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Culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method

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Summary. When four carbohydrates were tested against six commonly cited inorganic basal media, post-autoclave pH was highest for carbohydrate-free and sucrose- containing media, and progressively lower for maltose-, Plucose-, and fructose-containing media, respectively, post-autoclave pH for these media without carbohydrates was related to medium buffering capacity. Addition of gelling agents (10 of 11 tested) increased the postautoclave pH of MS medium containing sucrose. Neutralized and acid-washed activated charcoal also increased the post-autoclave pH of liquid and agar- solidified MS medium, and the pH changed further during 8 weeks of storage. Changes in medium pH caused by gelling agents, but not charcoal, could be alleviated by adjusting the pH after their addition but prior to autoclaving.

Key Words: Agar - Autoclaving - Fructose - Gellan gum - Ar-Carrageenan

Introduction

Culture medium pH affects nutrient availability and uptake (Minocha 1987) and has been shown to influence a number of plant developmental processes *in vitro,* including organogenesis (Zhang & Stoltz 1989), floral differentiation (Cousson et al 1989), micropropagation rate (Reeves et al 1983), secondary product formation (Hagimori et al 1983), cell division (Basu et al 1988), adventitious rooting (Williams et al 1985), somatic embryogenesis (Smith & Krikorian 1990a), xylogenesis (Khan et al 1986), and microspore culture (Barrow 1986). In some studies, very narrow pH optima (0.2 pH units) have been observed (Nesius & Fletcher 1973). Between pH S.5 and 6.0, significant differences nave been demonstrated for the uptake of plant growth regulators (Kaiser & Hartung 1981).

Most plant tissue culture media, however, are poorly buffered (Martin 1980) and, as such, are subject to significant changes in pH, depending on the specific medium formulation, medium sterilization method, and type of plant material cultured. Heat sterilization can significantly alter medium pH by denaturation of proteins, hydrolysis of carbohydrates (Schenk et al 1991), and dissolution of salts (Behagel 1971).

It is common practice in many plant tissue culture laboratories to adjust the pH of media prior to sterilization by autoclaving. In addition, medium pH is often adjusted prior to the addition of gelling agents and activated charcoal. This practice makes pH at the time of culture difficult to determine, and equivalent pH values between two or more media very difficult to obtain. Carbohydrate source (Batty & Dunwell 1989; Orshinsky et al 1990), gelling agents (Ichi et al 1986; Pasqueletto et al 1986; Morimoto & Murai 1989), and activated charcoal (Misson et al 1983; Zaghmout &

Torello 1988) have all been reported to influence plant developmental processes *in vitro.* In many instances, however, the effect of autoclaving of these compounds on the pH of the medium has not been examined. Thus, differences in medium pH between treatments may have influenced the results from some of these investigations.

The objective of this study was to determine the influence of inorganic basal medium formulations and medium components on post-autoclave pH values. This was accomplished by determining the following: (1) the effect of carbohydrates on post-autoclave pH of six common inorganic basal medium formulations, (2) the effect of gelling agents on post-autoclave pH, and (3) the effects of ictivatea charcoal and method of post-autoclave medium storage on the pH of liquid and agar-solidified media.

Materials & methods

For all experiments, 10 ml of medium were dispensed into 25 x 95 mm borosilicate glass shell vials and capped with aluminum foil or polypropylene closures (Bellco). Media were autoclaved for 15 min. at 15 psi using a Sterilmatic STME autoclave (Market Forge), and allowed to cool to room temperature (25C) prior to pH measurement. The pH of the contents of each vial was determined with a Corning 145 meter equipped with a Coming Calomel combination electrode. For pH measurement of semi-solid media, the electrode was pressed into the medium. There was good contact between the medium and the electrode and measurements were stable over time.

