The Yellowing of Dry-Powder Plant Tissue Culture Media

D. S. Hart[1], G.R. Seckinger[1], and K.C. Torres[1]

[1]Technical Services Department, *Phyto*Technology Laboratories, Overland Park, KS USA 66214

Overview

Dry powder plant tissue culture media are exposed to a variety of conditions when shipped around the world often because of the time spent in customs. We induced pristine white, dry powder MS (Murashige and Skoog) media to yellow through direct exposure to relative humidity (20-40% RH) and 40-44°C up to 24 hrs. We then tested the growth of a number of plant species and found no statistically significant differences in growth between plant tissue grown on pristine white media and yellow media.

The yellow color of aged MS basal media led us to hypothesize that the ferrous (II) ions input as ferrous (II) sulfate were being oxidized to ferric (III) ions. Evidence from ferrozine colorimetric assays suggests the color change is a result of oxidation of ferrous (Fe^{2+}) ions to ferric ions (Fe^{3+}).

Introduction

MS basal powder media in its standard formulation [1] contains 27.8 mg/L of ferrous sulfate which is known to complex with the 37.3 mg/L of disodium-EDTA. The equilibrium complexation constant (K_{MY}) of EDTA and iron is dependent on the oxidation state of iron [2]:

$$Fe(II) + EDTA \rightleftharpoons Fe(II)\cdots EDTA \qquad \log K_{MY} = 14.3$$
$$Fe(III) + EDTA \rightleftharpoons Fe(III)\cdots EDTA \qquad \log K_{MY} = 25.1$$

Since the equilibrium under standard conditions is favored almost exclusively to the complex in each oxidation state; very little free iron is present in aqueous solution (e.g. $10^{14.3}$ molecules of Fe(II)-EDTA complex per molecule of free Fe(II)). The ferrous or Fe(II)-EDTA complex is rapidly oxidized to Fe(III)-EDTA [3] in the presence of oxygen (which is soluble in aqueous solution at 8 mg/L [4]). Therefore upon dissolution of the ferrous (II) sulfate and disodium EDTA components of MS-basal media the overwhelming iron species present is Fe(III)-EDTA.

Though these equilibria are well understood in simple iron and EDTA component aqueous solutions, it is not known how this oxidation process occurs in the presence of the other metal salt components of standard MS-basal media. Therefore in addition to testing any potential growth differences between pristine white MS and yellow MS, we also investigated the oxidation state of the iron present of both white and yellow MS when dissolved in aqueous solution.

Materials and Methods

MS basal powder media samples (Prod. No. M524) were sealed in foil bags with as little air in the headspace as possible immediately after manufacture. To induce the yellowing of media samples, powder was stored in uncovered weigh boats in a incubator at 40-44°C and maintained at 20-40% RH.

Following the time-incubated yellowing, samples were sealed in foil bags again until media preparation or chemical analysis. Media was prepared with 30 g/L sucrose,1 mg/L BA, 0.1 mg/L NAA, 6 g/L agar to test the biological growth on yellow compared to white pristine powder. Tissue was grown on the media for 30 days at 25°C with a 16:8 hr light:dark regimen.

A ferrozine colorimetric assay for Fe(II) as outlined by Viollier *et al.* [5] was used to determine the ion form of iron dissolved in aqueous media. The dye 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid (ferrozine) binds specifically to ferrous (II) ions in aqueous solution and produces a chromophore which absorbs at 562 nm. Analysis of ferric (III) ions with ferrozine produced negligible absorbance suggesting low to potentially undetectable binding with ferric (III) ions.

The absorbance at 562 nm was measured with a Beckman Coulter DU 640 Spectrophotometer. Light scattering absorbance at 650 nm was subtracted for each of the samples to remove any artificial absorbance which could erroneously increase the apparent Fe(II) concentration.

To account for all of the iron present in solution all the Fe(III) was converted to Fe(II) through reducing with 1.4M Hydroxylamine HCl and then buffered ammonium acetate. This assay was shown to be linear with absorbance from 2-16 mg/L Fe(II), and ½ X MS-basal salts were used in these investigations.

Early work with the ferrozine assay showed that not all of the iron in the Fe(II) or Fe(III) form were able to be recovered when compared with Atomic Absorption spectroscopy. We believed that the EDTA chelated to each form was interfering with the binding of the ferrozine dye.

Therefore the EDTA was destroyed in the all of the MS basal media samples through a mild digestion protocol from the US EPA [6]. To 100 mL of ½ X MS basal salts, 0.5 mL of concentrated HNO_3 was added followed by 2.5 mL of concentrated HCl, and the samples were heated carefully not to boil (80-90°C) until water evaporated to make the final volume less than 20 mL. The pH of the solution was then brought up to 4.2 +/- 0.2, and diluted to the original sample volume of 100 mL and assayed.

