used for advanced breeding. Blueberry production in Oregon was 134 million pounds in 2018, making Oregon one of the top blueberry producers in the USA. Many of the 14,500 acres of blueberry plants were produced through micropropagation at commercial nurseries in Oregon.

New tissue culture growth media are typically developed by minor modifications to "standard" media formulations. This approach, though useful for species or cultivars that respond well to the "standard" formulations, is not useful for the more difficult cultivars and species where growth is suboptimal. What is required is a systematic approach to efficiently evaluate the large number of components in a typical formulation (there are thirteen essential plant mineral nutrients). Design of Experiment software can be used to improve many aspects of micropropagation media (Niedz and Evens, 2007). These types of studies allow observation of unique responses related to the genetics of the plants studied. This approach is now used to develop optimized tissue culture media for a wide range of plants. Early studies developed medium to grow pears that had long been difficult to culture in vitro (Reed et al., 2013, Wada et al., 2013, Wada et al. 2014). A similar process produced an optimized growth medium for hazelnuts that allowed widespread use of in vitro plants for expanding the Oregon hazelnut industry (Hand et al., 2014; Akin et al., 2018).
Our initial studies of blueberry media using computer aided design and analysis provided models for five blueberry cultivars that may be useful for wider use in blueberry micropropagation. That study evaluated the effects of five groups of mineral nutrients on five cultivars and provided models for optimized growth medium formulations. Therefore, validating the four optimized model formulations from our prior studies by comparing them with some commercially most used blueberry culture media such as Woody Plant Medium (WPM) and Preece's Blueberry Medium (PBM), would allow determining their suitability and productivity, as well as wider use of the model formulations for cultivars with diverse genetic backgrounds.

Project objectives

1. Test a wide range of commercially important blueberry genotypes on WPM, PBM and four experimental formulations from the models developed in earlier studies.
2. Publish finalized blueberry formulations for used by the industry.

Methods and timelines

Plant materials: Genetically diverse blueberry cultivars including hybrids were used for this study (Table 1).

Shoot cultures were grown in Magenta GA-7 containers with 50 ml medium/box with a transfer to fresh medium every 5 weeks. Growth room conditions at 24°C under a 16-hour photoperiod with an average of 76 $\mu Mm^{-2}s^{-1}$ radiation provided by cool white fluorescent lamps. Shoots were planted on each treatment medium (Table 2) in replicated with 4 boxes with 9 shoots per container (Figure 1). Cultures were grown for 5 weeks and transferred twice more to the same treatment medium for a total of 15 weeks of culture. Containers for each cultivar were randomized as four blocks on the growth room shelf. Data was taken at 15 weeks after three passages on each fresh medium and at the end of three passages, each representative four shoots were photographed for the relative visual comparisons of growth forms in six different media (Fig.2).

Data. Growth responses remeasured for 10 plants per box for each treatment (n=30) in five categories: quality (a subjective assessment of plant appearance (1 = poor, 2 = acceptable and 3 = good); shoot multiplication (shoots counted); shoot length (measured in mm); leaf color (1 = red/yellow 2 = light-green, 3 = dark green); leaf size (1 = large, 2 = medium, 3 = small).

Statistical analysis. Data taken was analyzed with analysis of variance (ANOVA: Table 4) and means were separated by Duncan's multiple range test and Dunnette's test for the significance (each medium was compared to the control medium WPM) using SAS program 9.4 ver. (SAS Institute, Cary, NC, USA).

Experimental process.

1. Blueberry explants were multiplied until establishment of enough numbers for the testing of the formulations with 6 chosen experimental mineral nutrition combinations (Table 2 and 3).

2. Experimental media of the combinations including WPM (as control), four formulations selected from our prior results on blueberry mineral nutrition trials by response surface methodology using the computer modeling programs (Design Expert 8) and PBM. Chemicals for 6 treatments were calculated for pipetting (Table 3.). With 6 (1,1-dimethylallyl-amino)-purine (2-ip) 3 mg/L, Sucrose 30 g/L, and hardened with agar (A1111, Phytochemicals) 6.5 g/L, pH adjusted as 5.7, then will be autoclaved 20 minutes.

