In Vitro Cell. Dev. Biol. 31:84–89, April 1995

1071-2690/95 $05.00 + 0.00

EFFECTS OF COMMON COMPONENTS ON HARDNESS OF CULTURE MEDIA PREPARED WITH GELRITE™

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(Received 9 August 1994; accepted 21 December 1994; editor M. K. Redenbaugh)

SUMMARY

Tissue cultures on properly solidified Gelrite media generally showed superior shoot proliferation and rooting, as well as shoot and root vigor and callus development to those on TC agar. Vitrification, or hyperhydricity, was observed in both Gelrite and agar media and minimized by increasing the gel concentrations. Rigidity of Gelrite media depended on combined levels of MS macrosalts, basal nutrient formulations, sucrose concentration, pH, and Gelrite concentration. Most MS macrosalts increased hardness of Gelrite gels; NH_4NO_3 had a decreasing effect. Rigidity of TC agar gels increased with reductions of MS macrosalts. A slightly softer Gelrite medium resulted when sucrose was excluded. Both Gelrite and agar media were softer at lower pHs and harder at higher pHs. Activated charcoal and mannitol increased gel hardness, and more noticeably of agar gels. NaCl addenda reduced rigidity, with their effects being more pronounced on Gelrite than on agar gels.

Key words: Gelrite; Phytagel; gellan gum; gelling agents; agar.

INTRODUCTION

A traditional problem with agar has been its variable quality and purity and the virtually prohibitive cost of its more highly purified grades. Furthermore, specific utility of each of the multitude of available grades is not known to most users. Unfortunately, the gelling agent is a major component that could significantly affect the performance of tissue culture media; thus, its inclusion must consider both quantitative and qualitative requirements.

The search for an economical agar substitute with essentially the same gelling characteristics, particularly thermal reversibility, has led to a bacterial gellan gum, Gelrite[2], now purchasable from retail suppliers by diverse trade names (e.g., Phytagel[2]). It has become the preferred gelling agent of many plant tissue culture media because of its high purity and consistent quality, with one grade satisfying a variety of needs and substantially smaller quantities producing gels of hardness comparable to agar. Nevertheless, in spite of the advantages, Gelrite has not supplanted agar as the universally employed gelling agent. This is partly because many potential users remain unaware of its existence. Some choose not to use it because gels of desired firmness have not been attainable by following the manufacturer's instructions. The recommended rate of 0.2% has resulted in softer or harder gels, depending on the basal nutrient formulation. And there are those that have been discouraged by reports that Gelrite causes or enhances vitrification, or hyperhydricity, of regenerating shoots (Pasqualetto et al., 1986; Zimmerman and Cobb, 1989).

We have now identified some major components that determine the hardness of Gelrite-containing media. We discovered that firmness of agar gels is similarly affected by some of the same components and that vitrification is equally a problem. It can be reported that Gelrite, when properly employed, is superior to standard grades of agar as gelling agent of plant tissue culture media.

MATERIALS AND METHODS

Plant tissue cultures. Comparisons of tissue and cell culture development in media solidified with Gelrite and agar were made with MS (Murashige and Skoog, 1962) basal medium containing appropriate phytohormone supplements. Unless specified otherwise, Gelrite was added at a rate of 0.2%, and TC agar[2] at 0.8%. The pH was set at 5.7 ± 0.1 prior to adding gelling agents. For most cultures, 25- × 150-mm glass culture tubes with polypropylene closures served as culture vessels, each containing 25 ml medium. Magenta GA7 plastic vessels with 100 ml medium were used for some shoot cultures. Cell plating was done in disposable 15- × 100-mm polystyrene petri dishes with 10 ml medium each. For most tissue cultures, the Gelrite and agar powders were added after pH adjustments and final dilution of media and dissolved by autoclaving at 1.05 $kgcm^{-2}$ for 5 min. Hot media were dispensed into culture vessels and sterilized by autoclaving an additional 10 min. For cell plating, the gelling agents were dissolved and the media sterilized by autoclaving for 10 min, cooled to 45° C, and poured into presterilized petri dishes. Observations were made of callus growth, shoot proliferation, root development, vitrification, and other aberrant development. All cultures, including those of plated cells, were maintained at about 27° C and under 16-h daily illumination with 45 $\mu Em^{-2}s^{-1}$ regular spectrum GroLux or FL-40SBR/38 Toshiba fluorescent lamps. At least 10 cultures were used per treatment and data were evaluated by calculating standard errors of means (Snedecor, 1957).

