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Stability of tissue culture medium pH as a function of autoclaving, time, and cultured plant material

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ABSTRACT

Autoclaving is a standard procedure for sterilizing nutrient media for plant tissue cultures. Most tissue cultures are grown at pH 5.2 to 5.8 with pH adjustments being made prior to autoclaving. This paper reports that there are significant differences between initial pH levels and pH levels following autoclaving, particularly in the pH range of 5.7 to 8.5. This effect is noted with and without agar. In addition, we report that with time the pH of the medium drifts into the acid range. When Cucumis callus was added to the medium, the pH was changed significantly within 48 hours. The amount and direction (increase or decrease of pH) was significantly correlated with the original pH. This suggests that researchers should be wary of the true pH situation in their medium. In addition, in publications authors should specify whether their medium pH value was determined before or after autoclaving.

INTRODUCTION

Plant tissue cultures are known to tolerate a wide range of pH's; a value between 5.2 and 5.8 is most often provided (7). The standard procedure for pH adjustment in tissue culture media is to correct the pH of the nutrient medium with acid and/or base prior to autoclaving. Although some researchers have measured pH after autoclaving and reported this "post-autoclave" pH value, the determination of this value has not been routine laboratory practice. There are reports that the high temperatures of autoclaving may cause the pH to drift (2,4,7,8,12). A recent study suggested that changes in pH after autoclaving are less pronounced with increasing agar levels (12). Behagel (2) discussed the dependence of post-autoclave pH on the course of temperature during autoclaving. He concluded that differences in post-autoclave pH were unavoidable due to the many chemical reactions that occur during media sterilization which are both temperature and pH dependent. Other factors which can influence media pH include the type of autoclave, position within the autoclave, quality of mineral nutrients, the quality of water used in the medium, and the duration of autoclaving.

In addition to physical factors which influence pH changes in vitro, it is also known that the presence of plant tissue affects the medium's ultimate pH. For instance, Pelet et al. (11) reported time related pH changes when Populus deltoides and Ulmus americana callus were grown at various post-autoclave pH's, ranging from 3.5 to 8.0. All media drifted towards a pH of 6.0 during a 4-week dark incubation. The drift was slow in control media without callus and accelerated in media with callus.

Dougall (5) has reviewed the literature associated with in vitro pH changes, and he believes that the cause of such pH changes is best explained in terms of ammonium (NH_4^+) and nitrate (NO_3^-) uptake from the medium. Dougall also presents evidence that initial medium pH can influence the ultimate pH of the medium by influencing the uptake rate of nitrate and/or ammonium.

In spite of all that is known about in vitro pH stability, most tissue culturists are unaware of the situation in their own medium. This study was initiated to examine the extent to which initial pH of a modified Murashige and Skoog (MS) (10) nutrient medium would be altered after autoclaving and to investigate pH stability with time with and without plant material.

MATERIALS AND METHODS

The medium used in this investigation was a modified Murashige and Skoog (MS) medium (13). Six batches of media were made. Each batch was adjusted to a different pH (5.0, 5.7, 6.4, 7.1, 7.8, and 8.5) prior to autoclaving with 0.5N KOH and HCl. Agar (DifCo Bacto, 6 g/liter) was added to half of the samples, heated, and then dispensed into 25 X 150 mm culture tubes at 10 mls of medium per tube. The samples without agar were dispensed directly into tubes. The tubes were covered with plastic caps. After autoclaving for 15 min at 15 psi, the tubes were cooled to room temperature. Post-autoclave pH was determined immediately after cooling and at weekly intervals for an additional 6 weeks with a Corning 125 pH meter using an Orion 91-35 combination electrode. The pH meter was standardized using 2 buffers (pH 7.0 and pH 4.01). The electrode was pushed into the medium and pH was recorded once the machine had equilibrated. For each treatment at each date, a sample of 10 different tubes was sacrificed to estimate pH changes over time.

In another experiment, MS media without agar were determined to have post-autoclave pH's of 3.33, 5.11, 6.63, and 7.98. About 100 mg of Cucumis melo callus (14) was added to the media. pH changes were monitored by harvesting 5 culture tubes of each pH every 2 hours for a 48 hour period. Some tubes were maintained without callus (control) to estimate the amount of drift that occurred during this period.

Comparisons of correlations between agar and liquid media at each pH were performed. The time studies were analyzed by regression analysis.

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RESULTS

Following autoclaving, pH changes were observed, particularly in the pre-autoclave range of 5.7 to 7.8 (Table 1). Post-autoclave pH readings were lower than the pre-autoclave pH readings. For example, pH 7.1 pre-autoclave medium changed to about 5.7 after autoclaving.

Table 1. pH changes after autoclaving on modified Murashige and Skoog medium adjusted to various pH's.

