Infection Unit Method of Vesicular-Arbuscular Mycorrhizal Propagule Determination

Raymond L. Franson and Gabor J. Bethlenfalvay*

ABSTRACT

A method is presented for quantifying infectivity of vesicular-arbuscular mycorrhizal (VAM) fungal inocula by directly counting infection units in roots. Inocula of five isolates of VAM fungi [*Glomus etunicatum* Becker and Gerd., *Glomus pallidum* Hall, and *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe from California, Florida and Nevada] were assessed. *Sorghum bicolor* L. was grown for 14 d under controlled conditions in 100 mL growth tubes containing 48, 12, 3, or 0.75 g of each inoculum (96, 24, 6, or 1.5 for *G. etuicatum*) mixed with sterile soil to provide a total growth medium of 96 g. Discrete infection units were found for all isolates at all inoculum density levels, except for *G. etunicatum* (no infection at lowest density) and *G. mosseae*-California (coalescing infection units at highest density). The relationship between inoculum density and discrete infection units per gram root fresh weight was linear ($P < 0.01$). This relationship is particularly suitable for determining amounts of inocula of equivalent potential for use in short-term experiments.

VESICULAR-ARBUSCULAR mycorrhizal (VAM) fungi are commonly propagated by means of soil inocula. The potential of such inocula (sensu Garrett, 11) to produce infection units (sensu Cox and Sanders, 6) and colonize the root is important in short-term experiments designed to compare the effects of VAM fungi on plants (1). Estimates of inoculum potential based on spore counts or root colonization have been found unreliable (1, 8, 14, 15), since propagules of VAM fungi (spores, multi-spore sporocarps, vesicles, hyphae, colonized root fragments) are difficult to quantify, and their viability (22) is equally difficult to determine.

Two procedures are often used to quantify inoculum potential, the most probable number (MPN) technique (10,17,18) and the mean percent infection (MPI) method (15). Both procedures use serial dilutions of the inoculum. In the MPN technique the numbers of propagules which cannot be counted directly are estimated from the dilutions. Thus, assessing only the occurrence and not the frequency of infection, the MPN technique is thought to eliminate differences in inoculum potentials due to variations in host-endophyte compatibility (9). Dilutions are used in the MPI method to provide direct comparisons between viable populations of propagules. Since the comparisons are based on the presence or absence, and not the intensity, of root colonization, the MPI method, like the MPN technique, only estimates propagule density.

Our method also employs serial dilutions, but only to delimit the linear range of the sigmoid relationship between inoculum potential and infection unit formation (2, 15, 26). The use of counts of infection units directly determines, rather than estimates, inoculum potential within a defined range of inoculum density.

USDA-ARS, Plant-Soil Symbiosis Group, Plant Development-Productivity Res. Unit, Western Regional Res. Ctr. (WRRC), Albany, CA 94710. Received 2 May 1988. *Corresponding author.

Published in Soil Sci. Soc. Am. J. 53:754–756 (1989).

MATERIALS AND METHODS

Five VAM-fungal isolates were cultured on *Sorghum bicolor* L. in a silty-clay loam soil (Typic xerorthent) of the Balcom Series (Yolo County, California). The soil, of pH 8 and limiting in $(NH)_4HCO_3$-extractable P (3.3 mg kg^{-1}), was collected to a depth of 30 cm. At least three successive cultures of 12 and 20 wk each were used to purify the strains. The isolates (WRRC no. 2, 3, and 4, respectively) were *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe, *Glomus pallidum* Hall, and *Glomus etunicatum* Becker and Gerd., all from Medell Flat, a site in northwestern Nevada (28). Also included were two other geographic isolates of *G. mosseae*, one (WRRC no. 1) from the Anza-Borrego State Park in Southern California (3) and one from the University of Florida Agronomy Farm from fine sand of pH 7.6. (International Cultural Collection of VAM Fungi, isolate no. 156, University of Florida, Gainesville). The five cultures were air-dried, homogenized and sieved (2 mm) prior to use. Root colonization in the inocula was determined in clear root fragments stained with trypan blue. Spore numbers were counted following wet-sieving and sucrose-gradient centrifugation, while sporocarps of *G. mosseae* were separated only by wet sieving (9).

Plants were grown in a growth chamber with day/night settings of 16/8 h, 28/21 °C, and 60/90% relative humidity. Light intensity was 800 μmol m^{-2} s^{-1}. Plants were watered three times a week with a nutrient solution equivalent to one-quarter strength Hoagland's solution, but without P (1 m*M* CaCl, 2 m*M* NH_4NO_3, 0.5 m*M* K_2SO_4, 0.25 m*M* $MgSO_2$).

