D2400
Media Optimization Kit (DKW)

Introduction

Since the development of DKW medium for walnut (Driver & Kuniyuki 1984), it has become an increasingly used plant tissue culture medium for various crops such as fruit, nut, and ornamental (Anna *et al.* 1998) trees, as well as cannabis (Page *et al.* 2021).

As such, there has been more interest in an optimized DKW-based media in a Design of Experiments (DOE) format (Niedz & Evens 2007). This product is similar to our Deconstructed MS Kit (Prod. No. M5630), yet it differs in the nutrient salts because of the original DKW formulation. The Deconstructed MS Kit allowed for direct modulation of NH_4^+, K^+, NO_3^-, and Fe^{3+}, as well as grouped ions in the meso- and micronutrients. This kit allows modulation of those ions (except Fe^{3+} outside of the micronutrients) and adds the ability to directly change Ca^{2+}, SO_4^{-2}.

There has also been work utilizing DKW individual nutrient solutions (Hand et al. 2014) similar to those in this kit. In this method the macronutrients, mesonutrients, and micronutrients, are split into different solutions so the amounts of each can be varied over a number of different experiments.

Table 1. This kit contains these mineral nutrients as different solutions (groups) formulated as:

Group	Product No.	Components	Conc. [g/L]	Conc. [mM]	X [DKW]	Volume for 1X [mL/L]	Volume Provided [mL]
I	M5631	NH_4NO_3	165	2061	116.5	8.6	100
II	D2401	$Ca(NO_3)_2{\cdot}4H_2O$	196	830	100	10	100
III	D2402	$CaCl_2{\cdot}2H_2O$	1.47	10	10	100	1000
		KH_2PO_4	2.65	15			
		$MgSO_4{\cdot}7H_2O$	7.39	30			
IV	D2403	H_3BO_3	0.048	0.78	10	100	1000
		$CuSO_4{\cdot}5H_2O$	0.0025	0.010			
		$Na_2EDTA{\cdot}2H_2O$	0.454	1.2			
		$FeSO_4{\cdot}7H_2O$	0.338	1.2			
		$MnSO_4{\cdot}H_2O$	0.335	2.0			
		$Na_2MoO_4{\cdot}2H_2O$	0.0039	0.016			
		$NiSO_4{\cdot}6H_2O$	0.00005	0.0019			
		$ZnNO_3{\cdot}6H_2O$	0.17	0.57			
V	D2404	K_2SO_4	77.95	10	50	20	200

Also described in our technical manual for Prod. No. M5630, DOE is preferred to changing concentrations in one-at-a-time experiments because it requires fewer total experiments for the same

precision in growth response estimation. A good introduction to DOE experiments which goes through these details is the book "DOE Simplified: Practical Tools for Effective Experimentation" by Mark J. Anderson and Patrick J. Whitcomb and is published by CRS Press. There are two main DOE approaches.

1.) 2-Level Factorial approach allows different mineral nutrients to be examined at high and low levels. This is not ideal for optimization, but can provide qualitative information to screen factors (groups).

2.) Response Surface Methods (RSM) can be used to define growth responses in a non-linear fashion with nutrient concentrations. This allows for an optimum concentration of a nutrient to be estimated along the growth response curve.

Inside each of these DOE approaches, the most important variables set at the beginning of these experiments are the concentration ranges for use of these solutions. Shown below (Table 2) are the general concentration ranges we are specifying for the example optimizations provided in this manual.

Table 2. Concentration ranges used in the DOE example optimizations.

			X [DKW]		Conc. [mg/L]		Conc. [mM]	
Group	Product No.	Components	Low-end Range	High-end Range	Low-end Range	High-end Range	Low-end Range	High-end Range
I	M5631	NH_4NO_3	0.3	1.5	424	2124	5.31	26.5
II	D2401	$Ca(NO_3)_2{\cdot}4H_2O$	0.5	1.5	980	2940	4.15	12.4
III	D2402	$CaCl_2{\cdot}2H_2O$	0.5	1.5	73.5	220	0.50	1.50
		KH_2PO_4			132	398	0.97	2.92
		$MgSO_4{\cdot}7H_2O$			370	1108	1.50	4.50
IV	D2403	H_3BO_3	0.5	2	2.4	9.6	0.04	0.16
		$CuSO_4{\cdot}5H_2O$			0.125	0.5	5.0E-04	2.0E-03
		$Na_2EDTA{\cdot}2H_2O$			22.7	90.8	0.06	0.24
		$FeSO_4{\cdot}7H_2O$			16.9	67.6	0.06	0.24
		$MnSO_4{\cdot}H_2O$			16.8	67.0	0.10	0.40
		$Na_2MoO_4{\cdot}2H_2O$			0.20	0.78	8.1E-04	3.2E-03
		$NiSO_4{\cdot}6H_2O$			0.0025	0.01	9.5E-06	3.8E-05
		$ZnNO_3{\cdot}6H_2O$			8.5	34	0.03	0.11
V	D2404	K_2SO_4	0.25	1.5	390	2338	2.24	13.4