3. First experiment with 6 treatments on 5 bluberry genotypes (Table 1) was set up, five genotypes × Six treatments (Table 2), a total 120 containers. A total 1,080 explants were planted. Experiment will be repeated three times for total 15 weeks.

4. Six media were formulated following Table 3.

5. Nine explants contains only 2 nodes were cut out and planted (Fig. 1) on each media combinations following Table 2 and 3 (50 ml/Magenta GA-7 container) for 4 replicates per genotype. Total 120 containers with 9 explants each, total 1,080 blueberry explants are planted.

6. After 5 weeks cultured on the six experimental media, the explants were transferred to each fresh media for another five week subcultures for two more times, aiming for achiving stabilizing effects on the five blueberry genotypes.

7. Data was taken and statistically analyzed using the SAS program (9.4 ver.) for the final report.

Results and Findings

1. ANOVA analysis (Table 4) indicated that tested media treatment effects on four genotypes (Draper, Misty, Ochlockonee, and Tophat) were highly significant with the lowest *P-value* <0.0001 while R^2 numbers closer to 1.0, as well as, the higher *F-value* more significant effects (Table 4). One genotype 'Texas tree' showed different outcome with higher p-values (less effects) in two categories (shoot length *P-value=0.2149*, leaf size *P-value= 0.5916*) of growth responses, but still had the lowest p-value in other two categories (overall quality and leaf color number).

2. Table 5 summarized the results of means separation by Duncan's Multiple Range Test and comparisons of the significance between each medium (experimental medium A,B,C,D, and PBM) compared to the control. These analysis indicated that the best experimental medium as of #4 medium where the most significant improvement effects were shown on quality of all five diverse buleberry genotypes.

3. The sample photo in Fig. 2 shows explants of 'Draper' planted on all six experimental media, medium A, B, C, D, WPM, and PBM (orders of GA-7 container from left to the right) at 4 weeks subcultured. The result of SAS analysis on 'Draper' for overall quality ratings (1. Poor - 3. good) in Table 5 also showed the difference among the six-tested media (A, B, C, D, WPM and PBM) evidently (WPM as the control).

4. Fig. 3 provides the visual comparisons on each growth morphologies of five blueberries subcultured on six media at the end of 15 weeks. Four shoots per container were taken out from predetermined positions and photographed. All the photos in this table has been

adjusted and modified as the same size to fitting into the table aiming in only visual references, however the actual growth measurements (mean numbers) used for the statistical analysis are presented in Table 5.

5. Box pot graphs for data distribution of five growth responses on all blueberry genotypes are presented in Fig. 4.

6. The chemical composition of the best medium '#4' determined from this study largely differs from WPM (control) for number of elements (see Table 3), and the most substantial differences identified as 2-3 times higher the concentrations of; NH_4 NO_3 and Ca $(NO)_3$ for 2 times, KH_2PO_4, $CaCl_2$, K_2SO_4, and $MgSO_4$ for 3 times, compared to the WPM control.

Summary

This is the final study concluding our series of research developing an optimal blueberry growth medium that enhance productivity of micropropagation and can also be widely used for different cultivars with diverse genetic backgrounds. Our prior studies of in vitro blueberry media using computer aided design that evaluated the effects of five groups of mineral nutrients on various cultivars and provided models for growth medium formulations. In this study the four model formulations chosen from prior studies and Preece Blueberry Medium (PBM) by comparing them with commercially most used Woody Plant Medium (WPM as the control) that allowed to determine the best in vitro blueberry growth medium as #4 as result.

