Vessel Volume, Gelling Agent, and Basal Salts Affect pH and Gel Strength of Autoclave Tissue Culture Media

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Abstract. Effects of autoclaving volume, gelling agent (Bactoagar, Gel-gro, Phytagar), and basal salts [Murashige and Skoog (MS); Woody Plant Medium (WPM); Gamborg B5 (GB)] on gel strength and pH of tissue culture media were tested. Gel strength was significantly affected by gelling agent and basal medium. MS media were generally softer than comparable WPM or GB media. As the vessel volume during autoclaving decreased, gel strength significantly decreased with Phytagar and Bactoagar gelling agents; Gel-gro had greater gel strength at the intermediate volume of medium autoclave. In all cases, autoclaving resulted in a pH decrease of 0.2 to 0.5 pH units. Lower pH values were associated with softer gels. The type of gelling agent did not greatly affect the postautoclave pH; mean values among gelling agents were within 0.05 pH units. Postautoclave pH of MS medium was lower than that of WPM or GB. This study verifies the need to observe uniform sterilization protocols to maintain consistency in the chemical and physical properties of media.

Protocols for preparing plant tissue culture media routinely incorporate adding inorganic and organic nutrients, growth regulators, a carbon source, and frequently a gelling agent to support and orient the tissue. Medium pH is usually adjusted before adding the gelling agent. The gelling agent is added, and the medium is melted and dispensed into culture tubes or vessels for autoclaving. Alternatively, media are not melted before sterilization but autoclave in bulk amounts and dispensed when molten, particularly if heat-labile medium amendments are required or if nonautoclavable containers (such as disposable petri plates) are used. Autoclaving at 121°C for 15 to 20 min is commonly indicated. Heat sterilization can induce profound changes in culture media components, including decomposition of heat-labile nutrients, reactions between sugars and amino acids, pH changes, and formation of toxic compounds (Hsiao and Bornman, 1989; Schenk et al., 1991; Schrauwen, 1971).

Medium pH has a pronounced effect on the growth of tissues in vitro and influences some plant developmental processes (Owen et al., 1991; Smith and Krikorian, 1990). The rates of heating and cooling, culture medium composition, and the sterilization temperature may affect poststerilization pH (Behagel, 1971; Owen et al., 1991). The response of cultured tissues to pH changes can be modified by other media components. For example, the pH optimum for adventitious bud production on cul-

tured needles of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] decreased with increasing agar concentration (Selby et al., 1989). Sarma et al. (1990) found that even the method of adding agar influenced postautoclave pH values.

The type and concentration of gelling agent have been shown to affect the growth of tissues in culture. Examples include somatic embryogenesis in cucumber (*Cucumis sativus* L.) (Ladyman and Girard, 1992), shoot apical meristems in Norway spruce [*Picea abies* (L.) Karst.] (Romberger and Tabor, 1971), rooting of sweetgum (*Liquidambar styraciflua* L.) shoots (Lee et al., 1986), shoot proliferation and growth in crabapple [*Malus baccata* (L.) Borkh. × *M. pumila* var. *niedzwetzkyana* (Dieck) Schneid.] and pear (*Pyrus communis* L.) (Singha, 1982, 1984; Turner and Singha, 1990), numbers of shoots in apple (*Malus domestica* Borkh.) shoot-tip cultures (Pasqualetto et al., 1986), and shoot growth and rooting in tobacco (*Nicotiana tabacum* L.) seedlings (Scherer, 1988). Vitrification of tissues can result from low concentrations of gelling agent or liquid medium cultures (Bornman and Vogelmann, 1984; Debergh, 1983; Pasqualetto et al., 1986; Turner and Singha, 1990; Zimmerman and Cobb, 1989).

In Sitka spruce, Selby et al. (1989) attributed enhanced organogenesis and elongation of shoots on media with relatively soft gels to better contact with the medium than in rigid gels, and resultant improved uptake of water, growth regulators, and nutrients from the culture medium. Debergh (1983) showed that a reduction in the availability of cytokinins occurred as agar concentrations increased. The rate of cytokinin uptake by tissues was reduced by increasing medium rigidity (Bomman and Vogelmann, 1984). Owens and Wozniak (1991) attributed the responses to gel type and concentration in sugar beet (*Beta vulgaris* L.)

leaf disks primarily to water potential differences; they identified matrix potential and expressibility of water from gel as factors that largely determine water availability from gels.

