TISSUE CULTURE GEL FIRMNESS: MEASUREMENT AND EFFECTS ON GROWTH

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1. Introduction

The gel in a solidified tissue culture medium can influence explant or callus growth and morphology in a concentration-dependent manner. Depending on the type and stage of culture, and the plant material, different mechanisms exist: some morphogenic effects appear to be common to all gels, while others are gel-specific. Isolating and interpreting the growth effects due solely to gel concentration and/or type (agar, gellan gum or other gel) is complicated due to interactions between gel hardness, the other tissue culture medium components, and medium preparation methods. Gel strength may be modified by any or all of: pH, basal salt recipe, carbohydrate type(s) and concentration, charcoal, the dissolution/autoclaving method, storage conditions and age of the medium [1,2,3,4].

The early phases of tissue culture i.e., the initial stages–often favour proliferation over differentiation, and rapid growth tends to be best in liquid media or at moderate-to-low gel concentrations. For example, early growth of spruce apical meristems [5] and tobacco shoot organogenesis [6] both increase as the agar concentration decreases, and similarly, using a low-to-intermediate gellan gum content can improve the induction and proliferation of early-stage conifer somatic embryogenic tissue [7,8].

The later cultural stages of differentiation and plantlet development are promoted by higher gel concentrations. For instance, in several different genera and species of conifers high concentration of gellan gum enhances maturation and conversion of somatic embryogenic tissue into plantlets [4], and elevated agar levels improve organogenesis from buds [9]. In some species either gel type works [10]. In others, however, simply increasing the gel concentration may not be sufficient to solve problems like vitrification (hyperhydricity), and gel type is important [11]. For instance, gellan cannot be substituted for agar in shoot culture of some species because agar contains specific low molecular weight, sulfated polysaccharide impurities which inhibit hyperhydricity [12,13].

For the more general case, in tissue culture systems where the gel type is not critical and minor gel components do not dominate the control of morphogenic response(s), it is the density of the gel matrix itself that modulates growth. There is general agreement that, in a concentration-dependant manner, both agar and gellan gum gels limit the access of the

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cultured tissue to water [10,14], nutrients [15,16], and hormones [9,17] dissolved in the medium, and may restrict the efflux into the gel of compounds from the tissue like enzymes as well [5].

In studies where both parameters have been measured, the gel water potential, Ψ, is correlated with gel concentration (more negative Ψ at higher gel concentration) [6,14,18], as is tissue Ψ and water content [4,10,19]. Several different investigators [14,20,21] have concluded that it is not the osmotic potential, Ψ_o, but the matric potential, Ψ_m, associated with gel structure and capillarity, that determines water and nutrient availability, and therefore morphogenesis, analogous to a "non-plasmolyzing" osmotic stress [4]. However, the matric potential component, Ψ_m, has been determined to be only a very small part of the total Ψ [14,21]. These observations have led to speculation that either plant tissue in culture is exquisitely sensitive to the small Ψ_m changes [19, 21], or there are additional unknown components which also contribute to the overall water potential. The suggested possibilities are vague: an explant/gel surface interaction involving mechanical pressure [14] and physical contact [17]; a "mechanical hindrance" [16]; or an undefined change "in some other gel property" [19]. Agar and gellan gum gels are structurally complex (see Section 4 below), and it is also possible that a basic water potential model simply cannot adequately describe water and/or nutrient and hormone availability to tissues cultured on a gel.

Although the physical mechanism remains unclear, the evidence is nonetheless compelling that gels limit explant or callus access to the other medium components which control growth in proportion to the gel concentration in a tissue culture medium. Therefore it is of interest to include gel firmness as yet another tissue culture parameter to be routinely monitored, and if required, manipulated by altering gel concentration. For this reason, a low-cost device designed to rapidly provide a simple empirical measure of gel firmness would be a useful tool.