Materials and Methods (cont.)

Atomic absorption (AA) spectroscopy was used to confirm in independent testing the total amount of iron present in dissolved MS basal media. It is important to note that AA cannot differentiate between the Fe(II) or Fe(III) oxidation state. Multiple lots of MS basal media were assayed to determine an appropriate baseline for what we could expect to recover after dissolution.

Samples were prepared by dissolving ½ X MS basal medium in aqueous solution containing 5% HNO_3. AA was performed on a AAnalyst 200 (Perkin Elmer). A LuminaTM hollow cathode lamp at 248.33 nm with the slit set to 1.8/1.35 mm was used to measure the flame absorbance. The flame was maintained with compressed air at 10L/min and acetylene at 2.5L/min. A calibration curve with known standards of $FeCl_3$ showed that the concentration-absorbance relationship was linear from 5-45 mg/L.

Results

Induction of yellowing MS-basal media

Immediately prior to exposing the powder media to the 40-44°C and 20-40% RH conditions, 4.33 g of M524 (MS basal powder media) was weighed in uncovered weigh boats. Shown below in Figure 1 is the resulting appearance of the powder following 7.25 hrs and 23.25 hrs of exposure.

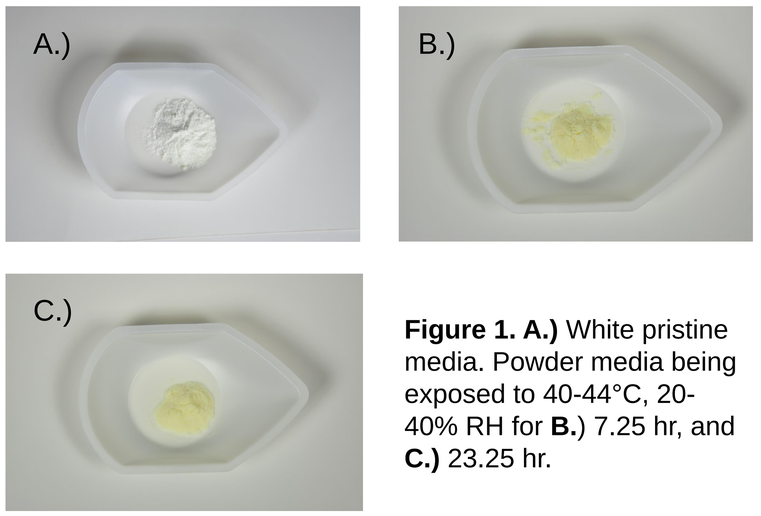

Figure 1. A.) White pristine media. Powder media being exposed to 40-44°C, 20-40% RH for **B.**) 7.25 hr, and **C.)** 23.25 hr.

This powder was then prepared into solid media as described in materials and methods and incubated with plant tissue for 30 days. The results are shown below in Figure 2.

Biological Testing showed no apparent difference between pristine white or yellow MS basal media

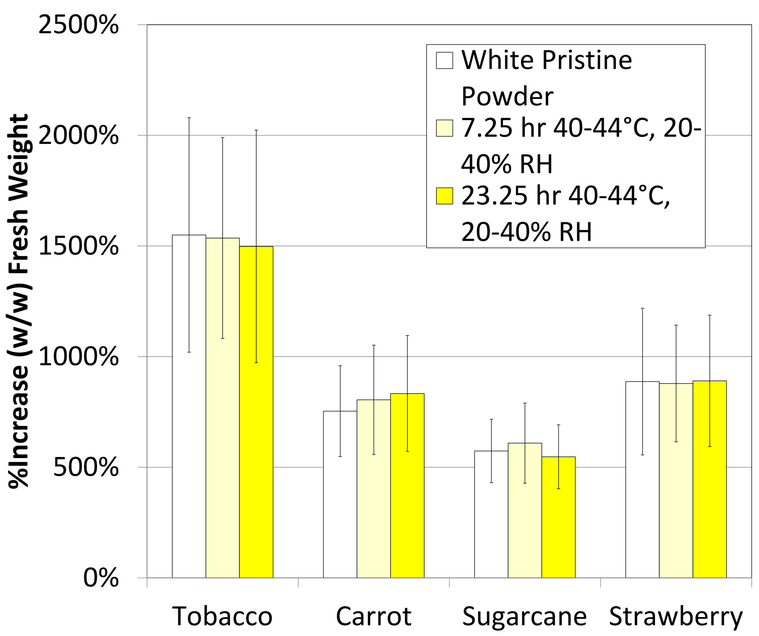

Figure 2. Plant tissue culture biological assay of pristine white and each of the treated yellow powder media.

Since no statistically significant difference in growth was seen in pristine white or yellow powder, we utilized the ferrozine colorimetric assay to determine if there was an oxidation state difference in prepared media.