Number of weeks after autoclaving	Pre-autoclave pH values[z] 5.0	5.7	6.4	7.1	7.8	8.5
Liquid medium (-Agar)						
0	4.2[y]	4.6	5.1	5.8	6.7	8.1
1	4.1	4.4	5.0	6.0	6.8	7.4
2	4.1	4.3	5.0	5.9	6.8	7.4
3	4.1	4.2	4.9	5.8	6.7	7.2
4	4.0	4.2	4.8	5.6	6.4	7.1
5	4.0	4.3	4.8	5.6	6.4	7.0
6	4.0	4.1	4.7	5.2	6.3	6.8
Semi-solid (+Agar)						
0	4.4	4.6	5.0	5.7	6.7	8.0
1	4.4	4.6	5.0	5.8	6.7	7.4
2	4.3	4.6	4.9	5.7	6.6	7.3
3	4.4	4.5	4.7	5.6	6.5	7.1
4	4.2	4.4	4.7	5.4	6.4	7.0
5	4.2	4.5	4.8	5.5	6.3	6.9
6	4.2	4.4	4.7	5.3	6.2	6.8

[z] pH adjusted with 0.5N KOH or HCl before autoclaving
[y] Each value is the average of 10 readings

The pH of each test medium drifted to a more acid condition over the 6-week test period (Table 1).

When Cucumis callus was added to the media, the pH changed rapidly (Figure 1). For all media with pH 5.11 or greater, there was a gradual acidification over the 48 hour test period. The very acid medium (pH = 3.33) became less acid in a linear fashion throughout the 48 hour period. After 48 hours, there were no significant differences among the pH's for any of the media (Table 2). Without plants, the pH drift encountered during the 48 hour period was negligible (Table 2).

Table 2. Changes in pH after 48 hours on tissue culture medium with and without Cucumis callus.

Original pH	Observed pH (48 hours) + plants	- plants	Predicted pH
3.33	4.87	3.27	4.82 ± 0.12[z]
5.11	4.55	4.99	4.52 ± 0.15
6.63	4.58	6.68	4.60 ± 0.40
7.98	4.71	8.00	4.42 ± 0.36

[z] Predicted pH ± 95% confidence intervals

DISCUSSION

Changes in pH do occur after autoclaving and these changes may be expected with or without the addition of agar. The extent of change was not the same at all pre-autoclave pH values. With time, the medium pH drifted to a more acid condition. As observed by Singha (12), agar slightly reduced media acidification. The amount of reduction depended upon the pre-autoclave pH. Of particular importance is the fact that autoclave-induced pH changes occur prominently within the pH range used by most tissue culturists, 5.2 to 5.8. It may be that for a particular species the combination of pH drift and autoclav-induced pH changes may be drastic enough to result in non-optimal growing conditions.

The addition of Cucumis callus to the media accelerated the rate of pH change regardless of original pH. The statistical equation that described the change in pH was different for each original pH (Figure 1). For instance, the post-autoclave pH of 3.33 increased to a final average pH of 4.87 while the 7.98 pH was reduced to 4.71 in the same 48 hour period (Table 2). These results correspond to those of Pelet et al. (11) who reported that the addition of poplar or elm callus to media of various pH's resulted in a drift towards a pH of 6.0.

Ion exchange, as a function of type of nitrogen in the medium, is a probable source of the extra hydrogen or hydroxide ions required to explain the pH changes (5). When the medium was only slightly acid to basic (pH 5.11 to 7.98), the addition of Cucumis callus resulted in more acidity (Table 2, Figure 1). It has been shown that as plant cells absorb ammonium, a hydrogen ion is exchanged. These H+ ions probably contributed to the pH decrease observed on post-autoclave pH 5.11, 6.63, and 7.98 media (Table 2). The pH changes also might have been due partially to intrusion of H+ ions into cell walls to create a pH optimum for cell wall loosening enzymes (6).

When Cucumis callus was added to the very acid medium, pH = 3.33, the medium became less acid (pH = 4.87) (Table 2, Figure 1). Acidity favors the uptake of nitrate (NO_3^-) ions. As nitrate is absorbed, bicarbonate (HCO_3^-) is extruded to the medium. The bicarbonate ion, in turn, joins with a proton (H+) (from ionized water) to yield carbonic acid (H_2CO_3). The decrease of H+ ions (and concomitant increase in OH- concentration), results in pH change to a more basic condition.

The prediction curve for the pH 3.33 medium was best fit by a linear, not quadratic, equation. On the basis of this equation, the predicted change in pH with increasing time would result in very basic medium. We have not found this to be the case (data not presented). On the contrary, with time the pH tends to stabilize near 4.5 to 4.8. Therefore, our prediction curve probably cannot be extrapolated past the first 48 hours. The curvilinear prediction equations for the other pH's are probably valid over a longer period of time.