Due to various nutrients containing multiple ions per molecule, it is useful to consider the total ion concentration range in these experiments. Table 3 below shows the total concentration range of each ion listed from the salt form Table 2.

Table 3. Ion concentration ranges used in the DOE example optimizations.

Ion	Ion Conc. [mM]	
	Low-end Range	High-end Range
NH_4^+	5.31	26.5
NO_3^-	13.6	51.4
K^+	5.45	29.7
Ca^{2+}	4.65	13.9
PO_4^{-3}	0.97	2.92
Mg^{2+}	1.50	4.50
SO_4^{-3}	3.90	18.5
Cl^-	0.50	1.50
Na^+	0.12	0.49
B	0.04	0.16
EDTA	0.06	0.24
Fe^{3+}	0.06	0.24
Mn^{2+}	0.10	0.40
Cu^{2+}	5.00E-04	2.00E-03
Mo^{2+}	8.10E-04	3.20E-03
Ni^{2+}	9.50E-06	3.80E-05
Zn^{2+}	0.03	0.11

Example Optimizations

The two example optimizations provided in this manual are:

1.) 5-factor (group), 2-level design at ½ fractional uses 16 treatments, which uses the 2-Level Factorial approach. This allows for high and low ends of the ion concentration range (Table 3.) to be evaluated. See Table 4.

2.) 3-factoral RSM design for high concentration components (Group I, II, and V). This design allows for a much more comprehensive look at a number of ions (e.g. NH_4^+, K^+, NO_3^-, Ca^{2+}, SO_4^{-2}) within each factor (group). Please see Table 5.

The contents in this kit allow for either the 5-factor (group) 2-Level design at ½ fractional or the 3-factoral RSM design to be performed. If additional nutrients are required for additional studies these solutions can be purchased individually. In DOE approaches the different variables are often referred to as factors. In the examples provided here we will refer to them as groups, since the different solutions are already categorized as that.

Table 4. 5-factor (group), 2-Level design at ½ fractional design.

Design points	Groups (all values are expressed in strength, X of DKW media)				
	I	II	III	IV	V
1	1.5	0.5	1.5	2	0.25
2	1.5	1.5	1.5	0.5	0.25
3	0.3	1.5	1.5	0.5	1.5
4	1.5	1.5	0.5	0.5	1.5
5	0.3	0.5	1.5	2	1.5
6	1.5	0.5	1.5	0.5	1.5
7	1.5	1.5	1.5	2	1.5
8	1.5	1.5	0.5	2	0.25
9	1.5	0.5	0.5	0.5	0.25
10	1.5	0.5	0.5	2	1.5
11	0.3	1.5	0.5	0.5	0.25
12	0.3	0.5	0.5	0.5	1.5
13	0.3	0.5	0.5	2	0.25
14	0.3	1.5	1.5	2	0.25
15	0.3	0.5	1.5	0.5	0.25
16	0.3	1.5	0.5	2	1.5

Protocol to prepare Design Point 1 in the 5-factor (group), 2-Level design at ½ fractional design

An Example Calculation of how to determine the volume of each nutrient is in the Appendix (Table A1)

1.) Obtain a container large enough to prepare 0.5L of the medium in
2.) Add 6.4 mL of Group I (NH_4NO_3) 116.5X DKW [Product No. M5631]
3.) Add 2.5 mL of Group II ($Ca(NO_3)_2$ 100X DKW [Product No. D2401]
4.) Add 75 mL of Group III Mesonutrients 10X DKW [Product No. D2402]
5.) Add 100 mL of Group IV Micronutrients 10X DKW [Product No. D2403]
6.) Add 2.5 mL of Group V K_2SO_4 50X DKW [Product No. D2404)
7.) Add any carbohydrates, plant growth regulators, gelling agents, or other supplements as necessary
    a. If gellan gum (e.g. G434, G3251) is used, the final Ca^{2+} and Mg^{2+} combined concentrations should be between 3-12 mM to form rigid gels
8.) Add additional tissue culture grade water to bring the medium to the final volume of 0.5L.