Results (cont.)

Ferrozine assay shows most Fe(II) converted to Fe(III) during media dissolution

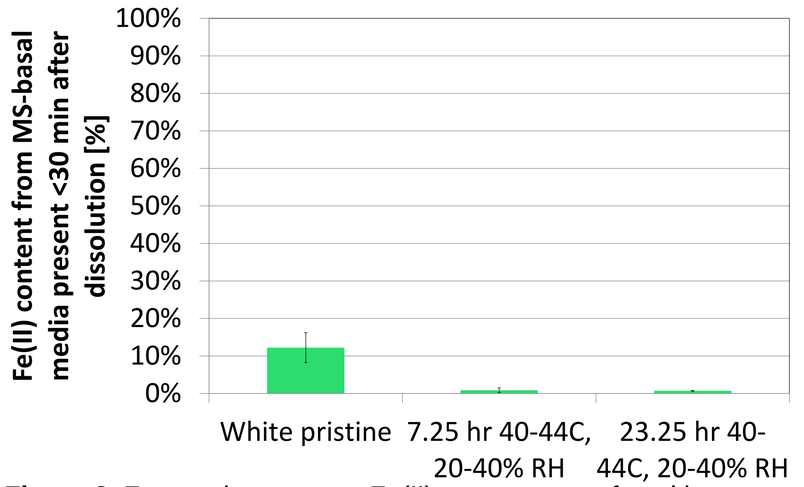

Figure 3. Ferrous ion content, Fe(II) as a percent of total iron content in MS basal media present within 30 minutes of dissolution

Figure 3 shows that approximately 90% of the Fe (II) ions input into MS basal media as ferrous (II) sulfate are converted into Fe(III) based on the nearly 100% recovery of iron in each oxidation state (Figure 4). Additionally upon dissolution almost all of the yellow powder media was in the Fe(III) oxidation state. The assays showed less than 1% of the total iron input in the MS basal powder was Fe(II) for these yellow powder samples. When assaying autoclave-prepared white-pristine media for Fe(II) content, all of the Fe(II) input into the powder had been converted to Fe(III).

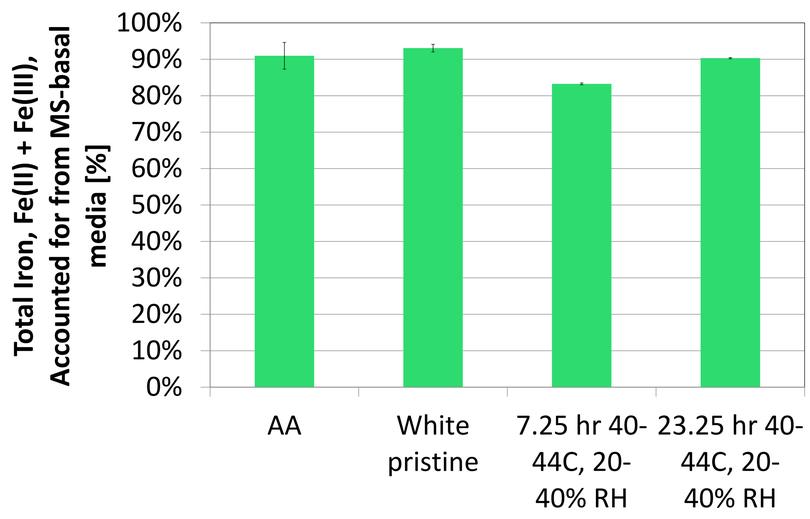

Figure 4. Recovered iron content, Fe(II)+Fe(III) as a percent of total iron content in MS basal media after dissolution

Conclusions

- The yellowing of powder media did not affect plant tissue growth for these species tested.
- The ferrozine assay depicted that most of the Fe(II) was converted to Fe(III) during just media preparation and completely after autoclaving.

Future Work

- Evaluate more plant species to show that whether the iron oxidation state is converted into the ferric form in powder (yellow) or upon dissolution (white pristine) that the growth is not impacted.

References

[1] T. Murashige, F. Skoog (1962) Physiol. Plant. Vol. 15 pg. 473-497.

[2] G. Schwarzenbach,"Complexometric Tritrations" (1957) pg 8.

[3] I.M. Kolthoff, C. Auerbach (1952) J. Am. Chem. Soc. Vol. 74 pg 1452-1456

[4] R. Battino, T. R. Rttich, and T. Tominaga (1983) J. Phys. Chem Ref. Data Vol. 12 (2) pg. 164-178.

[5] E. Viollier, P.W. Inglett, K. Hunter, A.N. Roychoudhury, and P. Van Cappellen (2000) *Applied Gecohemistry* Vol. 15 pg. 785-790.

[6] US EPA digestion protocol: Available www.hach.com/asset-get.download.jsa?id=7639984270