Inocula were mixed with autoclaved (2 h, 120 °C) Balcom soil at dilution ratios of 1:1, 1:7, 1:31, and 1:127 inoculum to soil, respectively, except for *G. etunicatum*, whose dilution ratios were 0:1, 1:3, 1:15, and 1:63. Inocula and autoclaved soil (96 g total) were mixed thoroughly and placed in 100 mL plastic, tapered growth tubes 3.5 cm in diam and 11 cm in height. The soil was wetted to field capacity and held at room temperature for 3 d. Seedlings were germinated on paper, selected for uniformity at 3 d and transplanted to the growth tubes. Four replications were used at each dilution, for a total of 16 test plants per fungal isolate.

Plants were harvested after 14 d, when soil became thoroughly permeated by roots without crowding and infection units had not yet coalesced. The roots were washed free of soil, weighed, sampled (sample size approximately 30% of total), cleared and stained with trypan blue. Discrete infection units per sample were counted with a dissecting microscope. The number of infection units per root system was calculated from the ratios of total root to sample mass and the results were expressed as infection units per g of root fresh mass. The relationship between inoculum density and infection units was determined by regression analysis.

RESULTS AND DISCUSSION

Infection units (6) originate from entry points (21) and represent the initial phase of fungal growth within the root cortex which may lead to general colonization of the root. The relationship between infection units per unit root fresh mass and inoculum density was linear over a 64-fold dilution range for all isolates except *G. mosseae* from Medell Flat (Fig. 1, Table 1). For the latter, the relationship was linear except for the highest level of inoculum density (lowest dilution). The deviation from linearity at high inoculum density

of the Medell Flat *G. mosseae* isolate was due to coalescing; adjacent infection units were scored as one since individual entry points could not be determined.

Two major sources of error complicate a direct count of infection units of VAM fungi. One arises from the geometry of the system used and the other from the sequence of events in the infection process and the timing of evaluation. If the soil volume is large and the distance between adjacent roots too great, propagules may not form entry points by harvest time and will remain uncounted. On the other hand, if more time is allowed for the roots to permeate the same volume to decrease propagule-to-root distances, secondary infection units originating from the same propagule may occur. The sequence of events in the colonization process has been modeled quantitatively and discussed elsewhere (21, 27) with reference to linear relationships between the formation of infection units and propagule density as determined by the MPN technique (27) or inoculum amount (4, 15). This linearity (within a presaturation range of inoculum density) indicated a relationship between early root colonization and inoculum potential and can be exploited as a rapid and direct method in comparing inocula for use in short-term experiments.

The dilution gradient of the infection-unit method (IUM) serves a different purpose than in the MPN or MPI techniques. In the latter, it defines limits for the presence or absence of propagules or serves as a basis for a direct comparison of inocula. In the IUM, it defines the linear portion of the inoculum-potential vs. infection-unit relationship, i.e., the range of inoculum density providing useable information for inoculum comparisons. Above this range, counts cannot be made due to the coalescence of infection units; below it, statistical scatter prevents meaningful comparisons.

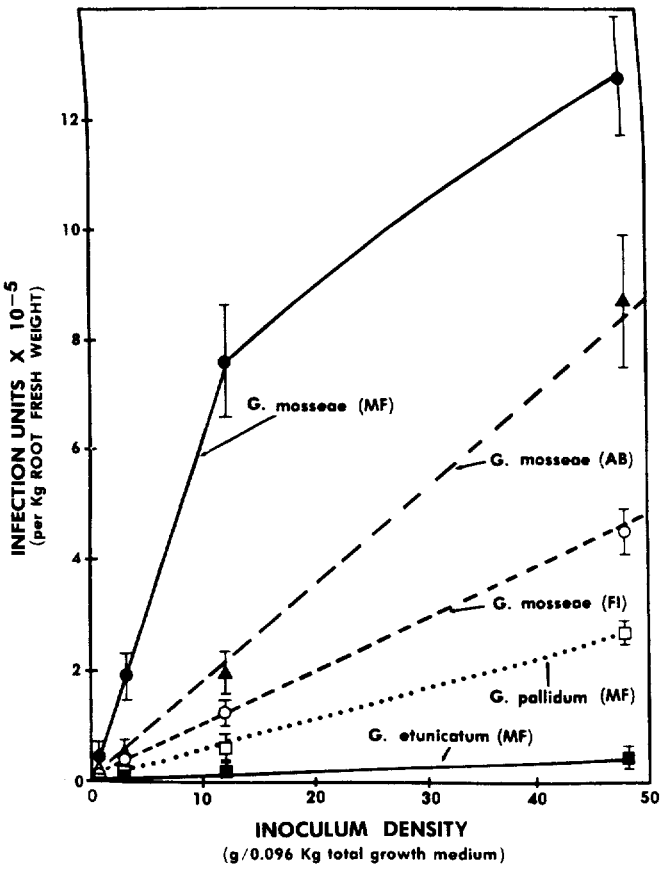

Fig. 1. Infection unit formation as a function of inoculum density. Inoculum density is given as the mass of inoculum per total mass (0.096 kg) of growth medium for all isolates except *Glomus etunicatum*. This latter isolate, whose inoculum density was twice that of the other isolates, is included for comparison with infection units proportionately adjusted (halved). Data points represent means of four replicates with standard error bars.

