New Culture Medium Containing Ionic Concentrations of Nutrients Similar to Concentrations Found in the Soil Solution†

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A new growth medium which closely approximates the composition of the soil solution is presented. This soil solution equivalent (SSE) medium contains the following components (millimolar): NO₃, 2.5; NH₄, 2.5; HPO₄, 0.005; Na, 2.5; Ca, 4.0; Mg, 2.0; K, 0.503; Cl, 4.0; SO₄, 5.0; ethylenediamine-di(*o*-hydroxyphenylacetic acid), 0.02; and MES [2-(*N*-morpholino)ethanesulfonic acid] (to maintain the pH at 6.0), 10, plus 0.1% arabinose. The advantages of the SSE medium are discussed.

Hundreds of media are available for the cultivation and maintenance of soil microorganisms. Media containing ill-defined components are frequently used for the initial isolation of microorganisms from soil. Soil extract agar, for example, contains an unknown mixture of nutritional components which support a wide spectrum of the soil population (6). Other media contain a variety of inorganic macronutrients as well as ill-defined components such as yeast extract or peptone. For maintenance of microorganisms following isolation, it is often desirable to use a well-defined medium in which the various components are fully characterized. Most often, well-defined media were originally formulated to produce a substrate on which the microbes exhibited a rapid growth rate. The primary problem with such a pragmatic approach to medium formulation is that the media do not resemble the environment from which the microorganisms were isolated. It is thus possible that both genetic and phenotypic properties of microorganisms growing on these media may be different from those of the same microorganisms growing in soil.

There is little doubt that the growth rate of microorganisms in soil is dramatically different from the growth rate of the same microorganisms on artificial media (11). There also exist numerous examples in the literature in which the composition of the medium upon which microorganisms are maintained affects phenotypic properties. To eliminate or reduce these concerns, it seems logical to utilize media with compositions that closely resemble that of the soil solution.

Until recently, there was a general lack of understanding of the composition of the soil solution which supported the growth of soil microorganisms. Recent advances in analytical instrumentation and computer modeling (14), however, have expanded our understanding of the concentration, activity, and interactions of nutrients within the soil solution (9).

To compare the compositions of commonly used biological growth media with that of the soil solution, ionic concentrations of several media are presented in Table 1. The HEPES-MES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesul-

fonic acid (Sigma Chemical Co.)–2-(*N*-morpholino)ethanesulfonic acid (Sigma)] medium (HM medium) of Cole and Elkan (3) is used to support the growth of rhizobia. This medium, of all of those reviewed, contains fewer abnormal concentrations of nutrients and was thus used for comparison in the subsequent growth rate study. The *Bacillus* medium is used for the enrichment of *Bacillus* species from soil and was originally developed by Hunger and Claus (5). The Czapek-Dox medium (12) is used to support the growth of soil fungi.

All media noted above contain concentrations of nutrients that are significantly different from those in the soil solution (Table 1). Although nutrient concentrations in the soil solution vary depending upon the soil type and moisture content, concentrations presented in Table 1 are averages for loamy agricultural soils with a neutral pH (13). They are also similar to those reported by Mengel et al. (10) and Fried and Shapiro (4). Complicating the discussion of the composition of the soil solution is the inherent heterogeneity of the solution. The composition can vary widely, for example, depending upon whether bulk or rhizosphere soils are assayed. Values reported in the current study are bulk soil averages.

The concentrations of nitrogen in media that have been previously used are consistently higher than concentrations found in the soil solution. Media are generally amended with either NH₄ or NO₃ but seldom with both forms of inorganic nitrogen. Whereas most species of soil microorganisms preferentially utilize NH₄ (7), a significant portion may also utilize NO₃ as an alternative nitrogen source (8). Hence, it is desirable to include both forms of inorganic nitrogen in a growth medium. A further advantage of including both inorganic nitrogen forms is that it will help to maintain the balance between protons and hydroxyl ions in the medium and thus stabilize the pH.