9.) While stirring, determine the pH. If necessary, adjust the medium to the desired pH using potassium hydroxide to raise the pH or hydrochloric acid to lower the pH. A pH of 5.6 to 5.8 is typically recommended for most plants.
10.) Sterilize the medium in a validated autoclave or pressure 9+cooker at 1 kg/cm2, 121 °C (15 psi, 250 ° F), for the time period described under "Sterilization of Media" below.
11.) Allow medium to gel for 3-4 hrs prior to use.

To perform the 3-factorial RSM design for the high concentration components (Table 5.), the mesos and micros should be constant throughout the experiment. Some preliminary experiments should be performed to determine what the meso and micro concentrations should be set to. Micronutrient optimization is very complex because the interactions of the elements vary with the plant species tested, but a wide range to test from would be 0.5 to 3X the DKW levels.

Table 5. 3-factorial (group) RSM Design.

Design points	Groups (all values are expressed in strength, X of DKW media)		
	I	II	V
1	0.3	1.5	0.5
2	0.9	0.5	1.5
3	0.9	1.5	1.5
4	0.3	0.5	0.5
5	0.9	1	0.75
6	1.5	1	1.5
7	1.5	0.5	0.5
8	1.5	1.5	0.5
9	0.3	1	1.5
10	1.5	1.5	1.5
11	0.9	1	0.75
12	0.9	0.5	0.5
13	0.3	1.5	0.5
14	0.3	0.5	1
15	0.3	0.5	1
16	1.5	1.5	0.5
17	1.5	0.5	0.5
18	0.3	1	0.5
19	1.5	0.5	1.5
20	0.3	1.5	1.5

Protocol to prepare Design Point 2 in the 3-factorial (group) RSM design

An Example Calculation of how to determine the volume of each nutrient is in the Appendix (Table A2)

1.) Obtain a container large enough to prepare 0.5L of the medium in
2.) Add 3.9 mL of Group I (NH_4NO_3) 116.5X DKW [Product No. M5631]
3.) Add 2.5 mL of Group II ($Ca(NO_3)_2$ 100X DKW [Product No. D2401]
4.) Add 100 mL of Group III Mesonutrients 10X DKW [Product No. D2402]
5.) Add 100 mL of Group IV Micronutrients 10X DKW [Product No. D2403]
6.) Add 15 mL of Group V K_2SO_4 50X DKW [Product No. D2404]
7.) Add any carbohydrates, plant growth regulators, gelling agents, or other supplements as necessary
    a. If gellan gum (e.g. G434, G3251) is used, the final Ca^{2+} and Mg^{2+} combined concentrations should be between 3-12 mM to form rigid gels
8.) Add additional tissue culture grade water to bring the medium to the final volume of 0.5L.
9.) While stirring, determine the pH. If necessary, adjust the medium to the desired pH using potassium hydroxide to raise the pH or hydrochloric acid to lower the pH. A pH of 5.6 to 5.8 is typically recommended for most plants.
10.) Sterilize the medium in a validated autoclave or pressure cooker at 1 kg/cm^2, 121 °C (15 psi, 250 ° F), for the time period described under "Sterilization of Media" below.
11.) Allow medium to gel for 3-4 hrs prior to use.

Note: There are many other 2-Level and RSM factorial designs that can be performed with this kit; only a couple of examples are provided here. **A spreadsheet which codes the actual X of DKW for each solution in 2, 3, 4, and 5-factorial (group) RSM design is available on the phytotechlab.com website. This spreadsheet will also allow the user to change the concentration ranges for each nutrient within these designs**.

Sterilization of Media

Plant tissue culture media are generally sterilized by autoclaving at 121°C and 1.05 kg/cm^2 (15 psi). This high temperature not only kills bacteria and fungi, but also their heat-resistant spores. Media can be sterilized in either an autoclave or pressure cooker with similar results. The time required for sterilization depends upon the volume of medium, type of vessel, and the type of autoclave. The suggested minimum times required for sterilization of different media volumes are listed below in Table 6. It is advisable to dispense medium in small aliquots after autoclaving whenever possible as many some media components can be broken down by prolonged exposure to heat.