Confirming the findings of others (1, 8, 14), we found no consistent relationship between inoculum potential as determined by bioassay (IUM) and root colonization or spore or sporocarp counts of the inocula (Table 2). We propose that a count of infection units is a more reliable measure of the number of viable propagules present than inferences drawn from other indicators. Nevertheless, the problems inherent in this method must also be considered. One limitation of the MPN technique is equally applicable to the infection unit method. It deals with the observation that root colonization, sporulation and the formation of extraradical mycelia is greatest when host species and soil diluent most closely match the native conditions of each inoculum to be compared (1, 12). Clearly, these conditions cannot be satisfied when several VAM fungi are to be tested for applicability to a situation in agroecology where both soil and host are different from those of the endophyte's native adaptation. Another problem is the temporal change in inoculum potential connected with dormancy (16, 23) and persistence (7, 24). Differences in infection unit counts may depend not only on the presence of viable propagules but also on conditions affecting the timing of spore germination (25).

Table 1. Relationship of infection unit (IU) formation and inoculum density (ID).†

Fungal isolate‡	Regression parameter $y = bx + a$	r
Glomus mosseae (AB)	$y = 18.2x - 6.1$	0.999**
Glomus mosseae (MF)	$y = 63.2x - 0.7$	0.999**
Glomus mosseae (FL)	$y = 9.2x + 9.9$	0.999**
Glomus etunicatum (MF)	$y = 0.8x + 0.5$	0.999**
Glomus pallidum (MF)	$y = 5.5x - 4.0$	0.999**

** Significant at the 0.01 probability level.

† The amount of inocula (x) needed to produce an equivalent number of IUs (y) per kg of root fresh weight may be calculated from the equations. Analysis of the *G. mosseae* (MF) data exclude the data point of highest inoculum density.

‡ AB, Anza-Borego; MF, Medell Flat; FL, Florida.

Table 2. Infection units (IU), spore counts, and root colonization of inocula. Fungal isolate identification as in Table 2.

Fungal isolate	IU†	Colonization, %	Spores‡	Sporocarps‡
Glomus mosseae (MF)	63	40	12	1
Glomus moseae (AB)	17	63	5	5
Glomus mosseae (FL)	11	56	9	20
Glomus pallidum (MF)	5	41	48	
Glomus etunicatum (MF)	1	19	24	

† Formation of IUs (10^{-3}) per kg fresh weight of host-plant root were determined after 14 d of growth.

‡ Spore and sporocarp counts (10^{-3}) per kg of inoculum and percent colonization refer to the diluted inocula.

The infection unit method assures the investigator only that infection units will be formed in subsequent experiments at levels comparable to those observed at harvest of the preceeding comparability determination. Sequential breaking of spore dormancy may alter this relationship, but the establishment of extensive root colonization early in the ontogeny of the symbiosis will minimize the effect of subsequent encounters between roots and germinating spores on roots already colonized. Given a sufficient number of propagules to produce infection units initially in emerging roots, a predetermined (maximum) level of infection is probably established early due to the spread of secondary infection units (20). A suitable range of dilutions can establish the propagule number falling within the exponential (linear) phase of a sigmoid relationship between inoculum density and infection unit formation (2, 26). Our method is therefore most applicable to short term experiments, since host-plant growth response is usually dependent on mycorrhizae formed early in growth (13, 19). It, like the MPN technique, employs 'common garden' conditions (5), in which all determinants of VAM development (new host, new soil, moisture, nutrients, light, growth period) are uniform, but it employs direct counts rather than an indirect probability estimation for the comparison of inoculum potential among fungal isolates.

ACKNOWLEDGMENTS

We thank K.L. Mihara for the maintenance of the VAM fungal cultures, and N.C. Schenck for providing a VAM-fungal isolate (no. 156, IN VAM, Florida).