Concentrations of P_i added to media are several orders of magnitude higher than that found in the soil solution. Excessive additions of HPO₄ to media cause many of the micronutrients in the media (often added as contaminants) to precipitate, thus limiting their availability (15) and the potential for using these media for testing micronutrient effects on microorganisms (1). With a smaller phosphate addition, added micronutrients and heavy metals will remain in solution to a greater extent compared with media in which phosphate additions are excessive. A further problem with

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TABLE 1. Ionic concentrations added to media used to cultivate soil microorganisms compared with concentrations reported for the soil solution

Ion	Ionic concn (mM) in:				Soil solution ^a
	Media				
	HEPES-MES	<i>Bacillus</i>	Czapek-Dox	SSE	
NO ₃			35.3	2.5	4.7 ^b
NH ₄	5.98	18.7		2.5	0.4 ^b
HPO ₄	0.88	7.35	5.74	0.005	0.005
Na	5.34		35.3	2.5	1.4
Ca	0.088	0.34		4.0	12.4 ^c
Mg	0.73	2.03	2.0	2.0	3.4
K		7.35	18.2	0.503	0.3 ^d
Cl	7.65	19.0	6.71	4.0	1.9
SO ₄	2.52	2.10	2.07	5.0	3.4
Fe	0.015	0.096	0.036	0.02 ^e	0.02

^a From Russell (13).^b Highly variable and dependent upon the fertility level of soil.^c According to Lindsay (9), this value is appropriate only for calcareous soils. Noncalcareous soils exhibit a Ca ion concentration of 2.5 mM.^d According to Lindsay (9), the K ion concentration is 1.0 mM.^e As FeEDDHA.

excessive HPO₄ additions to media is that the HPO₄ may react, during autoclaving, with carbohydrates in the media to form potentially toxic materials (15). Because the concentration of HPO₄ in many media is approximately 200 to 1,500 times greater than that found in the soil solution, it is necessary to add carbohydrates after autoclaving.

Sodium concentrations in media are also greater than those found in the soil solution, although only the Czapek-Dox medium is excessively high. On the other hand, concentrations of Ca in media are much smaller than those found in the soil solution. Although there is some controversy regarding the actual concentration of Ca in the soil solution (Lindsay [9] indicates that the Ca concentration is 2.5 mM for noncalcareous soils), it is evident that the HM and *Bacillus* media contain much lower concentrations than that found in the soil solution. The Czapek-Dox medium contains no added Ca.

Concentrations of magnesium are generally within the appropriate range for the soil solution. Potassium additions to media are variable, ranging from none added (in the case of HM) to 18.2 mM added (Czapek-Dox). Reported soil solution concentrations of K range from 0.3 (13) to 1.0 mM (9). Concentrations within the rhizosphere, however, may fall to several micromolar (1a). Hence, it is possible that HM supplies the appropriate requirement via contaminants, whereas additions to the other two media are excessive.

The concentrations of Cl in media are always greater than that found in the soil solution. This reflects the need to add an anion along with the required cations. Sulfate concentrations added to media are generally appropriate. Finally, additions of Fe added to media are within the appropriate range; however, Fe rapidly combines with other components of the media to form complexes which are not available for uptake (2). Thus, most media are likely to be lacking in available Fe.

With due consideration to the issues in the discussion above, we designed and tested a medium which was formulated to resemble the soil solution as much as possible. To some extent, we were constrained by the conflicting requirements to produce a medium which most closely resembled the soil solution and to use chemicals that are readily

available. We also strove to include chemicals in which at least two required ions were present in each chemical component. This minimized the number of individual chemicals added to the solution. Finally, solution pH and chemical solubility placed an additional constraint upon the composition of the medium.

The medium which we have developed contains the following ions at the following concentrations (millimolar): NO₃, 2.5; NH₄, 2.5; HPO₄, 0.005; Na, 2.5; Ca, 4.0; Mg, 2.0; K, 0.503; Cl, 4.0; SO₄, 5.0; and Fe [as ethylenediamine-di(*o*-hydroxyphenylacetic acid) (FeEDDHA)] 0.02. Ions were added as KH₂PO₄, CaSO₄, MgCl₂, and NH₄NO₃. To adjust the medium pH from an original level of 4.1 to 6.0, we added 0.5 mM K (0.5 ml of 1.0 M KOH), which supplied the appropriate amount of K, and 4.0 mM Na (4.0 ml of 1.0 M NaOH). Hydroxides were combined into a stock solution to facilitate addition to the medium. To maintain the medium pH at 6.0, MES was added at 10 mM. Although micronutrients are required for growth of soil microorganisms, none were added directly. Ultrapure water and analytical-grade chemicals, which have negligible micronutrient contamination, are excessively expensive for biological media. Instead, we relied upon contaminants present in water (distilled water in which complex micronutrients are present) and reagent-grade chemicals to supply the appropriate micronutrients. Arabinose was added to the medium at a concentration of 0.1%. We used arabinose because the subsequent evaluation of the medium used rhizobia as the test organisms; however, other carbohydrates could be used as deemed appropriate. Because the phosphate concentration in the medium was drastically reduced, the arabinose was added prior to autoclaving, thus simplifying preparation of the medium. The FeEDDHA was added after autoclaving and cooling (to 55°C) because EDDHA is heat labile. Both broth and agar (1.5%) were prepared.

Although this medium is a much better approximation of the composition of the soil solution than previous media, concentrations of several ions deviate from those in the soil solution more than we had hoped. The sodium concentration is slightly high; this resulted from the requirement to increase the pH of the medium from 4.1 to 6.0. Only bases containing K and Na hydroxides are required in the medium and are of adequate solubility to be of use. However, we could add only a small amount of KOH, not exceeding the K concentration of the soil solution. Hence, the pH of the medium was adjusted primarily with NaOH. In addition, the concentrations of both Cl and SO₄ are slightly higher than those found in the soil solution; however, these anions were required to counterbalance the cations.

Agar plates containing either the new medium (subsequently referred to as SSE, for soil solution equivalent) or HM medium, for comparison, were prepared and were streaked with log-phase cultures of the following rhizobial strains and species: *Rhizobium leguminosarum* bv. trifolii USDA 2227, USDA 2230, USDA 2234, USDA 2235, USDA 2237, and USDA 2238; *Rhizobium fredii* USDA 193; *Rhizobium meliloti* RCR 2011; *R. leguminosarum* bv. phaseoli RCR 3622; *R. leguminosarum* bv. viciae RCR 1001; and *Bradyrhizobium japonicum* RCR 3442. Mid-log-phase cultures (grown in HM medium) of these strains had been centrifuged, the supernatants had been discarded, and the pellets had been suspended in demineralized water. Growth of rhizobia, at 28°C, was noted daily.

No differences in the development of discrete colonies, visible biomass production, or colony appearance were observed between strains grown on the two agar media. A

FIG. 1. Growth of *R. leguminosarum* bv. *trifolii* USDA 2237 on SSE and HM media.

graph showing growth of *R. leguminosarum* bv. *trifolii* in broth of SSE and HM is presented in Fig. 1. Cell concentrations in both media, even at the time of maximum density, were low compared with those in rich media such as yeast extract-mannitol medium (1.0% mannitol), in which the carbon supply is not limited (16). The only significant difference in growth between SSE and HM was noted during the first 4 h. During this time, cell density increased slightly in the SSE, whereas it decreased in HM. The differential response in growth during the first 4 h may have been related to the osmotic potential of the two media. Cell density subsequently increased after this time for both media, and no differences were observed in growth between the two media throughout the remainder of the study. The cell density of *R. leguminosarum* bv. *trifolii* reached a maximum at 92 h and slowly declined after this time.

In conclusion, we have developed an outline for the preparation of media appropriate for the cultivation of soil microorganisms. The composition of the medium closely approximates that of the soil solution. The primary distinction between SSE and many previous media is that SSE contains very low concentrations of HPO_4 and high concentrations of Ca. The N source in SSE is equally split between NO_3 and NH_4 . As such, fewer genetic and phenotypic abnormalities should arise in microorganisms grown on this medium, unlike when soil microorganisms are grown on media that are atypical of the soil solution. This new medium

is only an outline and may be amended to resemble the particular soil from which a microorganism was isolated. For example, if organisms from calcareous soils were to be cultivated, it would be desirable to increase the pH and the Ca concentration in the medium. Microorganisms isolated from acidic soils should be grown on media with a lower pH. Each of these changes alters the ionic composition of the medium. A recognition of the composition of the soil solution and an attempt to approximate the composition of the soil solution to the greatest extent possible should enhance our ability to grow soil microorganisms under the most realistic conditions possible.

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