Due to inconsistencies in the literature, the salt formulations of some media were corrected as follows: Murashige & Skoog (1962) ZnSO^HjO corrected from $ZnSO_4$'$4H_20$, 37.2 mg I"[1] Na_2- EDTA'$2H_20$ (see Singh & Krikorian 1980); White (1963) NajSO, corrected from $NaSO_4$, 19 mg I'[1] NaH_2PO_4 H_20, ferric sulfate replaced with equivalent amount of ferrous sulfate in chelated form (see Singh & Krikorian, 1981); B5 (Gamborg et al 1968) iron replaced with MS iron formulation and $CuSO_4$'$5H_20$ corrected from $CuSO_4$ (see Gamborg et al 1976); Nitsch & Nitsch (1969) 5.57*g* $FeSO_4$'$7H_20$ in stock solution corrected from 0.557 g (see Nitsch 1977); Woody Plant Medium (Lloyd &McCown 1980) $MnSO_4$'$4H_20$ corrected from $MnSO_4$'H_20 (see Smith *&* McCown 1982/83). Schenk & Hildebrand! (1972) medium was prepared as originally cited.

Carbohydrates/Basal Media. To compare equal moles of hexose units for each carbohydrate, sucrose (0.1M), maltose (0.1M), glucose (0.2M), and fructose (0.2M) were added individually to each of the six basal media. The controls lacked carbohydrate. The pH of each treatment was adjusted with 0.1 N HC1 or NaOH according to the published value for each basal medium formulation. Treatments were dispensed into six vials each, autoclaved in two batches, and the entire experiment was repeated (12 vials/treatment total).

Gelling Agents. Difco Bacto agar, Difco Noble agar, Gibco Phytagar, Carolina Biological Supply Co. T.C. agar, Oxoid #1 agar, Sigma Agar, Sigma Purified agar, Merck Gelrite gellan gum. Research Organics Inc. fc-carrageenan, Sigma Agargel (an agar/gellan gum blend), and Research Organics Inc. Caragar (an agar/Jt-carrageenan blend) were added individually to Murashige and Skoog (MS) basal medium containing 0.1 M sucrose. The control was liquid MS + sucrose. Agars and *k*-carrageenan were added at 0.8%, agar blends at 0.4%, and Gelrite at 0.2% (w/v) to obtain similar gel strengths. The pH of each treatment was adjusted to 5.75 *before* gelling agent addition. Flasks were heated to dissolve the gelling agents. Each medium was dispensed into twelve vials and autoclaved in four batches. The entire experiment was repeated (24 vials/treatment total). The same experiment was conducted except the pH was adjusted to 5.75 *after* addition of the gelling agent, but before heating to dissolve the gelling agents.

Activated Charcoal/Storage Environment. Hydrochloric acid-washed activated charcoal and neutralized activated charcoal (Sigma Chemical Co.) were added individually at 0.5% (w/v) to MS basal medium containing 0.1 M sucrose and 0.8% Phytagar, and to MS basal medium containing 0.1 M sucrose only. Liquid and agar-solidified controls lacking activated charcoal were included. The pH was adjusted to 5.75 *after* charcoal and gelling agent additions. Flasks were heated to dissolve the gelling agent. Each medium was dispensed into 54 vials and autoclaved in three batches. 144 vials were placed on a shelf in a culture room (25C, 100 /imol m'^{2} s'^{1} PAR, 12 h photoperiod), and an equal number were wrapped in aluminum foil and plastic bags and placed in a walk-in cold room (4C), for sampling at 1,2,4, and 8 weeks after autoclaving. The remaining 36 vials were used for pH measurements the following day. The entire experiment was repeated at a later date (12 vials/treatment total). A similar experiment, except that pH was adjusted to 5.75 after addition of the gelling agents, but *before* charcoal additions, was also conducted.