2. Measurement of gel hardness

Full characterization of gels is accomplished through texture profile analysis, using instruments like an Instron® tester to measure hardness (rupture strength), firmness (resistance to compression), brittleness (compression distance to the gel's rupture point) and elasticity (height recovery after compression) [22]. The instrumentation is expensive, but provides a precise and comprehensive description of gel properties for applications like quality control and product development within the food industry. Simpler instruments with more modest capabilities also are or have been commercially available (e.g., the Marine Colloids™ gel tester).

In 2001 we described a simple, inexpensive device which can be built for laboratory use. (For construction details and operation see [23].) The prototype unit, which measures gel hardness expressed as peak force (g), is shown in Figure 1. It consists of a digital force gauge which is fixed in place but can be repositioned via a camera mount, a moveable platform, and a variable speed stepper motor. The latter was chosen so the best speed to use for measurements could be experimentally determined but, since platform speed was found to be not critical as long as it exceeds 10 mm min^{-1}, a reversible single-speed gear motor of 20-50 rpm could be substituted using the gearing specified in [23]. If a low-vibration synchronous motor is used, the tester could be

further simplified by eliminating the heavy polypropylene box and extra rubber tubing connection (used in order to damp vibrations from the stepping motor).

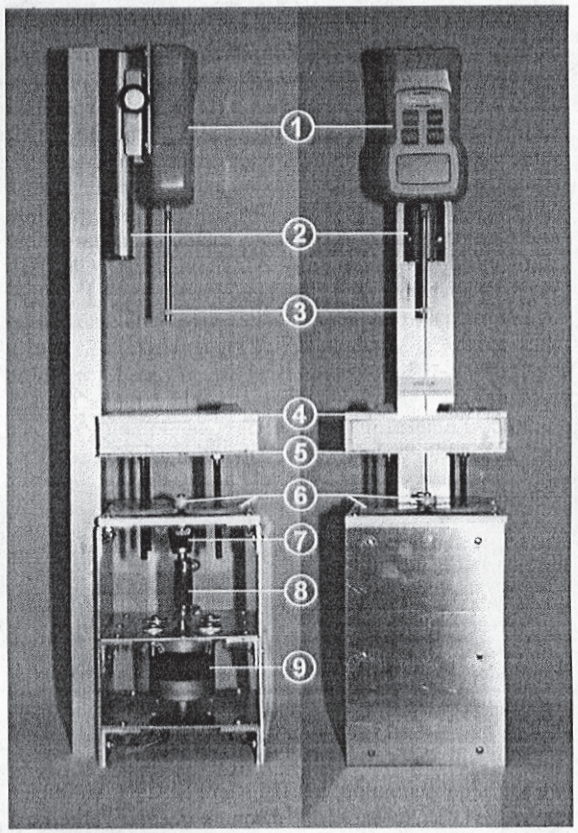

Figure 1. Prototype gel tester front and side views: (1) digital force gauge; (2) camera mount; (3) 5.4 mm diameter flat-bottomed probe; (4) polypropylene box containing lead shot; (6) movable platform; (7) flexible shaft coupler; (8) thick wall rubber tubing; (9) stepper motor. Reproduced from Cameron (2001) [23] with permission from the Society for In Vitro Biology, USA (formerly the Tissue Culture Association).

The gel rupture point readings from the force gauge (peak force in grams) are empirical, but allow comparison of gel firmness within a gel type as a function of various medium amendments and environmental conditions. The tester was designed to measure and compare gels in petri plates. For routine measurements, four plates from a batch pour (25 ml of medium per plate) are used. Four force gauge readings are made on each petri plate: one in the center and three others midway between the center and the wall of the

plate spaced equidistant (120° apart) from each other, for a total of 16 readings which are averaged. Readings may be made quite rapidly: less than 10 minutes per set of four petri plates.

The prototype gel tester was used to document the relation between firmness, gel concentration and three different media formulations commonly used in both our laboratory and elsewhere. Using both agar and gellan gum, sets of petri plates containing ½ Litvay (½LM), DCR and Murashige and Skoog (MS) media were made, and the pH was adjusted to 5.6 after autoclaving. Figure 2 demonstrates the very significant changes that result from either a change in gel concentration or medium formulation.

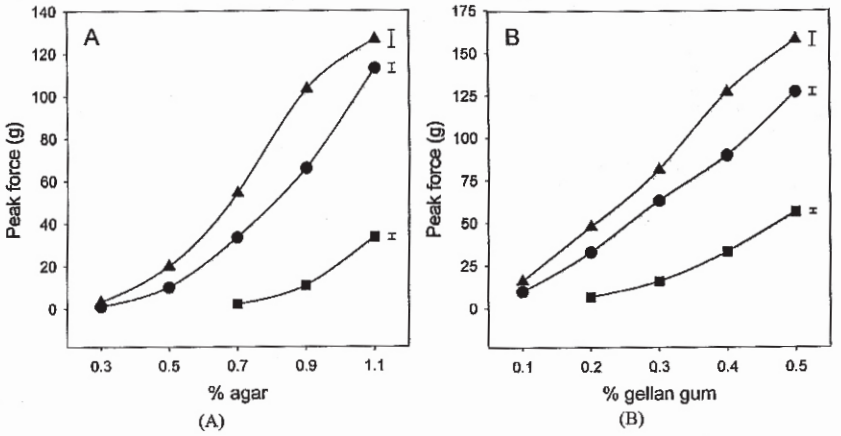

Figure 2. Hardness of three media, DCR (▲), ½ Litvay (●), and Murashige and Skoog (■) at different concentrations of agar (A) and gellan gum (B). Bars are the maximum SE encountered for each medium over a range of gel concentrations. Reproduced from [23] with permission.

Agar is insensitive to Ca concentration, but the summed Ca + Mg molarity is 3.9, 4.4 and 4.5 mM for ½ LM, DCR and MS medium, respectively-near the value of approximately 6 mM Ca which confers maximum hardness to gellan gum [23, 24]. Not surprisingly, however, all three media formulated with either agar or gellan gum are softer than a corresponding simple gel made to the same concentration with only Ca (see [23] and Figure 3). Indeed, MS, referred to as one of the "softer" media [2], does not even gel at either 0.3% or 0.5% agar content, or 0.1% gellan gum probably, as previously noted, because gel firmness is modified by other ions in the media [3]. For instance, agar firmness decreases with increasing NO_3 concentration, and the observed order of media hardness of DCR > ½ LM > MS in Figure 2A is consistent with increasing NO_3 molarities of 11.7, 14.4 and 39.4 mM in MS, ½ LM and DCR, respectively. Similarly, ions like NH_4 can decrease the gel strength of gellan gum-based media. The DCR > ½ LM > MS order of hardness in the gellan gum media in Figure 2B is the inverse of the MS, ½ LM and DCR NH_4 concentrations of 5.0, 15.6 and 20.6 mM, respectively.

3. Gel hardness and pH

The pH of an un-buffered gelled media, as measured prior to autoclaving, is a very dynamic variable whose value can change during most stages of medium preparation, even in the absence of a live culture on the medium. Post-autoclaving pH can vary as a function of: how the gelling agent is dissolved prior to autoclaving [25]; other media components (basal mineral salts, carbohydrate source, gelling agent and, in the case of agar, the brand, and charcoal) [26]; the length of time plates are in storage prior to use [27]; and whether storage is in the light or dark [26]. Placing live tissue on the gel accelerates pH changes as different media components are selectively taken up and metabolized, while organic exudates diffuse or are pumped back into the gel.

As described in the introductory section, gel hardness, regardless of the type, is independently affected by many of the same parameters even after adjusting post-autoclave pH values [3], so firmness might be expected to fluctuate in response to changes induced by some or all of autoclaving, pH and tissue growth.

To examine the effect of pH on gel hardness, simple gels were made consisting only of agar (agar, A-1296, Sigma) or gellan gum (Phytagel, P-8169, Sigma), with Ca and Mg gluconate (3mM and 1.5 mM, respectively), near the optimum concentration for solidifying the gellan gum [23, 24]. Hardness was measured using the gel tester shown in Figure 1 over three gel concentrations and five pH's within physiologically relevant ranges.

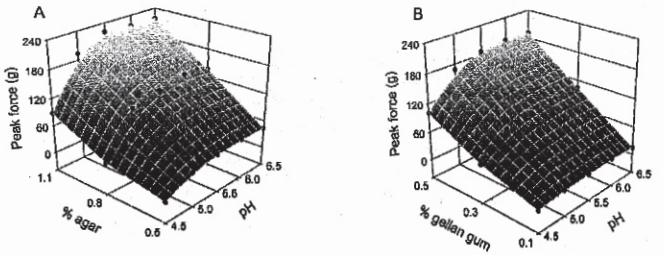

Figure 3. Hardness of agar (A) and gellan gum (B) gelled media adjusted to different pH values. Points(●) are the experimental values.

The data in Figure 3 show that gel hardness decreases at lowered pH, as has been noted previously by others [2, 3], especially between pH 4.5 and 5.0-5.5. The extent of the effect is surprising. At the highest concentrations used in this trial of gellan gum (0.5%) and agar (1.1%), an increase of just 0.5 of a pH unit from pH 4.5 to 5.0 increases hardness by 2.2- and 1.7-fold respectively. Although it is not obvious from Figure 3A and B, the increase in firmness is even sharper at the trial's lowest gel concentrations (7.2- and 2.6-fold at 0.5% agar and 0.1% gellan gum, respectively, between pH 4.5 and 5.0).

In preparing media for trials investigating the effects of pH on growth, it should be remembered that lowering the pH of a medium but retaining the same gel concentration may effectively increase the supply of water, some (but not all) nutrients [15], and

hormones [9] simply because the gel matrix is softened. Particularly at pH's of 4.5-5.5, compensatory effects of pH and gel concentration on growth have been observed where, for example, the optimum pH for adventitious bud production on spruce needles decreases as agar concentration increases [28]. The usefulness of being able to measure both pH and gel hardness, then adding sufficient gel to re-establish the medium's firmness at a lower pH is obvious. In matrix-type experiments set up to investigate the interaction between pH and concentrations of medium components whose availability is sensitive to pH, being able to maintain constant gel hardness at different pH's may minimize the risk of results being further complicated due to the secondary interaction of the opposing effects of gel texture and pH on nutrient and hormonal availability.

4. The dynamics of syneresis

Syneresis is the process of a liquid separation or "weeping" from the gel [29] due to contraction or structural changes of the gel matrix. Syneresis is often the result of events like cool or freezing temperatures and/or extended storage. All gels exhibit some degree of syneresis: agar is considered to be highly syneretic and gellan gum only slightly so [24].

Freshly poured tissue culture plates are usually "conditioned" or "dried" prior to use [30], often by leaving the petri plates either unsealed or uncovered in a laminar flow hood. Conditioning the media minimizes the formation of water on the surface of the gel and condensation on the petri plate lid.

The effect of conditioning on gel hardness is unclear, so a small trial with varied drying times was done. Petri plates were initially weighed, filled with 25 ml of 0.9% agar or 0.4% gellan gum gels, and then reweighed. As before, Ca and Mg gluconate (3 mM and 1.5 mM, respectively, adjusted to pH 5.7) were the only medium components other than the gels.

All petri plates were placed in a vertical laminar flow hood with the lids on but unsealed, and left to dry for 4-96 h. Four plates of each gel type were removed at specified intervals, reweighed, then measured for hardness (four readings per plate) using the gel tester. The time course of water loss, expressed as a percentage of the initial gel weight, and hardness changes are shown in Figure 4.