Benefit to the nursery industry

The beneficiaries are micropropagation nurseries, commercial nurseries and growers. The best in vitro growth media useful for diverse blueberries will allow faster propagation and make microplant production more profitable and the plants more available to the nursery industry. Findings from this study will strongly support improved blueberry micropropagation and enhanced productivity for commercial micropropagation laboratories and expanding nurseries.

Tables and Figures

Table 1. Plant materials (five diverse blueberry genotypes) used for this final experiment

Species	Cultivar	Type
Vaccinium arboretum Marshal	'Texas tree', 'Sparkleberry'	Huckleberry, Winterberry
V. ashei Reade	'Ochlocknee'	Rabbiteye
V. corymbosum L.	'Draper'	Northern High Bush
V. corymbosum × *V angustifolium* Aiton. hybrid	'Tophat'	Half High
V. corymbosum × *ashei* hybrid	'Misty'	Southern High Bush

Table 2. Test media –As a factor of Woody Plant Medium (WPM) concentration (×)

Experimental Formulations	Calcium Nitrate	Ammonium Nitrate	Potassium Sulfate	Mesos Components (KH_2PO_4, $CaCl_2$, and $MgSO_4$)
A*	1	1	3	2
B*	2	1	3	3
C*	2	2	1	1
D*	2	2	1	3
WPM **	1	1	1	1
PBM ***	3.3	2.3	1.3	1.3/1.7/3.1

** Four formulations (A to D) chosen from our prior mineral nutrition screenings with five diverse blueberry cultivars (Table 1).*

*** Woody Plant Medium (WPM)*

**** Preece Blueberry Medium (PBM) (factors shown as × WPM)*

Table 3. Chemical combinations of 6 experimental media: active ingredients calculated (mg/L)

Chemicals (mg/L)	A	B	C	D	WPM*	PBM
NH_4NO_3	400	400	800	800	400	908
$Ca(NO_3)_2$	386	772	772	772	386	1,262
KH_2PO_4	340	510	170	510	170	217.5
$CaCl_2$	145	217	72.5	217.5	72.5	122.5
K_2SO_4	2,297	2,970	990	990	990	1,274.5
$MgSO_4$	361.4	542.1	180.7	542.1	180.7	555
$FeSO_4$	27.9	27.9	27.9	27.9	27.9	30.8
EDTA, Na_2	37.3	37.3	37.3	37.3	37.3	41.4
$MnSO_4$	22.3	22.3	22.3	22.3	22.3	27.9
MoO_4, Na	0.3	0.3	0.3	0.3	0.3	0.3
H_3BO_3	6.2	6.2	6.2	6.2	6.2	5.5
$CuSO_4$	0.3	0.3	0.3	0.3	0.3	0.3
$Zn(NO_3)_2$	0.0	0.0	0.0	0.0	0.0	8.5
$ZnSO_4$	8.6	8.6	8.6	8.6	8.6	4.3
H_2NCH_2COOH - Glycine	2.0	2.0	2.0	2.0	2.0	2.0
$C_6H_5NO_2$- Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.3
$C8H11NO_3$- Pyridoxine	0.5	0.5	0.5	0.5	0.5	0.3
Thiamine	1.5	1.5	1.5	1.5	1.5	1.5
Myo-inositol	100	100	100	100	100	100
6 (1,1-dimethylallyl-amino)-purine, 2iP	3.0	3.0	3.0	3.0	3.0	3.0

*(*WPM as control)*

Table 4. ANOVA analysis by t-test (SAS)