Table 6. Autoclave times to reach 121°C for 15 min.

Volume of Medium per Vessel [mL]	Suggested Minimum Autoclaving Time [min]
25	15-20
50	25
100	28
250	31
1000	40
2000	48
4000	63

Please Note: Minimum autoclaving time includes the time required for the liquid volume to reach the sterilizing temperature (121°C) and 15 minutes at 121°C (Burger, 1988). Times may vary due to differences in autoclaves. Validation with your autoclave or pressure cooker is recommended. Use of a product such as Sterilization Indicator Strips, 3M™ Comply Plus™ [Product No. S7787] is useful for this purpose.

References:

Anna M, Mariella L, and Lorenzo M (1998). Elm Tissue Culture: Micropropagation of Clones Resistant to Dutch Elm Disease. *Acta Horticulturae*, (457), 235–242.

Burger DW (1988) Guidelines for autoclaving liquid media used in plant tissue culture. *HortScience* 23:1066-1068.

Driver JA, and Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. *HortScience* 19:507–509.

Hand C, Maki S, and Reed BM (2014) Modeling optimal mineral nutrition for hazelnut micropropagation *Plant Cell Tissue Org. Cult.* Vol. 119 pg. 411-425.

Niedz RP, Evens TJ (2007) Regulating plant tissue growth by mineral nutrition. *In Vitro Cell. Dev. Biol. - Plant* 43:370-381

Page SRG, Monthony AS, and AMP Jones (2021) DKW basal salts improve micropropagation and callogenesis compared with MS basal salts in multiple commercial cultivars of Cannabis sativa. *Botany*. 99(5)

Appendix:

Example Calculation:

$$V_{ADD} = V_{TOTAL}\left(\frac{X_{TABLE}}{X_{CONC.SOL.}}\right)$$

Where: V_{ADD} = Volume needed to add to media to achieve the X (of DKW) listed on the table

V_{TOTAL} = Total volume of media being prepared for the design point

X_{TABLE} = X (of DKW) prescribed for the group in the table

$X_{CONC.SOL.}$ = X (of DKW) of the concentrated solution

For design point 2 in the 3-factorial (group) RSM Method with Group I

X_{TABLE} = 0.9X (of DKW) prescribed for the group in the table

V_{TOTAL} = 500 mL (0.5L)

$X_{CONC.SOL.}$ = 116.5X (of DKW) for Group I

$$V_{ADD} = 500mL\left(\frac{0.9X}{116.5X}\right) = 3.9\ mL\ of\ Group\ I\ to\ 500\ mL\ of\ media$$

Table A1. Volume per design point in 5-factor (group), 2-Level design at ½ fractional design (Table 4).

Design points	Groups (volume of mL to add to Total Media Volume mL)				
	I	II	III	IV	V
1	6.4	2.5	75	100	2.5
2	6.4	7.5	75	25	2.5
3	1.3	7.5	75	25	15
4	6.4	7.5	25	25	15
5	1.3	2.5	75	100	15
6	6.4	2.5	75	25	15
7	6.4	7.5	75	100	15
8	6.4	7.5	25	100	2.5
9	6.4	2.5	25	25	2.5
10	6.4	2.5	25	100	15
11	1.3	7.5	25	25	2.5
12	1.3	2.5	25	25	15
13	1.3	2.5	25	100	2.5
14	1.3	7.5	75	100	2.5
15	1.3	2.5	75	25	2.5
16	1.3	7.5	25	100	15

Table A2. Volume per design point in 3-factorial (group) RSM Design (Table 5).

Design points	Groups (volume of mL to add to Total Media Volume mL)		
	I	II	V
1	1.3	7.5	5
2	3.9	2.5	15
3	3.9	7.5	15
4	1.3	2.5	5
5	3.9	5	7.5
6	6.4	5	15
7	6.4	2.5	5
8	6.4	7.5	5
9	1.3	5	15
10	6.4	7.5	15
11	3.9	5	7.5
12	3.9	2.5	5
13	1.3	7.5	5
14	1.3	2.5	10
15	1.3	2.5	10
16	6.4	7.5	5
17	6.4	2.5	5
18	1.3	5	5
19	6.4	2.5	15
20	1.3	7.5	15

Notes: