Title: Optimization and Production of Tissue Cultured Advanced Blueberry Selections for Multi-State Trialing

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Public Abstract:

Blueberry bushes grown "*in vitro*", i.e., from lab-based tissue cultures, are increasingly preferred over cutting-propagated bushes as they are potentially disease-free and can be rapidly multiplied from a single plant, allowing growers to purchase thousands of a novel cultivar soon after commercialization. Each cultivar responds differently to *in vitro* conditions, however. The NCSU Blueberry Breeding program is working on optimizing media for their most promising advanced selections, which will allow for trialing in more locations. This will speed up cultivar releases and increase the availability of plants to growers. In this experiment, standard tissue culture medium for blueberry were supplemented with either potassium nitrate (KNO3) or glutamine at 1x and 1/2x rates, or with KNO3 and glutamine at 1/4x and tested on 2 - 4 advanced selections. Overall, no supplement outperformed the standard base media, however within genotypes, glutamine had some significant effect while KNO3 caused mortality across all genotypes over the length of the experiment. There is evidence to suggest that supplementation with glutamine could improve *in vitro* growth in some genotypes.

Introduction:

Blueberry growers are increasingly purchasing juvenile crops grown "*in vitro*", i.e. from lab-based tissue cultures. Rapid reproduction means that a grower can purchase thousands of a novel cultivar to gain an edge over competitors, in contrast to traditional methods where newly released material would be scarce for several years while clonal plants were produced by cuttings. With this process in mind, the NCSU Blueberry Breeding program began a small tissue culture lab in 2015 in order to propagate promising selections more rapidly to create more and larger trials, which could in turn potentially speed up the breeding process. However, every genotype responds differently to various tissue culture media formulations[1, 2]. We have seen this first hand in our laboratory where one accession, NC5288, was most successful with 500+ clones rooted, while others in the same media failed to thrive or had only modest success. This issue stymies rapid propagation of the wide variety of advanced selections from our program.

Therefore our objective was to find one or several media formulations that can create optimal growth across multiple genotypes.
Our current base medium uses ½ McCown's Woody Plant media (WP) and ½ Anderson's Rhododendron media (AR) with Murashige & Skoog (MS) vitamins and supplemental calcium nitrate, which has proven more successful than wholly WP or AR medium alone. A recent study found that Olive Media (OM) was more successful than WP alone in O'Neal[3]. A ready-made source of OM could not be found, so media components were analyzed (Table 1) for potential differences, and potassium nitrate and glutamine were chosen to be part of this experiment.

					A	B	C	D	E	F
Component (mg/L)	***OM***	***WPP04***	***ARP05***	***MS vitamins***	***Standard formula***	***+1x KNO3***	***+1/2x KNO3***	***+1x Glutamine***	***+1/2x Glutamine***	***+1/4x KNO3 +1/4x Glutamine***
glycine	*2*	*2*		*2*	2	2	2	2	2	2
Ammonium nitrate	*412.5*	*400.0*	*400.0*		400.0	400.0	400.0	400.0	400.0	400.0
Calcium nitrate	*1300.0*	*556.0*			832.0	834.0	834.0	834.0	834.0	834.0
Potassium nitrate	*1772.0*		*480.0*		240.0	2012.0	1126.0	240.0	240.0	683.0
Calcium chloride anhydrous		*72.5*	*332.0*		202.2	202.2	202.2	202.2	202.2	202.2
Cobalt chloride • 6H2O										
Molybdic acid (sodium salt) • 2H2O	*0.3*	*0.3*	*0.3*		0.3	0.3	0.3	0.3	0.3	0.3
Na2-EDTA	*37.5*	*37.3*	*74.5*		55.9	55.9	55.9	55.9	55.9	55.9
Potassium iodide	*0.8*		*0.3*		0.2	0.2	0.2	0.2	0.2	0.2
Potassium phosphate monobasic	*340.0*	*170.0*			85.0	85.0	85.0	85.0	85.0	85.0
Sodium Phosphate			*330.0*		165.0	165.0	165.0	165.0	165.0	165.0
Cupric sulfate • 5H2O	*0.3*	*0.2*	*0.0*		0.1	0.1	0.1	0.1	0.1	0.1
Ferrous sulfate • 7H2O	*27.8*	*27.9*	*55.7*		41.8	41.8	41.8	41.8	41.8	41.8
Magnesium sulfate	*731.0*	*180.7*	*180.0*		180.4	180.4	180.4	180.4	180.4	180.4
Manganese sulfate • H2O	*16.9*	*22.3*	*16.9*		19.6	19.6	19.6	19.6	19.6	19.6
Potassium sulfate		*990.0*			495.0	495.0	495.0	495.0	495.0	495.0
Zinc sulfate • 7H2O	*14.3*	*8.6*	*8.6*		8.6	8.6	8.6	8.6	8.6	8.6
Biotin	*0.1*									
Boric acid	*12.4*	*6.2*	*6.2*		6.2	6.2	6.2	6.2	6.2	6.2
Folic Acid	*0.5*									
Glutamine	*1178.0*							1178.0	589.0	294.5
myo-inositol	*100.0*	*100.0*	*1886.0*	*100.0*	1043.0	1043.0	1043.0	1043.0	1043.0	1043.0
Nicotinic Acid	*5.0*	*0.5*		*0.5*	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine, Hydrochloride	*0.5*	*0.5*		*0.5*	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine	*0.5*	*1.0*	*0.4*	*0.1*	0.8	0.8	0.8	0.8	0.8	0.8

Materials and Methods:

Media preparation: Base media (A) was prepared with 2% sucrose, 2 mg/L trans-zeatin riboside, 1.3 g/L Woody Plant with Vitamins (Caisson Labs WPP04), 1 g/L Anderson's Rhododendron Basal Salts (Caisson Labs, ARP05), 554 mg/L calcium nitrate, 0.5 mg/L 1000x Murachige and Skoog Vitamin Mixture (Caisson Labs, MVL01). Potassium nitrate was added at the rates of 1,772 and 886 mg/L to the base media to create treatments B and C, respectively. Glutamine was added to the base media at rates of 1,178 and 589 mg/L to create treatments D and E, respectively. Treatments C and E were combined in equal measure to create treatment F of 1/4x KNO3 and 1/4x Glutamine. PH was adjusted to 5.2 across all treatments, and 7 g/L

Phytoblend (Caisson Labs, PTP01) was divided equally between 20 Magenta™ boxes (approximately .38 mg/box) before 50ml of each treatment was aliquoted into marked boxes. Treatments were autoclaved for 20 minutes and brought into a laminar flow hood to cool. Advanced selections NC5289 and NC5296 were chosen to receive all treatments; NC5289 historically performs well, while NC5296 has overall had less vigorous growth *in vitro.* NC5295 and NC5305 received the remaining treatments. Each box received 6 explants from plants established *in vitro* in September 2019. Lids were sealed with micropore tape and placed in our TC room with 12 hours of light, and maintained at 21.1 C.

Boxes were moved to a refrigerator at 4C to slow growth and contamination after 8 months and individual explants were removed from the media at 9 months to be weighed with and without callus. Shoots from each explant were divided and categorized by 10mm lengths (<10mm, >10mm, >20mm…>100mm)(Figure 1) . The potential number of cuttings was calculated with the assumption of viability being approximately 20mm in size, so shoots were divided accordingly with any excess discarded. For example, a >50mm shoot would produce a maximum of 2 viable cuttings.

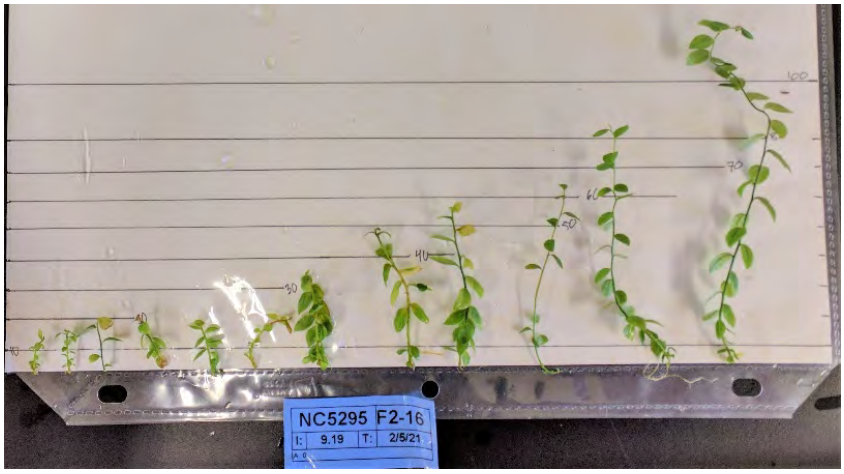

Figure 1: shoots from an explant of NC5295 treatment A divided and categorized by length.