Measurement of gel hardness. Gelrite and TC agar gels were prepared by adding prescribed quantities of powder to experimental solutions, dissolving

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Gelrite is a trademark of Kelco Division of Merck & Co. (San Diego, CA), manufacturer of the gellan gum. Phytagel is a trademark of Sigma Chemical Co. (St. Louis, MO) for repackaged Gelrite. TC agar used for comparisons in this investigation is plant tissue culture tested agar obtained from JRH Biosciences (Lenexa, KS).

by autoclaving 5 min at 1.05 $kgcm^{-2}$, mixing and dispensing 10-ml volumes into 15- × 100-mm polystyrene petri dishes, and cooling. At least four plates were used for hardness measurements of each variable. The measurements were made with a Marine Colloids Gel Tester, using the small plunger at slow speed. Instrument readings were recorded at the instant the plunger pierced the gel surface. To facilitate comparisons, the relative rigidity, or the ratio of means of instrument readings, was calculated, using the values obtained with gels containing MS salts and vitamins, 100 mg/liter inositol, 3% sucrose, and 0.2% Gelrite as common denominator. Where appropriate, mean instrument readings of gels prepared with 0.8% TC agar were also used as the denominator.

Results

Gelrite effects on plant tissue cultures. With only one exception, Gelrite used at the rate of 0.2% in MS-based media produced cultures that were superior to those by 0.8% TC agar. We had determined in earlier investigations that TC agar was better than Difco Bacto-agar and GIBCO Phytagar.

Callus cultures. More vigorous callus growth was obtained with every species examined, namely tobacco (*Nicotiana tabacum* and *N. glutinosa*); the bamboos *Bambusa multiplex*, *B. oldhamii*, *Phyllostachys aurea*, and *Sasa pygmaea*; carrot (*Daucus carota*); broad bean (*Vicia faba*); and lemon (*Citrus limon*). We also employed Gelrite successfully for plating bamboo and *N. glutinosa* cells; however, the optimum gel concentration for plating was lower, or 0.15% instead of 0.2%.

Plant regeneration. With the exception of jojoba (*Simmondsia chinensis*), shoot proliferation, shoot growth, root formation, and root vigor were substantially greater in Gelrite than in agar media. An example of the enhanced proliferation and growth of shoots is depicted in Fig. 1 by cultures of the bamboo, *P. aurea*. Palo Verde (*Parkinsonia aculeata*) shoot cultures for micropropagation were readily established in Gelrite media, but not in agar. Nodal segments after 4 wk on media solidified with TC agar barely produced a new Palo Verde shoot (0.27 ± 0.22) per explant, whereas those on Gelrite yielded nearly six new shoots (5.83 ± 1.04). Rooting of shoot separates of the orchid, *Paphiopedilum* sp., and of *Ficus benjamina* was more profuse and vigorous in Gelrite media.

Vitrification. Jojoba shoot cultures developed mainly callus in 0.2% Gelrite medium, and the few shoots that emerged were severely vitrified. The suggestion that Gelrite causes or enhances vitrification of shoots in vitro was specifically examined with shoot-apex cultures of a more easily cultured species (*Dianthus caryophyllus*). Hyperhydricity was evident on both Gelrite and agar media. The incidence of cultures with vitrified shoots was lowered, although not eliminated, by raising the Gelrite concentration to 0.6% and the TC agar to 1.4%. Unfortunately, the higher gel concentrations repressed shoot multiplication and growth.

Nutrient medium components and hardness of Gelrite gels. Our examinations focused on the effects of some major components of plant culture media on rigidity of Gelrite as well as agar gels.

Effects of reduced levels of total MS macrosalts. The manufacturer's recommended 0.2% level of Gelrite for plant culture media was based on our experiments with the MS medium, using all salts at full strength and supplementing with 100 mg/liter inositol, MS vitamins, 3% sucrose, and appropriate phytohormones. The pH was set at 5.7 prior to autoclaving. As apparent in Fig. 2, lowering the total macrosalt content of MS medium significantly lowered gel rigidity. The reduced rigidity was observed at all levels of Gelrite, but more noticeably at the higher concentrations. TC agar displayed the reverse trend, the hardness of its gels increasing with decreasing macrosalt content. An agar medium with no macrosalts produced a gel that was nearly eight times more rigid than that containing the standard level of MS salts. In another experiment (data not shown), relatively firm Gelrite gels were obtainable with water alone, but only by employing gel concentrations as high as 1%.

FIG. 1. Proliferation and growth of *P. aurea* in media solidified with Gelrite (*left*) and TC agar. *Note* clarity of gel prepared with Gelrite.

Specific salts and hardness of Gelrite media. Further experiments with MS macrosalts revealed that the rigidity variations of Gelrite media were due to the combined effects of all salts (Fig. 3). Reduced levels of individual salts, but not NH_4NO_3, showed only slight lowering of gel rigidity, except when completely excluded. Even then, only the exclusion of $CaCl_2$ or KNO_3 resulted in substantive lowering. NH_4NO_3 showed the reverse relationship; lowering its concentration increased gel rigidity, and raising it above the standard MS level produced much softer gels.

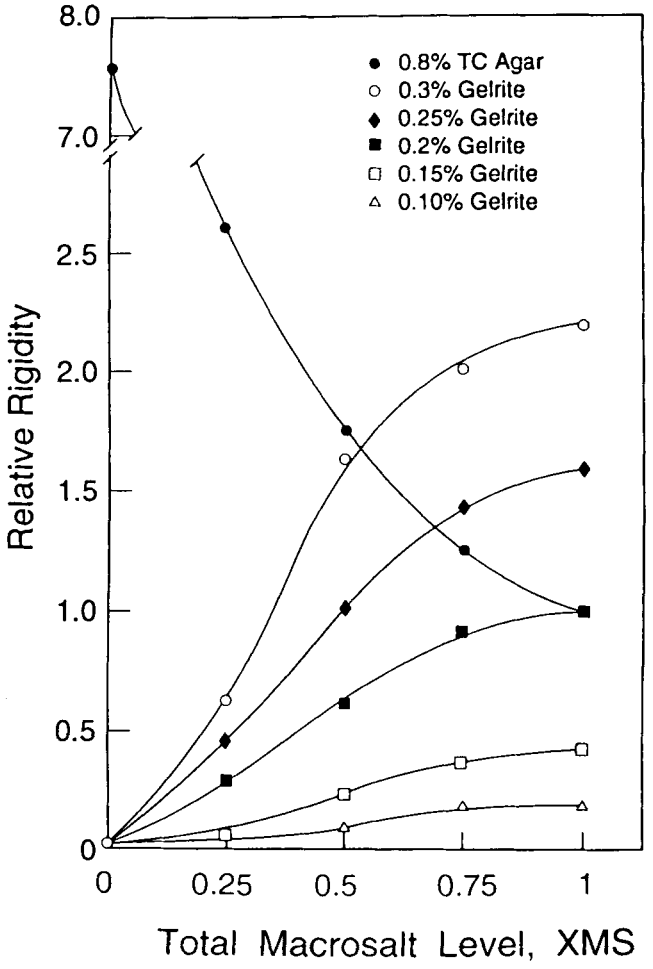

FIG. 2. Rigidity of Gelrite gels as influenced by levels of MS macrosalts and Gelrite in medium. Relative rigidity of 1 is based on full strength of all salts.

Variations of Gelrite hardness in different basal media. Anticipated variations in hardness of gels prepared with prescribed concentrations of Gelrite in diverse nutrient formulations were confirmed. The data in Fig. 4 show very clearly that Gelrite levels based on the MS medium were not immediately applicable to other media. In this investigation, the optima, or Gelrite concentrations the relative rigidities of which approached 1, for the various basal formulations were: B_5, slightly above 0.2%; N_6, slightly above 0.3%; Knudson, considerably below 0.2%; Anderson, about 0.25%; Schenk and Hildebrandt, about 0.2%; Nitsch and Nitsch, about 0.5%; and Vacin and Went, about 0.6%.

Gel hardness as influenced by medium pH. In addition to the thermal reversibility, Gelrite gels were similar to agar by a dependence of their hardness on nutrient medium pH. Gels were progressively softer at lower pHs and firmer at higher pHs (Fig. 5). But the variations were not as pronounced as those displayed by agar, particularly at pHs above 5.

Other commonly employed addenda. Fig. 6 shows the effects of sucrose, the most widely employed carbon source. No difference was observed between media containing the more standard 3% and a higher 6% sucrose levels. Slightly softer gels were evident when

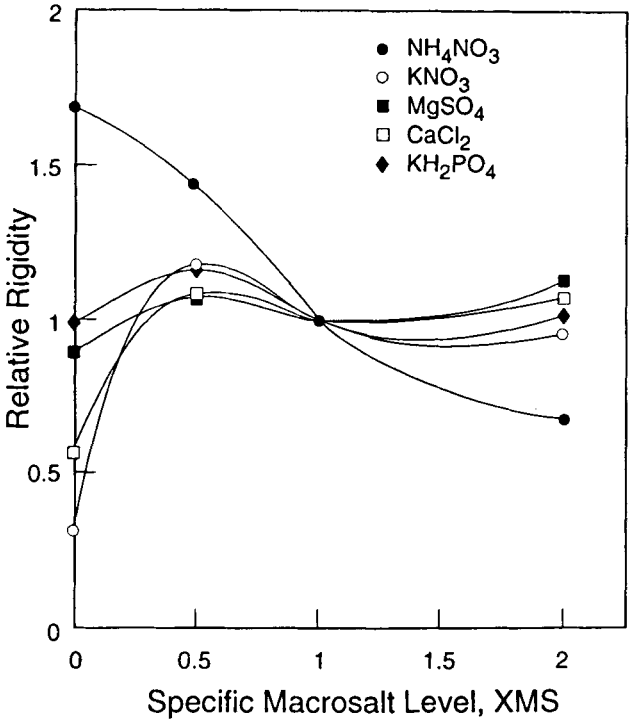

FIG. 3. Rigidity of Gelrite gels as influenced by level of individual MS macrosalt in medium.

media contained less, 1.5%, or no sucrose. The activated charcoal that is frequently used to overcome browning of medium and tissue and adsorb growth inhibiting substances increased hardness of Gelrite media, especially at higher gel levels (Table 1). But its more conspicuous effects were observed in media solidified with TC agar. The usually employed concentration range, 0.1–1%, produced relative rigidities that were as much as 4 ½ times higher than agar media without the addendum. Mannitol, the extensively used osmoticum of protoplast cultures, also showed gel hardening effects (Table 2). The more commonly employed concentrations, 0.4 and 0.7 *M*, caused Gelrite media to be 1 ½ times more rigid than those lacking mannitol. Here also, the hardening effects were more severe on TC agar, with 0.4 and 0.7 *M* levels of mannitol doubling rigidity. NaCl is often added to culture media to detect salt-tolerant somaclonal variants. This salt showed gel rigidity effects that were the reverse of charcoal and mannitol. NaCl addenda generally decreased rigidity of Gelrite as well as agar gels (Table 3). But in further differing from those of activated charcoal and mannitol, the NaCl effects were more pronounced on Gelrite than on agar gels.

DISCUSSION

The superiority of Gelrite over agar as a gelling agent of media for plant callus cultures has been confirmed. Earlier, Ichi et al. (1986) reported that tobacco (*N. tabacum*) and pokeweed (*Phytolacca americana*) callus growth rates were increased 30 and 45%, respectively, by using Gelrite in place of purified agar. Huang and Chi (1988) obtained a 400% higher yield of banana (*Musa sapientum*) callus on Gelrite than on TC, Difco Bacto- or Difco Bacto-purified agar media. Gelrite further prevented the tissue and medium discoloration that

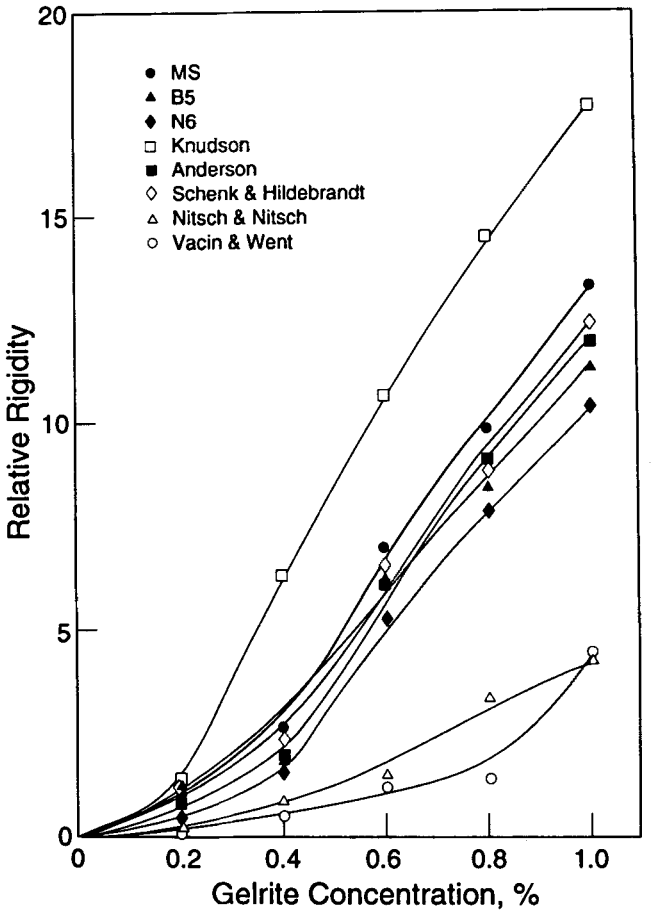

FIG. 4. Variations in gel hardness of media prepared with different nutrient formulations and Gelrite concentrations. MS = Murashige and Skoog (1962); B_5 = Gamborg et al. (1968); N_6 = Chu et al. (1975); Knudson = Knudson (1946); Anderson = Anderson (1984); Schenk & Hildebrandt = Schenk and Hildebrandt (1972); Nitsch & Nitsch = Nitsch and Nitsch (1969); and Vacin & Went = Vacin and Went (1949).

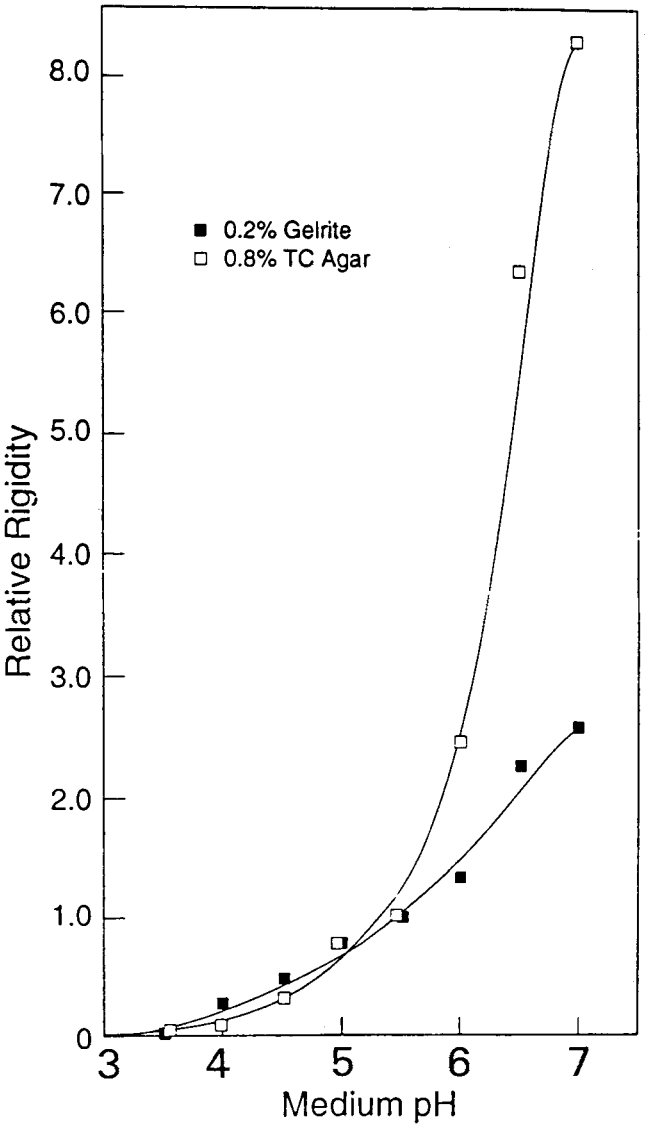

FIG. 5. Relationship of hardness of Gelrite and agar gels to pH of nutrient medium. Basal medium contained MS salts, 100 mg/liter inositol, and 3% sucrose.

normally occurs in banana cultures (Huang and Chi, 1988). In this investigation, we also employed Gelrite successfully to plate bamboo and tobacco cells.

With the exception of jojoba, we further confirmed the superiority of Gelrite in micropropagation by tissue culture of diverse species. Shoot proliferation, shoot growth, rooting, and root vigor were substantially better in Gelrite than in agar media. Other investigators have reported enhanced adventive embryogenesis (somatic and androgenetic) by Gelrite [e.g., mango (*Mangifera indica*) by DeWald et al. (1989a) and rice (*Oryza sativa*) by Koetj et al. (1989)]. DeWald et al. (1989b) additionally reported that Gelrite helped to prevent developmental abnormalities and precocious germination of mango embryos.

The suggestion that Gelrite causes or enhances vitrification of shoots in vitro is not supported by the evidence. Our *Dianthus caryophyllus* experiment confirmed that vitrification can occur in either Gelrite or agar media. We lowered the incidence of hyperhydricity by utilizing firmer gels prepared with higher concentrations of gelling agents. Zimmerman and Cobb (1989) were also able to reduce vitrification of *Petunia hybrida* leaves by increasing the Gelrite concentration. In apple (*Malus domestica*) shoot cultures, the problem has been partially resolved by additionally solidifying Gelrite media with small amounts of agar (Pasqualetto et al., 1986). Unfortunately, an avoidance of vitrification by simply increasing the gel concentration is usually accompanied by suppressed shoot proliferation and growth. Satisfactory resolution of the problem will require elimination of its causes, namely excessively moist medium, unsuitable nitrogen provision, imbalance among phytohormonal addenda, and possibly poor gas exchange.

Gelrite is a clarified grade of gellan gum, an exocellular heteropolysaccharide obtained from cultures of the bacterium, *Pseudomonas* (Kang et al., 1982). Because Gelrite is a product of a single-celled organism cultivated in a controlled laboratory environment, consistency of quality can be better assured than is possible for agar. With most basal media, gels of suitable firmness can be obtained

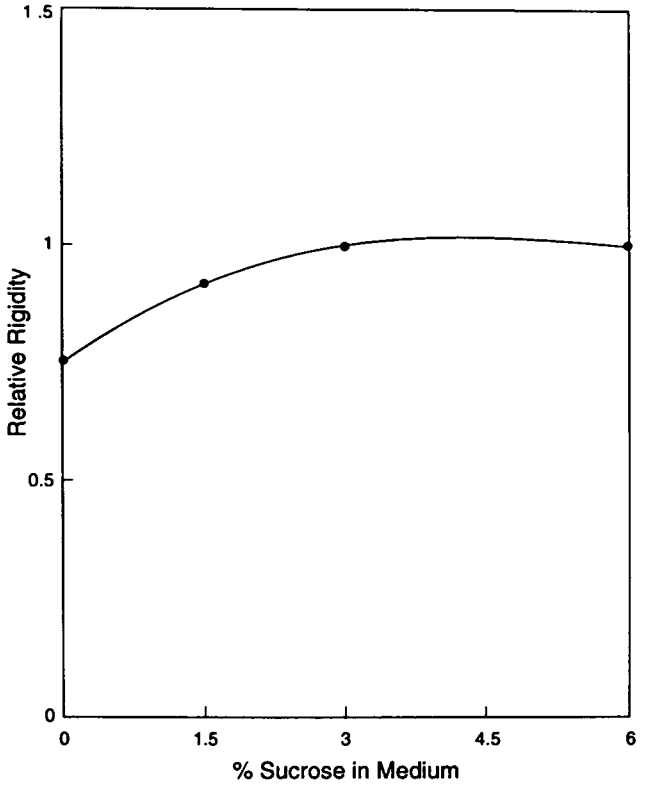

FIG. 6. Hardness of Gelrite media as influenced by sucrose addenda. Basal medium contained MS salts, 100 mg/liter inositol, and 0.2% Gelrite.

TABLE 1

EFFECTS OF SIGMA C5385-ACTIVATED CHARCOAL ADDENDA ON RIGIDIGY OF GELRITE AND AGAR GELS. BASAL MEDIUM CONSTITUENTS WERE MS SALTS AND VITAMINS, 3% SUCROSE, AND 100 mg/L INOSITOL; pH OF MEDIA WAS 5.7. GELRITE WAS EMPLOYED AT 0.2% AND TC AGAR AT 0.8%

	Relative Rigidity of Gel	
% Charcoal Addendum	TC Agar	Gelrite
0	1.00	1.00
0.03	1.05	0.97
0.1	1.52	1.02
0.3	2.33	1.37
1.0	4.26	2.00

with substantially smaller quantities, rendering Gelrite even cheaper than most common and relatively unpurified grades of agar. Media prepared with Gelrite are also clearer, thus, facilitating detection of microbial contaminants and observation of root development. Perhaps because of the lower levels of impurities that accompany the gelling agent into culture media, by virtue of its higher purity and lesser required quantity, tissue cultures on Gelrite media are generally healthier and more vigorous than those on agar media. The instances of Gelrite gels the hardness of which is inferior to those of agar gels are most probably attributable to unfavorable balances between gelling agent and other media ingredients, especially ingredients that are included in relatively large amounts. Although of

TABLE 2

MANNITOL EFFECTS ON HARDNESS OF AGAR AND GELRITE MEDIA. BASAL MEDIUM CONSTITUENTS WERE MS SALTS AND VITAMINS, 100 mg/L INOSITOL, AND 3% SUCROSE; pH OF MEDIA WAS SET AT 5.7. TC AGAR WAS EMPLOYED AT 0.8% AND GELRITE AT 0.2%

	Relative Rigidity of Gel	
M Mannitol Addendum	TC Agar	Gelrite
0	1.00	1.00
0.1	1.70	1.33
0.4	2.23	1.53
0.7	2.60	1.58
1.0	2.60	1.68

TABLE 3

RIGIDITY OF NUTRIENT GELS AS INFLUENCED BY NACL ADDENDA. BASAL MEDIUM COMPONENTS WERE MS SALTS AND VITAMINS, 100 mg/L INOSITOL, AND 3% SUCROSE; pH WAS SET AT 5.7. TC AGAR WAS INCLUDED AT A RATE OF 0.8% AND GELRITE AT 0.2%

	Relative Rigidity of Medium	
% NaCl Addendum	TC Agar	Gelrite
0	1.00	1.00
0.5	1.02	0.94
1.0	0.94	0.84
1.5	0.83	0.56
2.0	0.73	0.42

similar thermal reversibility, Gelrite differs from agar by being more dependent on solutes in the medium for its gel hardness. Most nutrient constituents promote hardness; but others such as NH_4NO_3 are depressive.