Because the pH of all four media were so similar after 48 hours (Table 2), it is tempting to suggest that the plant material has an active role in establishing an optimum pH environment. It also might be that the direction and extent of the pH change might be influenced by the parent plant's in vivo pH optimum. Such a hypothesis can only be verified by comparing species with known in vivo requirements. For instance, if this is true, then members of the Ericaceae (Heath family), which require acid soils, should acidify the medium more than a species that prefers basic soils. We leave these investigations to others.

The pH changes associated with our modified MS should not be assumed to be identical for all media. It is suggested that similar experiments be conducted in every tissue culture laboratory under the particular growing conditions available in that laboratory. In addition, we suggest that all tissue culture researchers should take care to measure the pH of

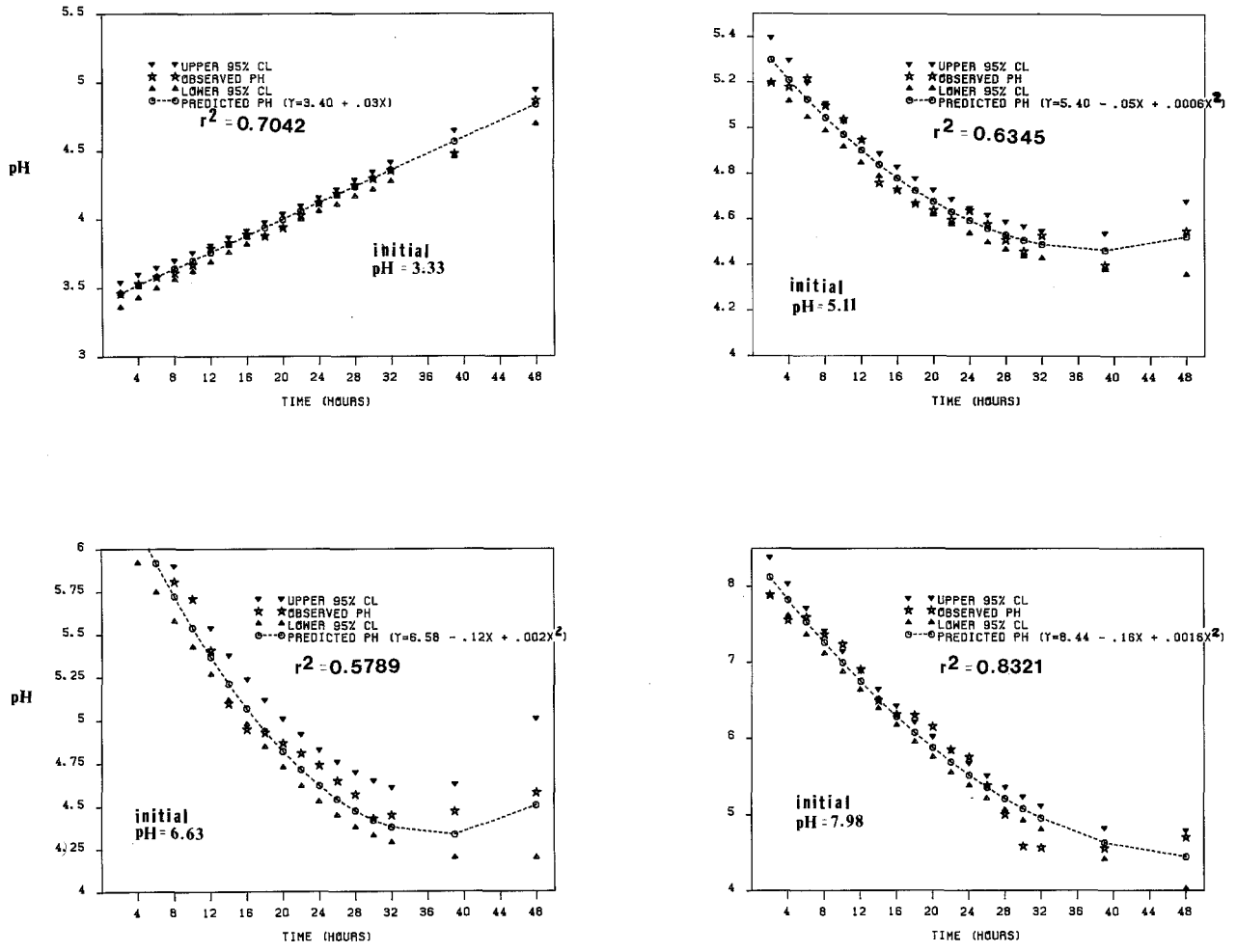

Figure 1. pH changes which have been induced in vitro by the addition of Cucumis callus to Murashige and Skoog media of various initial pH's.

nutrient medium both before and after autoclaving as well as at various times during culture.

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