Results and Discussion:

Differences were readily apparent (Figure 2), with potassium nitrate causing initial growth but eventual plant mortality. The calculated potential number of cuttings was jusdged the best method of deciding media effectiveness and was used for statistical analysis. ANOVA test showed that there was significant differences between treatments, between genotypes, and treatments within genotypes. Differences between and within genotypes and treatments were seen when least means squares were analyzed using Tukey's HSD test (Table 2). NC5289, the base media outperformed all other treatments, and in NC5305 the 1x glutamine was significantly superior to other treatments. In NC5295 and NC5296, while there was no significant difference between treatments, there was a very slight improvement in number of shoots and shoot weight seen with glutamine in NC5296. The contamination of NC5289 1x glutamine should also be considered as this might have led to a slight skewing of the data. Although repeated experiments will give a definitive answer, since glutamine supplementation

showed no deleterious effects overall indicates that it could be trialed in those genotypes that fail to thrive in base media alone.

Figure 2- 5 accessions tested on 4-6 medias: A) ½ WPM+ ½ ARP (Standard); B) +1x K(NO)3; C) +½x KNO3; D) +1x Glutamine; E) + ½x Glutamine; F) + ¼x KNO3 + ¼x Glutamine. Note that NC5289 (D) became contaminated.

Table 2. Least Squares Means Differences by Tukey HSD Test

Treatment	Potential Number of Cuttings				Explants Only Weight (mg)			
	NC5289	**NC5296**	**NC5295**	**NC5305**	**NC5289**	**NC5296**	**NC5295**	**NC5305**
A) Base Media	**41 a**	2.5 c	**13.2 bc**	6.1 bc	**431 a**	107.3 c	**424.2 a**	221.1 abc
B) +1x KNO3	3.8 c	0.8 c	5.9 bc	2.4 c	53.3 c	41.2 c	188 abc	94.7 c
C) +½x KNO3	9.8 bc	1 c			153.3 bc	69.2 c		
D) +1x Glutamine	7 bc	1.2 c	7.7 bc	**18.4 b**	114 c	106 c	383.2 ab	**391.9 ab**
E) +½x Glutamine	7.5 bc	**4.5 bc**			207 abc	**236.7 abc**		
F) +¼x KNO3 + ¼x Glut.	8.8 bc	1.2 c	12.9 bc	8 bc	184.2 abc	67.5 c	414.9 a	197.3 abc

Levels not connected by same letter are significantly different. Maximum least squares mean value by genotype is in bold.

Limitations and future plans:

Ideally, this experiment would have run for a much shorter length of time. The effects of explants grown in KNO3 may have been clearer if plants had been removed at 2-3 months, as is standard. The start of blueberry season coinciding with a sudden shortage of temporary employees meant the experiment could not be completed until much later in the year. The length of time and decision to use micropore tape for sealing also reduced moisture within the

boxes and allowed contaminants to enter one (NC5289 D). Efforts will be made in the future to ensure a more complete design, and to finish experiments to better timing in commercial laboratory settings.

In 2022, we will continue our media optimization experiments by trialing potassium phosphate and magnesium sulfate supplementation, and potentially increasing calcium nitrate as well. In addition, we will be trialing responses to liquid media using We Vitro (wevitro.magentallc.com) gravity wells and PlantForm (www.plantform.se) temporary immersion bioreactors as research has shown favorable responses of *Vaccinium spp.* to these methods of tissue culture[4, 5]. Using established cultures, six explants of each accession will be subjected to media modified with a similar experimental design as seen above After two months in culture, explants will be removed to weigh callus and shoots and measure shoot length and number. Explants will then either be placed in new media for continued growth or transplanted into 50 cell liners for rooting and later trialing with growers and other researchers to further our collaborative efforts within the southern region.

References:

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