Cultivar	***Response***	***P-value***	***R squared***	***F-value***
Draper	Quality	<0.0001	0.9691	112.90
	Shoot number	<0.0001	0.9003	32.51
	Shoot length	<0.0001	0.8890	28.86
	Leaf color	<0.0001	0.9794	171.34
	Leaf size	<0.0001	0.9735	132.16
Misty	Quality	<0.0001	0.9425	59.08
	Shoot number	<0.0001	0.8201	16.41
	Shoot length	<0.0001	0.9133	37.93
	Leaf color	<0.0001	0.9831	209.46
	Leaf size	<0.0001	0.9287	46.89
Ochlockonee	Quality	<0.0001	0.7832	12.29
	Shoot number	<0.0001	0.8328	16.94
	Shoot length	<0.0001	0.8305	16.66
	Leaf color	<0.0001	0.9138	36.02
	Leaf size	<0.0001	0.8599	20.88
Texas tree	Quality	<0.0001	0.9238	43.64
	Shoot number	0.0021	0.6215	5.91
	Shoot length	0.2149	0.3056	1.58
	Leaf color	<0.0001	0.7937	13.85
	Leaf size	0.5916	0.1739	0.76
Tophat	Quality	<0.0001	0.9218	42.41
	Shoot number	<0.0001	0.7721	12.20
	Shoot length	<0.0001	0.7565	11.18
	Leaf color	<0.0001	0.9419	58.34
	Leaf size	<0.0001	0.7244	9.46

The P-values, R^2 and F-values presented in this table were calculated by t-test(α=0.05).

Table 5. Means separation by Duncan's Multiple Range Test and Comparisons of Significance between each medium to the control (WPM) calculated by Dunnett's test

Cultivar	***Medium*** (6 media)	***Quality*** (1 poor–3 good)	***Shoot number***	***Shoot length*** (mm)	***Leaf color*** (1 poor–3 good)	***Leaf size*** (1 poor– 3 good)
Draper	A	1.00 d*	1.50 c	29.2 c	1.00 d	1.00 c
	B	1.05 d	1.40 c	29.3 c	1.05 d	1.00 c
	C	1.85 b	2.40 b	42.3 b	1.78 b**	1.35 b**
	D	2.87 a**	4.85 a**	57.7 a**	2.95 a**	2.90 a**
	WPM	1.43 c	1.80 bc	40.8 b	1.35 c	1.00 c
	PBM	1.30 c	2.35 b	39.7 b	1.50 c	1.25 b**
Misty	A	1.15 d	1.70 c	23.0 c	1.00 e	1.00 d
	B	1.28 d	1.95 c	26.4 c	1.00 e	1.00 d
	C	2.31 b**	3.75 b**	55.0 a**	2.60 b**	2.00 b**
	D	2.77 a**	4.65 a**	61.5 a**	2.90 a**	2.55 a**
	WPM	1.78 c	1.78 c	36.6 b	1.23 d	1.23 d
	PBM	1.90 c	1.90 c	5.3.7 a**	1.85 c**	1.55 c**
Ochlockonee	A	1.47 cd	2.27 d	33.2 c	1.43 c	1.27 c
	B	1.20 d	2.30 d	31.1 c	1.35 c	1.25 c
	C	2.13 b	4.20 bc	50.5 b	2.90 a**	2.15 b
	D	2.58 a**	6.55 a**	65.4 a**	2.85 a**	2.83 a**
	WPM	2.03 b	3.50 cd	45.8 b	2.08 b	1.98 b
	PBM	1.88 bc	5.40 ab**	63.4 a**	2.00 b	2.80 a**
Texas tree	A	1.93 c	2.75 bc	42.5 ab	2.20 b	2.38 a
	B	2.15 c	3.30 ab	42.3 ab	2.35 b	2.35 a
	C	2.85 a**	3.65 a	48.5 a	2.90 a**	2.68 a
	D	2.53 b**	3.35 ab	47.0 ab	2.48 b	2.48.a
	WPM	2.00 c	3.30 ab	40.0 b	2.08 b	2.35 a
	PBM	1.48 d	2.10 c	43.5 ab	1.35 c	2.55 a
Tophat	A	1.45 d	4.65 b	41.3 d	1.45 d	1.33 c
	B	1.70 cd	5.15 b	44.0 cd	1.70 d	1.25 c
	C	2.38 b	5.80 b	58.5 ab	2.68 b**	2.43 a**
	D	3.00 a**	8.15 a**	63.1 a**	3.00 a**	2.50 a**
	WPM	1.95 c**	5.45 b	50.9 bc	2.08 c	1.68 bc
	PBM	1.78 c	5.35 b	56.5 ab	1.70 d	1.88b

Numbers in each raw/column are average number of total samples in gradings 1-3, actual counts, or measured numbers in millimeter. WPM as control

** Means separation are calculated by Duncan's Multiple Range Test for five growth response categories, numbers with same letters are not significantly different (α=0.05) among the treatments.*

*** Comparisons (between each treatment to the control) significant (α=0.05) by Dunnette' s test are indicated with ** after numbers.*

Figures (1-5)

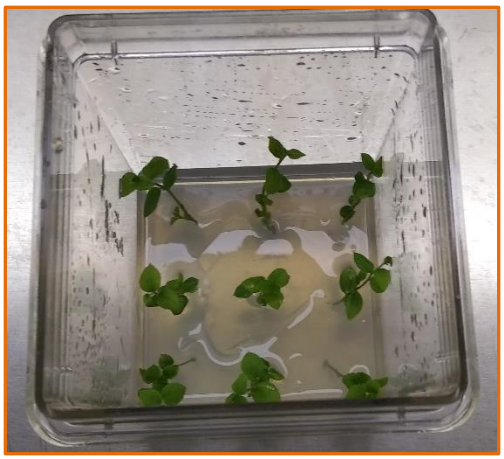


Figure 1. *Nine blueberry explants per GA-7 container at the beginning of the experiments.*

Fig. 2. *'Draper' subcultured on four mediua A, B, C, and D, WPM (as control), and PBM (the order of GA-7 container in photo from left to the right).*

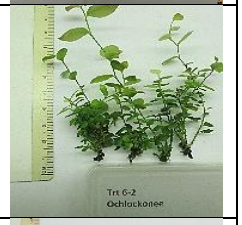
Genotype	Medium A	Medium B	Medium C	Medium D	WPM	PBM
Draper						
Misty						
Ochlocknee						
Texas Tree						
Tophat						

Fig. 3. *Visual comparisons for the growth morphologies of five blueberries subcultured on six media (5 weeks All the photos in this table has been adjusted and modified as the same size to fitting into the table aiming in only visual references, the actual growth measurements (mean numbers) used for the statistical analysis are presented in Table 5.*

