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CHANGES IN MEDIA WATER STATUS DURING PREPARATION AND STORAGE

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TOLEDO, J. U., SPOMER, L. ART, and SMITH, M. A. L. *Changes in Media Water Status During Preparation and Storage.* BIOTRONICS **26**, 59–66. 1997. Significant water loss from plant tissue culture media occurs during its preparation and storage. Because medium water status has a recognized influence on culture response, any water loss and resulting increase in gelling agent and other component concentrations should be allowed for or at least accounted for during media preparation, to ensure the final media composition is that specified or expected. Two formulations of gelled-agar plant tissue culture media were prepared according to standard protocol and 42 ml portions of the prepared media were dispensed into GA7 vessels for autoclaving and storage. A sample of each mixture was removed and its fresh and oven dry mass measured to determine postpreparation concentration. Likewise the postautoclave and poststorage concentrations were measured to determine the water loss and subsequent concentration increase at each step. The observed water loss indicated about a 3% increase in media agar concentration from water lost during mixing, 6% after autoclaving, and over 25% after 30 days storage in lab conditions. The loss of water under refrigerated storage resulted in considerably less change (<10%) in agar concentration over the same period.

Key words: agar concentration, medium concentration, medium preparation, water availability, water loss, water status.

INTRODUCTION

Although covered or enclosed during preparation, storage, and use to preclude contamination and dehydration, sterile plant tissue culture media are rarely perfectly sealed against water loss to the atmosphere. Without extraordinary precaution, water will be continually lost from the media and its dry matter (gelling agent plus solutes) concentration will thereby continually increase. Increase in gelling agent concentration means a decrease in media water availability through a corresponding decrease in water *matric potential.*

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Increase in solute concentration means a decrease in water availability through a decrease in *osmotic potential.* Water loss may also affect media water availability through change in gel physical characteristics influencing water movement (*15, 17*). Accordingly, the observed effects on plant tissue culture growth and development from small variations in media gelling agent concentrations have been attributed to changes in water availability (*3–6, 8, 9, 12, 14–23, 26, 29*). In addition to affecting water availability, loss of water from media may influence culture response through increase in nutrient and growth regulator concentration and activity.

Water is lost from media during preparation and storage by evaporation and diffusion or convection and bulk flow out of its enclosure. Evaporation from the media occurs in response to the enormous vapor concentration (vapor pressure) gradients normally existing between the media and surrounding atmosphere. These water vapor pressure gradients are enhanced whenever media temperature is higher than ambient such as during its preparation. Rate of diffusion water loss from the media and its enclosure is limited by diffusion path length and vessel closure efficacy. The enclosed atmosphere (headspace) vapor pressure quickly equilibrates with that of the media (atmosphere becomes virtually saturated) when the vessel is closed. Some of the enclosed water vapor condenses on the vessel walls as the surrounding temperature fluctuates around the dew point. This condensed water may subsequently remain on vessel surfaces, evaporate, or fall back to the surface of the medium where it remains as *free water,* is absorbed, or evaporates (*24*). Water withdrawn by evaporation and condensation can significantly affect medium water concentration, depending on the nature of the vessel, relative volume of the medium, and the culture room thermal environment. Water is lost from the enclosure by vapor movement through the air gap between the vessel and its closure. The rate of loss is proportional to the total cross section of the air gap between the vessel and its closure and the water vapor pressure differential across the gap (determined primarily by room humidity).

Bulk flow is the movement of volumes of gas molecules in bulk volumes as opposed to molecular diffusion of individual molecules. It enhances diffusion gas exchange directly through convection within an enclosure and through movement or 'pumping' gas in and out of the enclosure and indirectly by turbulence in the surrounding air which maintains steep humidity gradients between the vessel and room. Mass air flow occurs in response to bulk pressure differences resulting from meteorological phenomena (barometric pressure) and environmental control processes (turbulence resulting from forced or convective circulation). The drying effects of the surrounding unsaturated atmosphere are therefore exacerbated by loosely fitting vessel closures, high media surface to head space ratios, media temperature higher than ambient, and increased air flow in the surrounding research labs, growth rooms, and storage rooms (*11, 24*). The rate and degree of media water loss in any particular circumstance depends on the complex integrated effects of media (formulation, gelling agent, etc.), vessel (headspace, closure, etc.), ambient environmental conditions (temperature,

relative humidity, airflow, radiation, etc.), and duration of the specific circumstances. Different degrees of media water loss create different media concentrations and water availability to cultures, even when initial formulations are identical (*24*).

It is practically impossible to prevent media water loss during preparation, storage or use. Some tactics for assessing the subsequent changes in media water availability or matric potential have been reported (*1, 16, 17, 24*) ; yet, the extent and significance of water loss during routine media handling have not been characterized. The purpose of this study was to characterize the degree of media water loss and subsequent influence on media formulation during preparation and storage.

MATERIALS AND METHODS

The degree of water loss during media preparation and storage was determined by monitoring decrease in media mass (Fig. 1).