Results and Discussion

Based on phosphate concentration, White's medium is the least buffered, and Schenk & Hildebrandt medium is the most buffered formulation, and they exhibited the highest (0.16) and lowest (0.03) standard errors for post- autoclave pH values, respectively. In addition, total deviation from initial pH of the control treatments (carbohydrate-free media) was highest for White's medium (1.05 pH units) and lower tor die other media (0.03-0.35 pH units; Fig. 1). Autoclaving was not found to be a significant variance component.

With the exception of Nitsch & Nitsch medium, postautoclave pH values for basal liquid media without carbohydrates were not significantly different from the same basal medium containing sucrose. Post-autoclave pH values were highest for sucrose-containing media and progressively lower for maltose, glucose, and

fructose- containing media, respectively (Fig. 1). pH differences between sucrose-containing ana fructose-containing media were significantly different for all basal medium formulations. Sucrose-containing and glucose-containing media exhibited significantly different post-autoclave pH values for all basal medium formulations except MS. Sucrose-containing media and maltose-containing media exhibited significantly different post-autoclave pH values for all basal medium formulations except MS and Woody Plant Medium.

Investigators have reported significant effects of alternative carbohydrates on several morphogenic processes *in vitro;* however, the possible contributing ractor of autoclaving-induced differences between carbohydrate treatments has only seldomly been examined and reported (Hildebrandt & Riker 1949; Hsiao & Bomman 1991). Researchers have tested the effect of autoclaving versus sterile filtration of culture media, to minimize autoclaving-induced degradation of medium components (Mathes et al 1973). Growth inhibition was observed when fructose-containing media are autoclaved and was attributed to toxicity of some of its degradation products (de Lange 1989; Redei 1974). Shaw et al (1967) have shown, however, that fructose degradation is increased under acid conditions. The present data, together with the observation that fructose degrades more under acidic conditions, suggest that by increasing the pre-autoclave pH of fructose-containing media to obtain post-autoclave pH values equivalent to post-autoclave values for sucrose-containing media, fructose degradation, and thus its toxicity, may be reduced.

In the second experiment, when the pH of MS medium was adjusted to *5.75 before* gelling agent additions, all gelling agents significantly alteredpost-autoclavepH, up to 0.23 units from the control (Table 1). In contrast, when the pH of the media is adjusted to 5.75 *after* gelling agent additions, only Phytagar and Sigma Purified agar exhibited significantlv higher (0.09) and lower (0.08) post-autoclave pH values from the control, respectively. The minor post-autoclave differences observed when pH was adjusted *after* addition of the gelling agent indicate that most of the pH differences between gelling agents can be eliminated simply by adding them to the culture medium *before* pH adjustment.

Gelling agents have "been shown to influence plant growth *in vitro,* depending on their type (Jaramillo & Summers 1990), manufacturer (Debefgh 1983), and concentration (Bomman & Vogelmann 1984). Relatively few studies, however, have examined the effects of gelling agents on culture medium pH (Singha 1982, Selby et al 1989). Sarnia et al (1990) demonstrated that the method of agar addition can affect post-autoclave pH. The method used in this study (heating media to the point of boiling to dissolve the gelling agent and then autoclaving) was found by them to have a minimal affect on postautoclave pH. Phytagar exhibited a post-autoclave pH closest to the initial pH of MS medium, and thus was selected as the agar to be used in the third part of this study.