REFERENCES

1. Adelman, M.J., and J.B. Morton. 1986. Infectivity of vesicular-arbuscular mycorrhizal fungi: Influence of host-soil diluent combinations on MPN estimates and percentage colonization. Soil Biol. Biochem. 18:77–83.
2. Baker, R. 1971. Analyses involving inoculum density of soil-borne plant pathogens in epidemiology. Pytopathology 61:1280–1292.
3. Bethlenfalvay, G.J., S. Dakessian, and R.S. Pacovsky. 1983. Mycorrhizae in a Southern California desert: Ecological implications. Can. J. Bot. 62:519–524.
4. Carling D.E., M.F. Brown, and R.A. Brown. 1979. Colonization rates and growth responses of soybean plants infected by vesicular-arbuscular mycorrhizal fungi. Can. J. Bot. 57:1769–1772.
5. Clausen, J., D. Keck, and W.M. Hiesey. 1948. Experimental studies on the nature of species. III. Environmental responses of climatic races of *Achillea*. Carnegie Inst. Washington Publ. 581. Washington, DC.
6. Cox, G., and F. Sanders. 1974. Ultrastructure of the host-fungus interface in a vesicular-arbuscular mycorrhiza. New Phytol. 73:901–912.
7. Daft, M.J., D. Spencer, and G.E. Thomas. 1987. Infectivity of vesicular-arbuscular mycorrhizal inocula after storage under various environmental conditions. Trans. Br. Mycol. Soc. 88:21–27.
8. Daniels, B.A., P.M. McCool, and J.A. Menge. 1981. Comparative inoculum potential of spores of six vesicular-arbuscular mycorrhizal fungi. New Phytol. 89:385–391.
9. Daniels, B.A., and H.D. Skipper. 1982. Methods for the recovery and quantitative estimation of propagules from soil. *In* N.C. Schenck (ed.) Methods and principles of mycorrhizal research. Am. Phytopathol. Soc. Publ., St. Paul, MN.
10. Fisher, R.A., and F. Yates. 1963. Statistical tables for biological, agricultural and medical research. p. 8–10. Hafner Publ. Co., Inc., New York.
11. Garrett, S.D. 1956. Biology of root-infecting fungi. Cambridge Univ. Press, Cambridge.
12. Graw, D., M. Moawad, and S. Rehm. 1979. Untersuchungen zur Wirts- und Wirkungsspezifität der VA-Mykorrhiza. Z. Acker- und Pflanzenbau. 148:85–98.
13. Haas, J.H., A. Bar-Tal, B. Bar-Yosef, and J. Krikun. 1986. Nutrient availability on vesicular-arbuscular mycorrhizal bell pepper (*Capsicum annuum*) seedlings and transplants. Ann. Appl. Biol. 108:171–179.
14. Haas, J.H., and J. Krikun. 1985. Efficacy of endomycorrhizal-fungus isolates and inoculum quantities required for growth response. New Phytol. 100:613–621.
15. Moorman, T., and Reeves, F.B. 1979. The role of endomycorrhizae in revegetation practices in the semi-arid West. II. A bioassay to determine the effect of land disturbance on endomycorrhizal populations. Am. J. Bot. 66:14–18.
16. Nemec, S. 1987. Effect of storage temperature and moisture on *Glomus* species and their subsequent effect on *Citrus* rootstock seedling growth and mycorrhiza development. Trans. Br. Mycol. Soc. 89:205–212.
17. Porter, W.M. 1979. The 'Most Probable Number' method for enumerating infective propagules of vesicular-arbuscular mycorrhizal fungi in soil. Aust. J. Soil Res. 17:515–519.
18. Powell, C. L1. 1980. Mycorrhizal infectivity in eroded soils. Soil Biol. Biochem. 12:247–250.
19. Sanders, F.E., P.B. Tinker, R.L.B. Black, and S.M. Palmerley. 1977. The development of endomycorrhizal root systems: I. Spread of infection and growth-promoting effects with four species of vesicular-arbuscular endophyte. New Phytol. 78:257–268.
20. Lopez-Aguillon, R., and B. Mosse. 1987. Experiments on competitiveness of three endomycorrhizal fungi. Plant Soil 97:155–170.
21. Smith, S.E., and N.A. Walker. 1981. A quantitative study of mycorrhizal infection in *Trifolium*: Separate determination of the rates of infection and of mycelial growth. New Phytol. 89:225–240.
22. Sylvia, D.M. 1988. Activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. Soil Biol. Biochem. 20:39–43.
23. Tommerup, I.C. 1983. Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Trans. Br. Mycol. Soc. 81:37–45.
24. Tommerup. I.C. 1984. Persistence of infectivity by germinated spores of vesicular-arbuscular mycorrhizal fungi in soil. Trans. Br. Mycol. Soc. 82:275–282.
25. Tommerup, I.C. 1985. Inhibition of spore germination of vesicular-arbuscular mycorrhizal fungi in soil. Trans. Br. Mycol. Soc. 85:267–278.
26. Vanderplank, J.E. 1963. Plant diseases: Epidemics and control. Academic Press, London.
27. Walker, N.A., and S.E. Smith. 1984. The quantitative study of mycorrhizal infection. II. The relation of rate of infection and speed of fungal growth to propagule density, the mean length of infection unit and the limiting value of the fraction of root infected. New Phytol. 96:55–69.
28. Young, J.A., and R.A. Evans. 1974. Population dynamics of green rabbitbrush in disturbed big sagebrush communities. J. Range Manage. 27:127–132.