Our investigation disclosed that alterations of the macrosalt levels of MS medium necessitated changes in the Gelrite concentration for gels of comparable hardness. Media prepared with half-strength or other dilutions of the MS formula must contain proportionately larger amounts of Gelrite addendum.

Our investigation further disclosed that, for each of the several basal nutrient formulations that are available to plant tissue culturists, the optima with respect to Gelrite levels must be specifically established (i.e., for N_6, B_5, Nitsch and Nitsch, etc.). To establish the optima, consideration should be given to Gelrite concentration as it affects gel hardness and manipulability and, more importantly, development of cultured tissues.

Finally, like agar, the firmness of Gelrite media was shown to be affected by pH and additions of activated charcoal, mannitol, and NaCl. The effects observed of these special addenda suggest a desirability of systematic examination of any substance that is included in notable quantity, establishing its effects on hardness of Gelrite as well as agar media prior to routine utilization. The information could be helpful in more accurately interpreting results of experiments that employ any of these additives.

ACKNOWLEDGMENTS

Çelrite samples for the investigation were generously provided by Mr. Paul Ulrich of Kelco Division of Merck & Co., San Diego, CA. We thank the Kelco

Division also for allowing the use of its Marine Colloids Gel Tester. TC agar was a gift of JRH Biosciences, Lenexa, KS. The investigation was supported in part by grants from the National Science Council and the Council of Agriculture of Taiwan, ROC.

References

Anderson, W. C. A revised tissue culture medium for shoot multiplication of Rhododendron. J. Am. Soc. Hortic. Sci. 109:343–347; 1984.

Chu, C. C.; Wang, C. C.; Sun, C. S., et al. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scientia Sinica 18:659–668; 1975.

DeWald, S. G.; Litz, R. E.; Moore, G. A. Optimizing somatic embryo production in mango. J. Am. Soc. Hortic. Sci. 114:712–716; 1989a.

DeWald, S. G.; Litz, R. E.; Moore, G. A. Maturation and germination of mango somatic embryos. J. Am. Soc. Hortic. Sci. 114:837–841; 1989b.

Gamborg, O. L.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151–158; 1968.

Huang, L.-C.; Chi, D.-L. Pivotal roles of picloram and gelrite in banana callus culture. Environ. Exp. Bot. 28:249–258; 1988.

Ichi, T.; Koda, T.; Asai, I., et al. Effects of gelling agents on in vitro culture of plant tissues. Agric. Biol. Chem. 50:2397–2399; 1986.

Kang, K. S.; Veeder, G. T.; Mirrasoul, P. J., et al. Agar-like polysasccharide produced by a *Pseudomonas* species: production and basic properties. Appl. Environ. Microbiol. 43:1086–1091; 1982.

Koetje, D. S.; Grimes, H. D.; Wang, Y.-C., et al. Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J. Plant Physiol. 135:184–190; 1989.

Knudson, L. A new nutrient solution for the germination of orchid seeds. Am. Orchid Soc. Bull. 15:214–217; 1946.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Nitsch, J. P.; Nitsch, C. Haploid plants from pollen grains. Science 163:85–87; 1969.

Pasqualetto, P.-L.; Zimmerman, R. H.; Fordham, I. Gelling agent and growth regulator effects on shoot vitrification of "Gala" apple in vitro. J. Am. Soc. Hort. Sci. 111:976–980; 1986.

Schenk, R. U.; Hildebrandt, A. C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199–204; 1972.

Snedecor, G. W. Statistical methods. 5th ed. Ames: Iowa State College; 1957:73–74.

Vacin, E. F.; Went, F. W. Some pH changes in nutrient solutions. Bot. Gaz. 110:605–613; 1949.

Zimmerman, T. W.; Cobb, B. G. Vitrification and soluble carbohydrate levels in *Petunia* leaves as influenced by media Gelrite and sucrose concentrations. Plant Cell Rep. 8:358–360; 1989.