The rate of moisture loss was constant throughout the drying period. Both gel types had lost approximately 9% of their initial mass after 96 h of drying. However, during the first 8-12 hours gel hardness rapidly increased, then leveled off to relatively constant values for the remainder of the test period. The practical conclusion from such measurements is that covered petri plates should be dried for no less than 12 h or more than 24 h (with the particular style of laminar flow hood used in our laboratory). The results also suggest that water in the gel may exist in two compartments, both of which are equally accessible for diffusion into the airflow above the gel's surface. As a result of the loss of the first fraction the contraction of gel occurs. However, the second fraction does not seem to induce continued gel shrinkage.

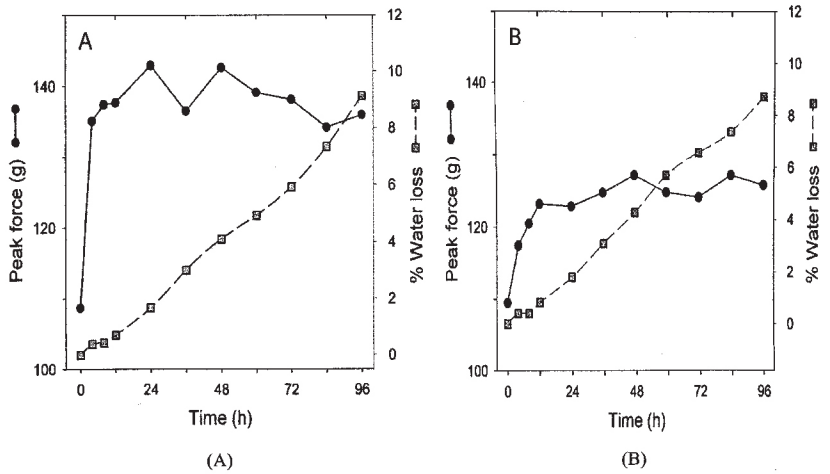

Figure 4. Water loss (B) and gel hardness (α) of agar (A) and gellan gum (B) gelled media as a function of 0, 4, 8, 12, 24, 36, 48, 60, 72, 84 or 96 h of drying time under a vertical laminar flow hood.

Though the mechanism controlling diffusional water loss is poorly defined, both water flux and hardness are likely related to pore size. Though their microstructure differs, both gellan gum and agar gels are heterogeneous. Gelled agar is reported to have "a polydispersity of bundle thickness (3 to 20 nm) and pore size (30 to 600 nm)" [16]. Similarly, gellan gum gels are observed to have two or more discrete size classes (0.1μ and 1μ), with thick and thin gel strands associated with the large and small pores, respectively. Maximum gel hardness, produced by using 6-8 mM Ca, corresponds to the minimum large pore size and higher water-holding capacity. [24]. The authors propose that in gellan gum the large pores formed from thick strands determine gel hardness, but with a water-holding capacity more sensitive to external forces. The thin-stranded, small pores (whose dimensions do not depend on Ca concentration) have a greater capacity to stably hold water due to their higher capillarity, so gellan gum exhibits low syneresis during long term storage.

5. Conclusion

The small tests documented above have been presented to demonstrate that simple, rapid empirical estimates of gel hardness are practical using peak force measurements from a low-cost, easily constructed device. Even though the functional mechanism remains largely unknown, agar and gellan gum gels have well-documented, concentration-dependant effects on growth and morphology in tissue culture systems. The structure and density of the gel matrix are thought to restrict access of the explant or callus to the nutrients, hormones and/or water in the gelled medium. This is independent of other

gel-concentration-dependant factors-e.g., the low molecular weight carbohydrate substances or the sulfate content of agar, or the high cation concentration in gellan gum [31]. Since gel firmness itself can modify, or be modified by, many of the cultural parameters which tissue culturists attempt to manipulate experimentally, we believe a simple assessment tool providing very basic information on gel rigidity and structure may be of value for routine use.

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