Media

Media were prepared according to standard protocol by blending components in a 2,000 ml Erlenmeyer flask on a Corning PC-351 hot plate stirrer at 97–99°C. Two different formulations were prepared—a grape callus medium containing Gamborg's B5 macro and microelements (*7*), 10 ml L^{-1} coconut water, 5.4 μM naphthaleneacetic acid, and 1.4 μM kinetin, and a cranberry shoot medium containing WPM macro and micronutrients (*13*) and 0.98 μM 6-(γ, γ-dimethylallyamino) purine. Both media also contained 0.1 g myoinositol, 0.5 mg

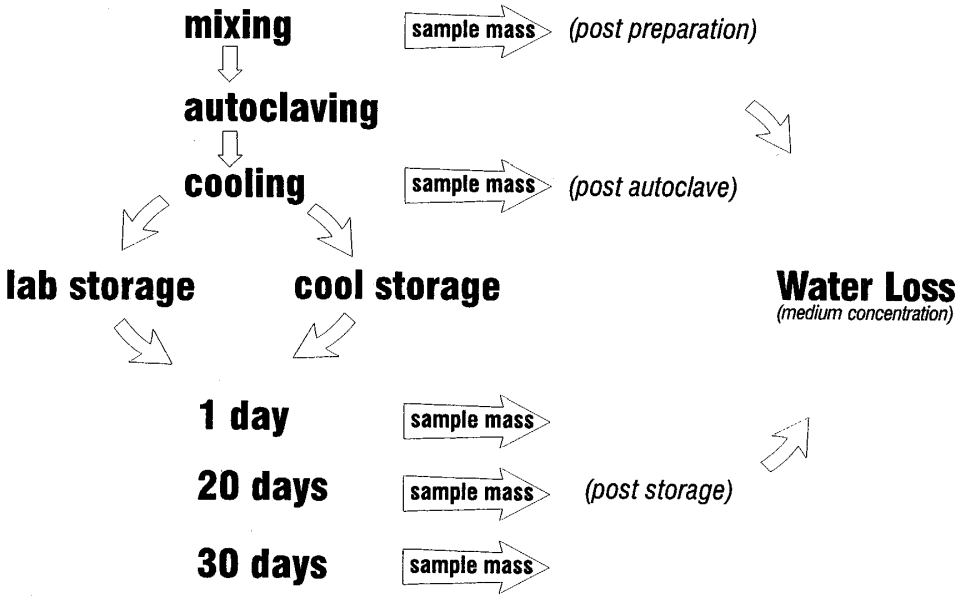

Fig. 1. Overview of experimental plan indicating medium preparation, storage, and evaluation.

thiamine, 0.5 mg pyridoxine, HCl, 0.5 mg nicotinic acid, 2 mg glycine, 250 mg casein hydrolysate, 0.1 g polyvinylpyrrolidone, and 200 μM Fe as $FeNa_2$ EDTA per liter. Components' masses were determined to a precision of $\pm$0.00005 g on a Sartorius 1601 AMP8–1 balance. The cranberry and grape media were pH–adjusted to 4.8 and 5.8 respectively by adding KOH or HCl (0.1–1.0 M). Both media were solidified with 7 g·L^{-1} of Sigma (St. Louis, MO) agar. A sample of each mixture was removed, its fresh mass measured, oven dried, and its dry mass measured (Sartorius 1412 MP8–1 balance to $\pm$0.0005 g), to determine *postpreparation* media concentration. Then 42 ml portions were dispensed (Wheaton Instruments Unispense II autodispenser) into each GA7 (Magenta Corp. Chicago, IL) treatment vessel. After capping, the mass of each vessel plus sample was recorded as the baseline for monitoring subsequent water loss (empty vessel was previously measured to determine its tare mass). After liquid cycle sterilization for 18 minutes at 140℃ (250°F) in an AMSCO 2023 Vacamatic steam sterilizer (American Sterilizer, Erie, PA), media samples were moved to a sterile Baker Edgegard laminar flow hood and allowed to equilibrate with lab temperature (20$\pm$2℃) and fully solidify (approximately 3 hr). The mass of each vessel was again measured to determine *postautoclave* agar concentration prior to storage.

Experimental treatments

Media were exposed to dark storage treatments of 24 hours (fresh), 20 days, and 30 days (aged) in growth room laboratory (24$\pm$2℃) or cold room (5$\pm$2℃) temperatures. Although the storage cabinets were not tightly sealed, the atmosphere surrounding the samples was stagnant—no turbulence. Relative humidity (Airguide HCS hygrothermometer, Chicago, IL) was about 60$\pm$10% in the lab and about 45$\pm$5% in the cold room. Change in media water content was monitored by measuring vessel mass at selected intervals (Fig. 1).