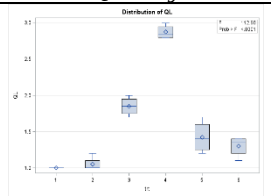
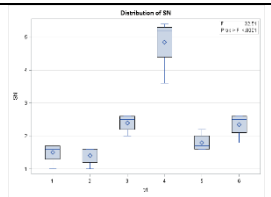
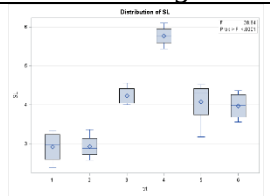
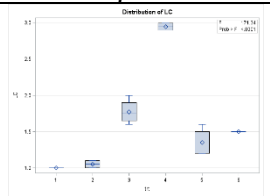
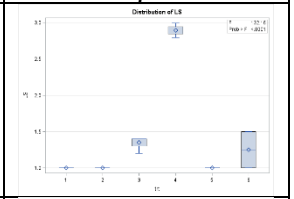
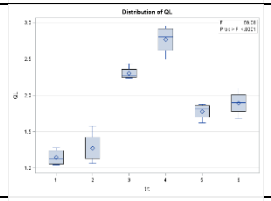
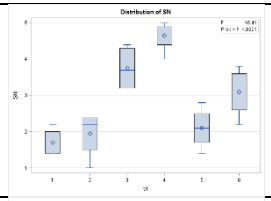
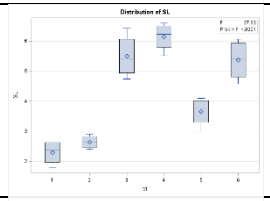
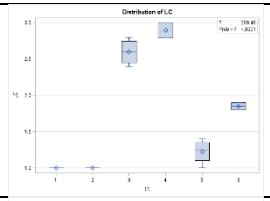
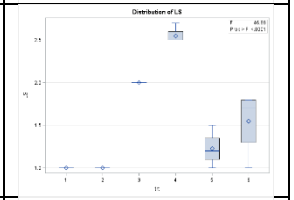
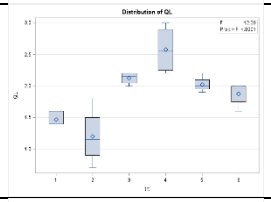
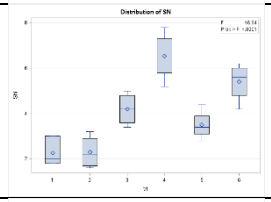
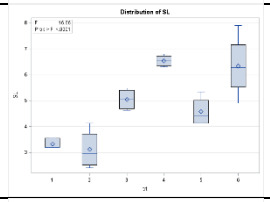
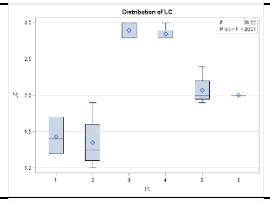
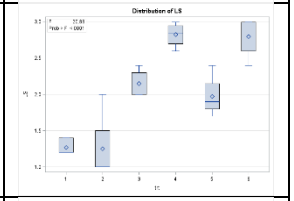
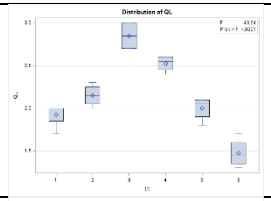
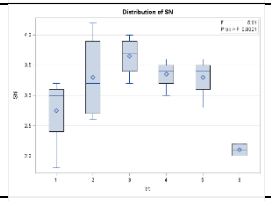
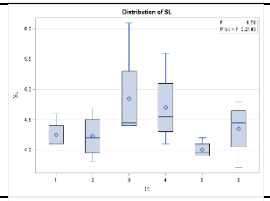
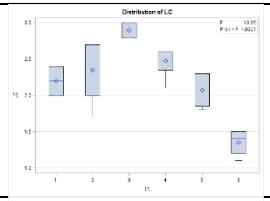
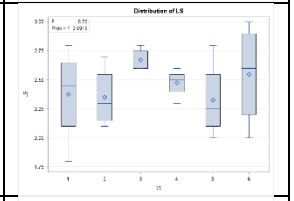
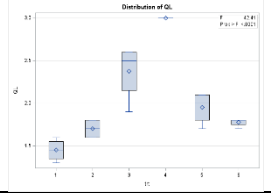
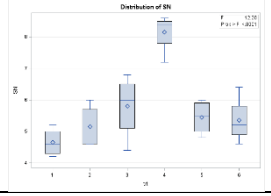
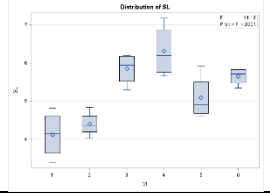
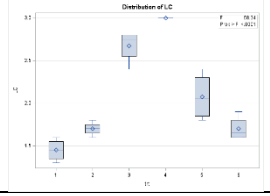
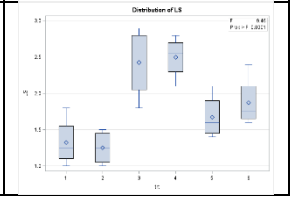
	Quality	*Shoot number*	*Shoot length*	*Leaf color*	*Leaf size*
Draper					
Misty					
Ochlockonee					
Texas tree					
Tophat					

Fig. 4. Box pot graphs on data distribution for the growth responses, overall plant quality, shoot number, shoot length, leaf color and leaf size, for all genotypes are presented in this figure.