During routine culture experiments, we have noticed differences in gel strength dependent on the volume of medium autoclave (i.e., predispensed in test tubes vs. bulk autoclave, then distributed). Differences were more noticeable with certain types of basal salts, such as Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). It was unclear whether the type of gelling agent was a contributing factor. This study was thus conducted to evaluate the effects of medium volume during autoclaving, gelling agent, and basal medium composition on gel strength and pH of gels obtained.

Materials and Methods

The effects of three basal media, three gelling agents, and three medium autoclaving volumes were studied. MS medium (1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), and Gamborg B5 (GB) medium (Gamborg et al., 1968), all with 2% sucrose, were used. Solidifying agents were Phytagar (Gibco Lab., Gaithersburg, Md.) at 0.8% (w/v), Bactoagar (Difco Lab., Detroit) at 0.8% (w/v), and Gel-gro (ICN Biochem., Irvine, Calif.) at 0.4% (w/v). Media were prepared by adding the appropriate major, minor, and Fe stock solutions and 2% sucrose to purified water from a Modulab analytical water purification unit (Continental Water Systems, Norcross, Ga.). The pH was adjusted to 5.7 with either NaOH or HCl. The appropriate gelling agent was added, and the media were heated on a stirring hot plate until melted. Media were dispensed in three volumes: 1000 ml in 2-liter beakers, 100 ml in 250-ml beakers, and 10 ml in 25 × 100-mm test tubes for each basal salt and gelling agent. Media were autoclave (Amsco, Medallion Series, Erie, Pa.) simultaneously in two loads in beakers and test tubes for 20 min at 121°C, 2.9 MPa, then redispensed while still molten into containers for texture and pH measurements. There were four replications per 27 treatments in a completely randomized design. Data were subjected to analysis of variance procedures of the Statistical Analysis System (SAS Institute, 1988).

Gel strength. Following autoclaving, 30 ml of medium from each treatment was dispensed into individual 100 × 15-cm plastic petri dishes for texture measurements. There were four replications per treatment, with three petri dishes per replication. Firmness was measured with a Volland Stevens Texture Analyzer (Volland Corp., Hawthorne, N. Y.) using a 0.5-cm-diameter, flat probe, 2 mm·s⁻¹ penetration speed and 4-mm penetration distance. Measurements were made at four points in each petri dish, avoiding edge effects, i.e., in the center and at three places midpoint from the center and edge of each plate. Data were taken for penetration force in gram-force and converted to newtons. Values are an indication of the texture of a medium, with higher

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values representing media with greater gel strength.

Media pH. After autoclaving, 10 ml of each medium were dispensed into 22 × 150-mm test tubes and allowed to solidify. There were four replications per treatment, with three test tubes per replication. Media were homogenized with a Virtis tissue homogenizer (Virtis Co., Gardiner, N. Y.) for 15 sec with 10 ml of purified water from a Modulab analytical water purification system. The pH was measured with a Corning Ion Analyzer 150 (Corning Science Products, Halstead, Essex, U.K.).

Results and Discussion

Gel strength was influenced by gelling agent, basal medium, and volume of medium autoclave (Table 1). Phytagar gels generally had greater gel strength than Bactoagar gels, although both were used at 0.8% Gel-gro media were of intermediate strength at 0.4%. Comparing agar sources, Debergh (1983) found that agar brand and concentration affected the solidity of gels; his results had a range of more than 40 J difference among agar strengths at a concentration of 10 g.liter⁻¹. We used two agar-based gelling agents: Phytagar and Bactoagar. Differences in gel strength between the two may have been associated, in part, with the proportion of agarose in the agars, which affects gelling ability in agar gels. Agarose concentration varies with the species of red algae from which an agar is derived, as well as processing method (Matsuhashi, 1990).

Autoclaving volume had a pronounced and significant effect on gel strength in general, declining as volume of the vessel autoclave decreased (Table 1). Burger (1988) showed that solutions autoclave in smaller volumes are subjected to longer exposure times to higher temperatures; liquid volumes of 10 and 4000 ml differed by 40 min in their time to reach 121°C. In a study evaluating autoclave-induced medium toxicity, Sawyer and Hsiao (1992) found that media autoclave in smaller aliquots were more toxic than the same media autoclave in larger aliquots. Loss of medium gel strength may be associated with heating, at least for the two agar-based media, in which

gel strength declined with smaller medium volumes. Media solidified with Gel-gro differed from the two agar-solidified media (Table 1). Media autoclave in 10-ml volumes had greater gel strength. However, Gel-gro media consistently had stronger gels at the intermediate autoclave volume. Others have reported fundamental differences between gellan gum and agar gelling agents in embryo production, shoot proliferation, culture fresh weight, and vitrification (Ladyman and Girard, 1992; Pasqualetto et al., 1986; Turner and Singha, 1990).

Formulation of basal salts significantly influenced gel strength (Table 1). This effect was particularly apparent with media using Phytagar, where there was a greater difference between treatments in texture values than with the other gelling agents. MS media were significantly softer than those formulated with WPM or GB in both the agar gelling agents. MS and GB media were significantly softer than WPM medium when solidified with Gel-gro. In addition, MS media were particularly sensitive to autoclave volume effects, where differences in gel strength between larger and smaller volumes were marked. In every case, gels formed from media autoclave in volumes of 10 ml were less rigid than those autoclave at 1000 ml.

In a study of the effect of agar addition method on tissue culture media, Sarma et al. (1990) observed that gel strength of media that were autoclave twice (first to dissolve the agar and again after distribution into tubes) was lower than that of media prepared by any other method. Predissolving the agar on a stir hot plate or preautoclaving without other media nutrients resulted in more rigid gels. This result suggests that high heating in the presence of other medium constituents may be a factor affecting gel strength.

Qualitative differences in the ions present reportedly affect gelation of agar gels: K⁺, SO₄²⁻, and Cl⁻ promote gelation rate, whereas NO₃⁻ retards gelation rate (Matsuhashi, 1990). The concentrations of these gel-promoting and gel-retarding ions in the media we used are MS: 27.4 mM and 39.4 mM; GB: 27.8 mM and 24.7 mM; and WPM: 21.1 mM and 9.7 mM. The high retarding : promoting ion ratio in MS

medium may explain, in part, its lower strength compared to the other media. It is well established that cations and concentration influence the strength of gels of gellan gums such as Gel-gro (Sanderson, 1990). Divalent cations produce a stronger gel than monovalent cations at the same concentration. In the media we used, divalent cations are at the same concentration for WPM and MS, whereas K⁺ levels are much higher in MS than WPM. For GB, the cation concentration is less than half that of MS medium; K⁺ is about the same. Colloids are subject to destabilization by high salt concentrations, which may possibly contribute to the lower gel strength of MS medium.

In all cases, autoclaving resulted in a decrease in pH, ranging from ≈0.2 to 0.5 pH units (Table 2). Numerous studies have likewise reported a drop in pH associated with autoclaving (e.g., Owen et al., 1991; Sarma et al., 1990; Selby et al., 1989; Singha, 1982). Singha (1982) found a decrease in medium pH of ≈0.5 pH units; pH reduction was less pronounced with increasing agar concentrations. Selby et al. (1989) found that decrease in pH varied depending on the pH range used (0.8 to 0.9 of a pH unit at pH 6 vs. 0.1 to 0.2 of a pH unit at pH 4).

In comparisons within a gelling agent, MS generally exhibited a lower postautoclave pH than the other two media (Table 2). The pH of MS was significantly lower than that of WPM or GB solidified with Gel-gro or Bactoagar; in Phytagar-solidified media, the pH of MS was lower than GB. Although the pH of Phytagar and Gel-gro was significantly lower than that of Bactoagar when comparing overall means of the three gelling agents, mean postautoclave pH values were within 0.05 units. Such a small difference in pH among the gelling agents unlikely would directly cause differences in physiological growth of cultures, as medium pH has been shown to fluctuate widely with both storage and tissue growth (Owen et al., 1991; Sarma et al., 1990; Skirvin et al., 1986). Marked interactions between basal media components and carbohydrates, which may affect medium pH, occur during autoclaving. Precipitation and dissolution of salts; neutralization or esterification of acids; hydrolysis of proteins, polysaccharides, and esters; reactions between carboxyl and amino groups; and polymerization or depolymerization of alcohols, aldehydes, and saccharides result in changes in pH values (Behagel, 1971). Precipitation and carbohydrate breakdown during autoclaving may form toxic compounds (Schenk et al., 1991).

Media volume showed variable effects on postautoclaving pH. In the two agar-based gelling agents (Phytagar and Bactoagar), pH values mostly were lower with media autoclave in the smaller volumes (Table 2). The greater gel strength of Gel-gro media at the intermediate autoclave volume concurred with generally higher pH values for those samples. Selby et al. (1989) found that the rigidity of media solidified with the same agar concentration increased with pH. However, the differences in pH that we obtained, although statis-

Table 1. Effect of gelling agent, basal salts, and medium volume during autoclaving on gel strength as measured using a Volland Stevens Texture Analyzer. Data are for penetration force in millinewtons. Data are means of 48 observations.

Gelling agent and concentration	Basal salt	Volume (ml)			Mean	Grand mean
		10	100	1000		
Phytagar 0.8%	MS	134	182	210	175 c ^a	260 a
	WPM	236	295	312	281 b	
	GB	299	323	352	325 a	
	Mean	223 c	267 b	291 a		
Gel-gro 0.4%	MS	147	288	192	209 b	230 b
	WPM	220	330	266	272 a	
	GB	159	256	216	210 b	
	Mean	175 c	291 a	225 b		
Bactoagar 0.8%	MS	78	135	241	151 b	181 c
	WPM	126	196	269	197 a	
	GB	143	210	235	196 a	
	Mean	116 c	180 b	248 a		
Grand mean		171 c	246 b	255 a		

^aMean separation in columns or rows by Duncan's multiple range test, $P \leq 0.05$.

Table 2. Effect of gelling agent, basal salts, and medium volume during autoclaving on medium pH. The pH was adjusted to 5.7 before autoclaving. Data are means of 12 observations.

Gelling agent and concentration	Basal salt	Volume (ml)			Mean	Grand mean
		10	100	1000		
Phytagar 0.8%	MS	5.29	5.30	5.32	5.30 b'	5.35 b
	WPM	5.27	5.31	5.34	5.31 b	
	GB	5.38	5.40	5.51	5.45 a	
	Mean	5.31 c	5.36 b	5.39 a		
Gel-gro 0.4%	MS	5.19	5.26	5.33	5.26 c	5.36 b
	WPM	5.35	5.43	5.37	5.38 b	
	GB	5.37	5.50	5.41	5.42 a	
	Mean	5.30 c	5.40 a	5.37 b		
Bactoagar 0.8%	MS	5.26	5.32	5.40	5.33 b	5.38 a
	WPM	5.36	5.41	5.44	5.40 a	
	GB	5.47	5.26	5.47	5.40 a	
	Mean	5.36 b	5.33 c	5.44 a		
Grand mean		5.33 c	5.36 b	5.40 a		

'Means separation in columns or rows by Duncan's multiple range test, $P \leq 0.05$.

tically significant, were small. Values as affected by volume fluctuated within ≈ 0.2 pH units. The observed pH differences may be contributory, but are unlikely to be the major causative factor in controlling gel strength.

Mean pH values of the different treatments varied from pH 5.19 (10 ml MS solidified with Gel-gro) to pH 5.51 (1000 ml GB solidified with Phytagar). Changes in pH near this magnitude have been shown to have a pronounced effect on cell differentiation in some culture systems. Morphogenesis of carrot (*Daucus carota* L.) somatic embryos was controlled by external pH of the culture medium. Cultures retained their morphology as preglobular stage proembryos at pH 4, but developed into later embryo stages at pH 4.5 or above (Smith and Krikorian, 1990). The growth of cultured buds of Sitka spruce and the incidence of vitrification were markedly affected by differences of 0.5 pH units, when a range from pH 4.0 to 6.0 was evaluated (Selby et al., 1989). In scarlet plume (*Euphorbia fulgens* Karw. ex Klostsch) cultures, the optimal medium pH was 5.3 for shoot proliferation; lower pH values stimulated shoot growth (Zhang and Stoltz, 1989).

Effects of medium pH and gel strength on plant differentiation and development in vitro are well documented. We have shown that autoclaving can induce differential effects on gel strength and pH, contingent on the type of basal salts, autoclaving volume, and gelling agent. In this light, it is apparently important to control autoclaving conditions carefully. This work verifies the need to observe uniform sterilization protocols, particularly within an experiment. For example, in studies where heat-labile substances may be added to some media (i.e., media components are bulk-autoclaved then combined before dispensing), it is important to sterilize all other media similarly. Likewise, physical changes and varying pH values in the culture medium should be considered as influencing factors in studies evaluating effects of basal salts or gelling agents on in vitro growth and development.

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