Measurements

Final dry mass was determined by drying samples still contained in the vessels in a Blue–M OV–490A–2 Stabil–Therm forced–air oven at 80$\pm$2.5℃ until they reached a constant mass (within 16 hrs). Medium dry mass was determined by subtracting individual vessel tare mass from the final combined dry mass. Media concentrations were calculated as the ratio of dry mass to the difference between fresh and dry mass. Final media gel concentrations were calculated by multiplying dry matter by the original ratio of gel mass to total non–aqueous ingredient mass.

RESULTS AND DISCUSSION

Media water content decreased during preparation (Table 1). This resulted in an agar concentration increase from the original 0.7 to 0.72 and 0.73 g·L^{-1} for cranberry shoot proliferation and grape callus media respectively. The further increase in concentration due to water loss during autoclaving was to 0.74 and

Table 1. Cranberry shoot proliferation and grape callus media mass and agar concentration measured prior to and after preparation and storage.

Stage of Media Preparation and Storage	Cranberry Medium (fresh mass, g)*	Agar ($g \cdot L^{-1}$)	Grape Medium (fresh mass, g)*	Agar ($g \cdot L^{-1}$)
Original		0.70		0.70
Post-Preparation	40.85[a]	0.72	40.30[a]	0.73
Post-Autoclaving	39.50[b]	0.74	38.90[b]	0.75
Post-Lab Storage, days				
1	39.10[b]	0.75	38.50[b]	0.76
20	35.10[d]	0.83	35.60[d]	0.82
30	33.00[e]	0.89	33.50[c]	0.88
Post-Cool Storage, days				
1	39.20[b]	0.75	38.60[b]	0.76
20	38.80[cb]	0.76	38.20[cb]	0.77
30	38.50[c]	0.76	38.00[c]	0.77

*Numbers followed by different letters are significantly different at the 5% level.

0.75 $g \cdot L^{-1}$, respectively.

There was no measurable water loss from lab or cool stored media over the first 24 hours—their subsequent concentrations were 0.75 and 0.76 $g \cdot L^{-1}$ for cranberry and grape media respectively (Table 1). Significant water loss, however, occurred during longer term storage. Lab-stored media concentration dramatically increased from 0.75 and 0.76 $g \cdot L^{-1}$ to 0.83 and 0.82 $g \cdot L^{-1}$ after 20 days and to 0.89 $g \cdot L^{-1}$ after 30 days for cranberry and grape callus media respectively. Media in cool storage, on the other hand, changed relatively little from the starting 0.75 and 0.76 $g \cdot L^{-1}$ to 0.76 and 0.77 $g \cdot L^{-1}$ after 20 days and remained the same after 30 days for cranberry and grape media respectively.

This study quantified the time course of water lost from the plant tissue culture media and subsequent media concentration increase throughout standardized routines of preparation and storage. The results confirm that significant discrepancies can exist between the expected or reported gelled plant tissue culture medium concentrations and the actual concentrations to which the cultures are exposed. As suggested previously, changes in relative water status can be recorded coincident with changes in the order of mixing components (*2*). Variations in preparation and storage protocols are further likely to alter media water availability, with consequent influence on cultured plant performance (*24, 25, 27*). A parallel increase in concentration of the other non-aqueous ingredients also occurs. Even though this media water loss is practically unavoidable, knowledge of the extent of loss makes it possible to compensate for it through manipulation of the original formulation to ensure cultures are initially established in media of a known, desirable agar concentration; especially for species or varieties most susceptible to gel concentration effects (*8, 28*). This

approach will only work as long as preparation protocol and storage conditions are consistent. Above all, the results of this sutdy especially indicate the importance of measuring agar concentration in research studies to ensure accurate application of media treatments.

Desiccation was considerably reduced in long term cool storage. This method would seem preferred for medium stored for longer than 24 hours; however, effects of the low temperature on gel structure and subsequent water availability is unknown. For this particular reason, and because of the observed drying, ensuring consistent formulations and properties for media needed at different stages of an experiment probably requires the media to be prepared in batches which allow the same media storage period and conditions for each of the stages. Repeat runs of an experiment can only be expected to result in similar trends if media of the same age is used for each run. If, for example, the same batch of media is split and used at different times, microculture plants from one trial may be subjected to a different degree of water stress than supposedly parallel replicates from an earlier run. Rapid use of the medium is also recommended. According to studies recently reported, not only medium physical properties change during preparation and storage but important chemical reactions take place as well during the same period, diminishing medium quality and causing performance variations (*10*). Other studies have indicated that fresh prepared medium outperforms stored medium when both media are used for the same cultures (*16*).

An important disadvantage of using low temperature storage to preserve gelled-medium is the condensation of water on the vessel's inside walls and on the gel surface when the vessel is returned to lab temperature. As a consequence, the free water introduces problems of hyperhydricity in the in vitro tissue of shoots and callus in culture. This problem is exacerbated for gelled-media solidified with lower concentrations of gelling agent. An alternative is to remove the free water by removing it from the vessel. This 'extracted' water also means medium gel concentration has increased; it is important to account for this increase in research applications.

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