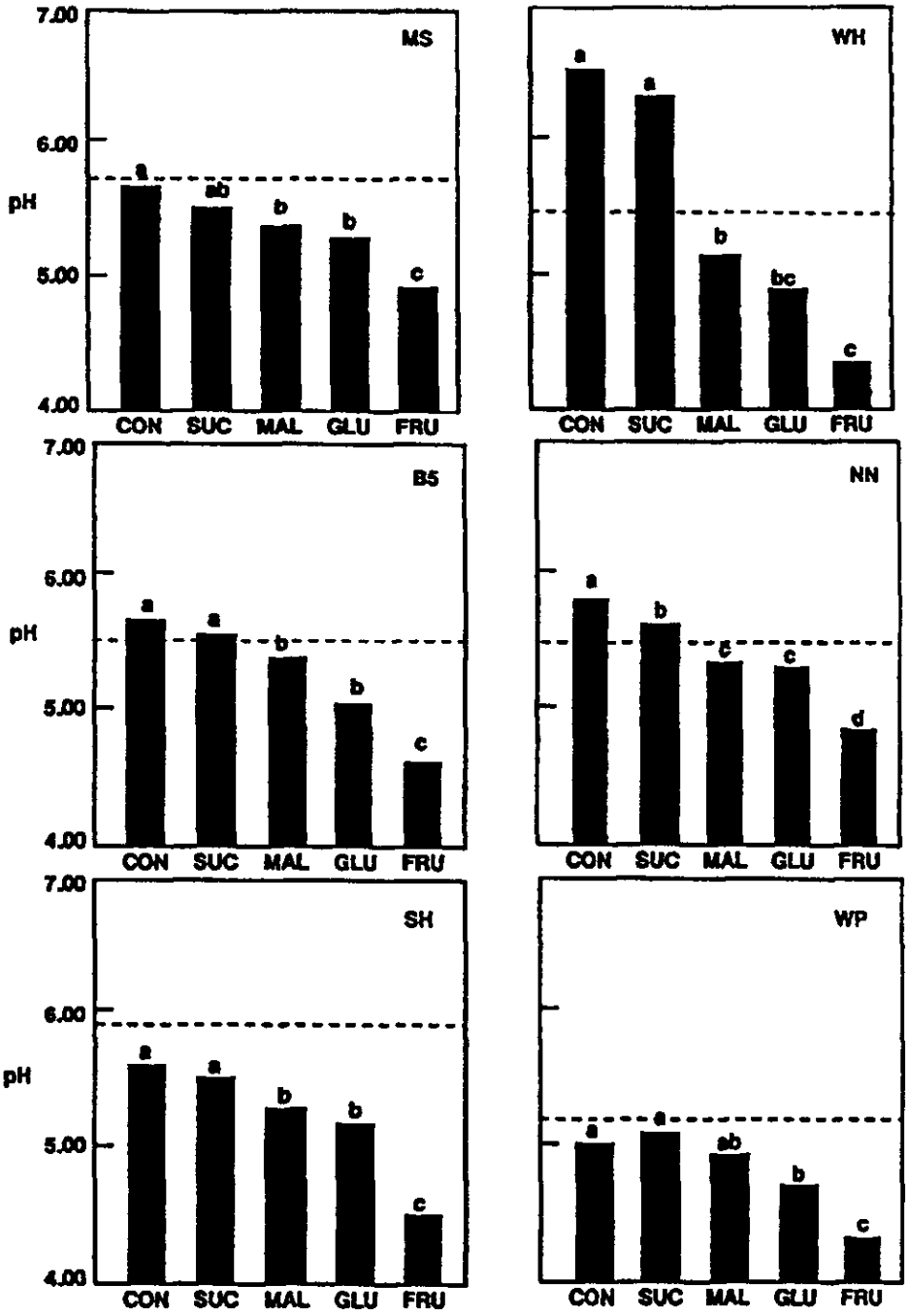

Fig. 1. Influence of inorganic medium formulation and carbohydrate source on post-autoclave pH. Con= control, Suc= sucrose, Mal= maltose, Glu= glucose, Fru= fructose. MS= Murashige & Skoog 1962, WH= White 1963, B5= Gamboig et al 1968, NN= Nitsch & Nitsch 1969, SH= Schenk & Hildebrandt 1972, WP= Woody Plant Medium (Lloyd & McCown 1980). Dashed lines indicated pH before autoclaving. Mean separation by Duncan's New Multiple Range Test, 0.05 level, by medium. Values with the same letter are not significantly different.

Table 1. Influence of gelling agent on post-autoclave pH of MS medium containing 0.1M sucrose[a]

	post-autoclave pH	
Gelling Agent	pH adjusted to 5.75 *before* adding gelling agent	pH adjusted to 5.75 *after* adding gelling agent
T.C. agar	5.84a	5.58bc
Difco Bacto	5.82a	5.58bc
Sigma agar	5.78b	5.58bc
k-carrageenan	5.77b	5.56bc
Agargel	5.75bc	5.59bc
Phytagar	5.72cd	5.67a
Gelrite	5.71cde	5.56bc
Oxoid #1	5.70de	5.60b
Caragar	5.69de	5.56bc
Difco Noble	5.67e	5.58bc
none	5.61f	5.58bc
Sigma Purified	5.56g	5.50d

[a]pH was determined 24h after autoclaving. Mean separation by Duncan's New Multiple Range Test, 0.05 level, by column. Values followed by the same letter are not significantly different.

In the third experiment, post-autoclave pH of all charcoal-ammended treatments adjusted to 5775 *before* addition of the charcoal was considerably higher than the same treatments adjusted to 5.75 *after* addition of the charcoal (0.15 to 0.42 units, Table 2). With the exception of acid-washed charcoal in an agar-solidified medium, post-autoclave pH values for all charcoal-ammended media are closer to their respective controls when the pH of the media is adjusted *after* charcoal addition. These data indicate that charcoal addition alone influences culture medium pH.

Based on the above results, only the results from media adjusted to pH 5.75 *after* charcoal addition are shown for the time-course and storage study (Fig. 2 & 3). In liquid MS + 0.1M sucrose medium, treatments containing acid- washed or neutralized activated charcoal exhibited higher post-autoclave pH values than the control regardless of method or length of storage (Fig. 2). One day after autoclaving, the pH of solidified media containing acid- washed activated charcoal was lower than the control, whereas the pH of solidified media containing neutralized

activated charcoal was higher than the control (Fig. 3). This interaction between type of medium (agar-solidified vs. liquid) and charcoal treatment, however, was not significant one week after autoclaving, when all media containing acid-washed or neutralized activated charcoal had pH values significantly higher than controls and medium type (agar-solidified vs. liquid) was no longer a significant variance component. Thus, checking and recording the pH of charcoal-ammended media one week after autoclaving should give a more representative value for medium pH.

Table 2. Influence of activated charcoal on post-autoclave pH of liquid and Phytagar-solidified MS media containing 0.1M sucrose[a]

	post-autoclave pH	
	pH adjusted to 5.75 *before* adding charcoal	pH adjusted to 5.75 *after* adding charcoal
Liquid MS Medium		
control	5.64a	5.59a
+ acid-washed charcoal	6.24b	5.95b
+ neutralized charcoal	6.64c	6.49c
Phytagar-solidified MS medium		
control	5.71a	5.66b
+ acid-washed charcoal	5.81a	5.39a
+ neutralized charcoal	6.27b	5.90c

[a]pH was determined 24h after autoclaving. Mean separation by Duncan's New Multiple Range Test, 0.05 level, by medium and column. Values followed by the same letter are not significantly different.

Acid-washed activated charcoal and neutralized activated charcoal significantly affected post-autoclave pH, regardless of the type of medium (agar-solidified vs. liquid), and storage method. Both types of charcoal were produced from the same source and the neutralized charcoal was produced from hydrochloric acid-washed charcoal that was subsequently neutralized (Kenneth Torres, Sigma Chem Co., personal communication). Thus, some residual effect of the neutralization procedure may have caused a fixed increase in post-autoclave pH, but required exposure of the charcoal to autoclaving conditions to cause this increase.

Photochemical changes may also occur in culture media (Stasinopoulos and Hangarter 1990). Therefore, we hypothesized that storage environment may also affect medium pH. We compared pH changes over time of media stored in two different environments (at 25C in the light and at 4C in darkness). Similar to Skirvin

et al (1986), we found that both liquid and agar-solidified culture media stored in the light acidified over time. Storage in darkness at 4C was shown to decrease medium acidification compared to media stored under a standard plant culture environment (at 25C in the light), irrespective of gelling agent addition, type of activated charcoal, or length of storage. These data suggest that media should be stored under refrigeration in darkness to minimize its acidification and illustrate the importance of re-checking the pH of a culture medium if it has been stored.

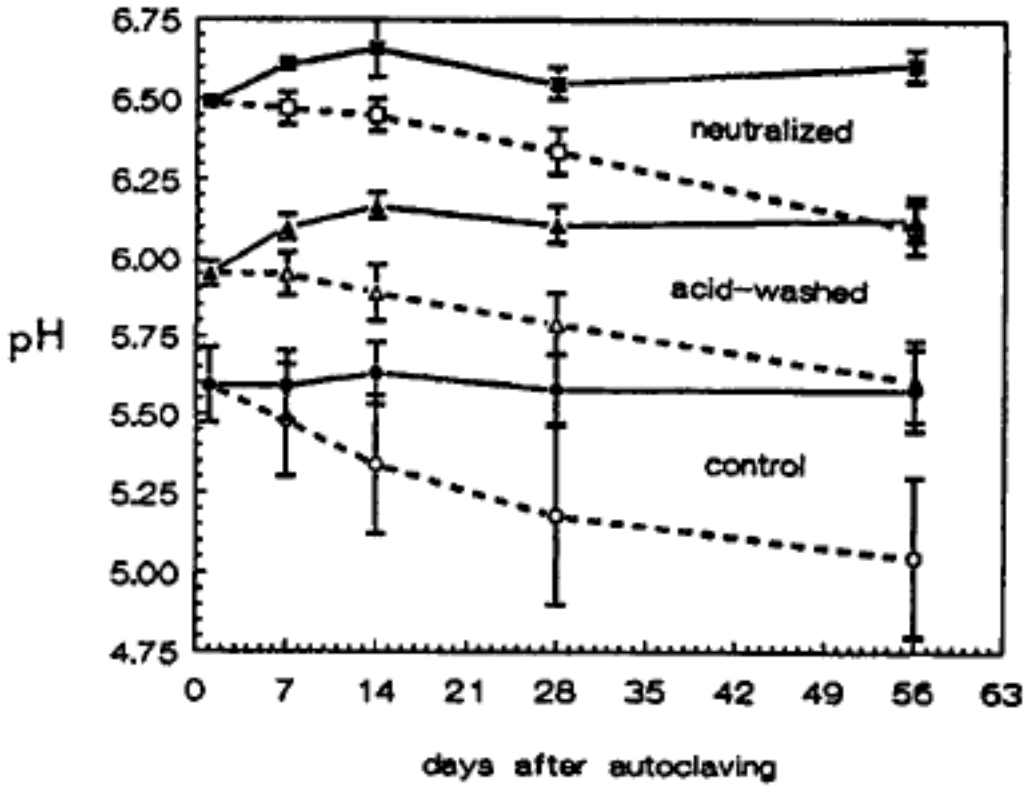

Fig. 2. Influence of acid-washed and neutralized activated charcoal and storage method on post-autoclave pH of liquid MS medium containing 0.1M sucrose. pH was adjusted to 5.75 after charcoal additions, but before autoclaving. Dashed lines indicate storage at 25C in the light. Solid lines indicate storage at 4C in darkness. Vertical lines = S.E.M.

Activated charcoal has been added to plant tissue culture media formulations because of its promotive effect on androgenesis (Johansson 1986), embryogenesis (Buccheim et al 1989), and organogenesis (Zagnmout & Torello 1988). It has been shown to adsorb a number of compounds, including culture metabolites, inhibitors, and growth regulators (Fridborg et al 1978). Few investigations, however, have taken into account its influence on plant tissue culture medium pH. Rahbar & Chopra (1982) and Smith & Krikorian (1990b) observed that activated charcoal

increased medium pH, similar to our results, and demonstrated the promotive effect solely of an increase in pH on moss gametophyte fertility and carrot somatic embryo development, respectively. Langowska (1980) concluded that activated charcoial suspensions raised culture medium pH by the adsorption of cations from the medium.

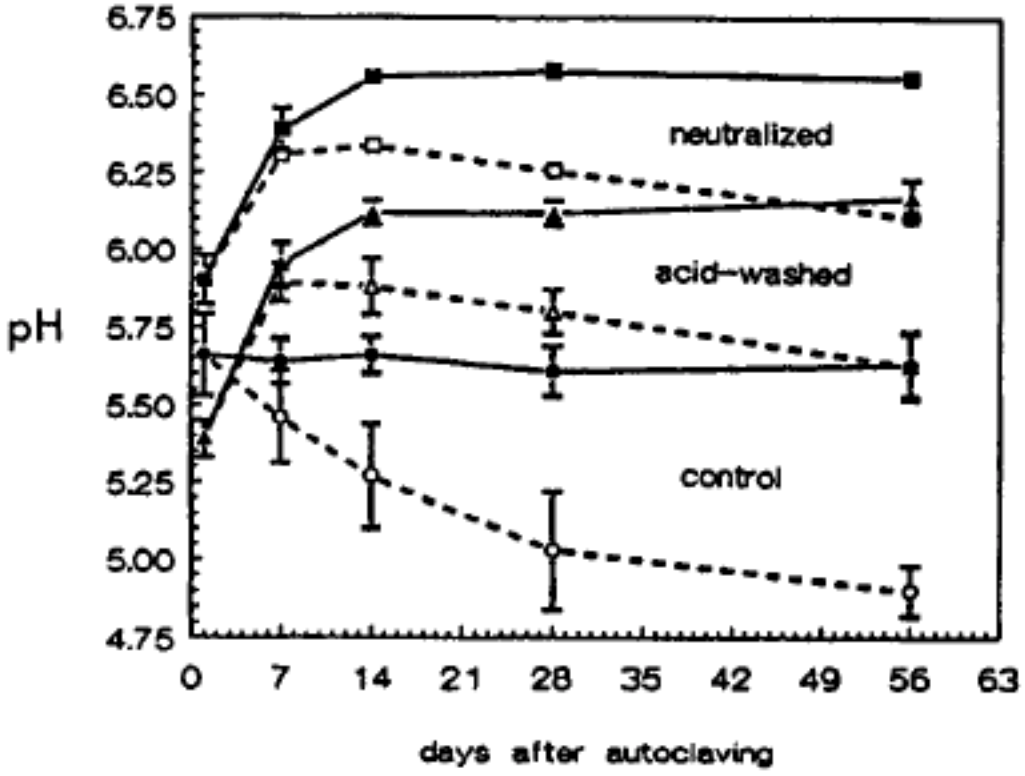

Fig. 3. Influence of acid-washed and neutralized activated charcoal and storage method on post-autoclave pH of Phytagar-solidified MS medium containing 0.1M sucrose. pH was adjusted to 5.75 after agar and charcoal additions, but before autoclaving. Dashed lines indicate storage at 25C in the light. Solid lines indicate storage at 4C in darkness. Vertical lines = S.E.M.

This study has demonstrated that common culture medium components (inorganic, organic, and complex) can influence culture medium pH, which in turn may result in alterations of plant growth and development *in vitro.* It is recommended that pH values be determined after autoclaving, as well as during critical developmental junctures *in vitro,* in order to describe more accurately the environmental conditions under which the plant material is being cultured. In addition, the type as well as the manufacturer of complex components should be reported in the literature and the pH should be adjusted after their addition. Development of plant tissue culture media with increased buffering capacity, compared to current, poorly buffered formulations, could alleviate the pH changes observed in this study.

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