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Measurement of the extraradical mycelium of a vesicular-arbuscular mycorrhizal fungus in soil by chitin determination

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Summary Development of a vesicular-arbuscular mycorrhizal (VAM) fungus in association with soybean was determined in a greenhouse soil mix by chitin assay. Samples were sieved to eliminate hexosamine-containing contaminants. This preparation reduced the interference caused by extraneous soil substances and permitted quantitative measurement of extraradical VAM fungal mycelium in the soil mix by colorimetric assay. Recovery of added chitin, used as an internal standard, was greater in the soil mix than in an inert medium indicating that some hexosamine was stabalized from chemical degradation by other soil components.

Introduction

The extent of colonization of roots by VAM fungi is customarily estimated by staining the roots[16] to determine the proportion of VAM and non-VAM plant tissue. Various histological methods currently in use have recently been reviewed[7], and new, improved procedures to standardize the evaluation of infection are proposed[4,8]. One shortcoming of histological methods[7] is the difficulty in obtaining unbiased estimates of the intensity of infection[24] without an inordinate expenditure of time and effort. The intensity of infection can be determined by measuring the amount of fungal biomass by chemical analysis of certain cell wall constituents[17,26]. One method which does not require expensive instrumentation[17] is based on the determination of chitin [poly-beta-(1,4)-N-acetylglucosamine] from the cell walls of VAM fungi[2,9]. This method has been criticized based on the finding in other fungi that chitin content varies with age and environmental conditions[20]. This phenomenon has yet to be confirmed in VAM fungi.

The chitin assay is particularly useful when a determination of total VAM fungal biomass is of interest[1]. The extraradical mycelium of VAM fungi is of paramount importance to the symbiotic fungus-root association, as it is the major interface between plant and soil. Yet, its measurement has rarely been attempted[18,19]. As a consequence, estimates of VAM fungal biomass as a percentage of total symbiotic tissue may be low, as they are based on the intraradical mycelium alone[25]. The measurement of VAM fungal chitin derived from extraradical mycelium in an inert (sand-perlite) rooting medium has been reported earlier[1]. The purpose of this study was to determine if the measurement of VAM fungal biomass by the chitin assay was feasible in a soil mixture containing large amounts of organic matter.

Materials and methods

Growth conditions

Soybean [*Glycine max*, (L.) Merr. cv. Kent] plants were grown in association with the VAM fungus *Glomus fasciculatum* (Thaxt. *sensu* Gerd.) Gerd. and Trappe or as uninoculated controls in a greenhouse potting mix in pot cultures under conditions described elsewhere[3]. The rooting medium was a University of California Type C soil mix[11,12] (50% peat moss, 50% fine sand), modified to consist of 33% clay loam, 17% Canadian sphagnum peat moss, 17% Sacramento River Delta peat soil,

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25% fine (0.1 to 0.25 mm) sand, and 8% coarse (0.5 to 1.0 mm) sand. The soil mix (pH 6.8) was steam sterilized and amended by 1.3 kg/m^3 oyster shell lime ($CaCO_3$), 0.6 kg/m^3 dolomitic lime [$MgCa(CO_3)_2$], 0.5 kg/m^3 single superphosphate (18 to 20% available P_2O_5), and 0.2 kg/m^3 KNO_3. Pots were flushed once a week with deionized water and five times a week with a P-free nutrient solution (pH 6.7) equivalent to one-quarter strength Johnson's solution[10].

Sample preparation

At harvest, the roots of the control plants or of the mycorrhizae were thoroughly washed over a sieve of 43 μm pore size. This procedure separated the extraradical mycelium and spores from the mycorrhizae. The fungal structures were retained in the sieve, and the mycorrhizae were inspected microscopically (20 ×) to insure complete removal of fungal hyphae. Soil particles clinging to control plant roots or mycorrhizae were also washed off, and much of the clay and silt fraction passed through the pores of the sieve. The material retained by the sieve, including spores and extraradical mycelium, was returned to the soil mix. This moist soil was stirred to approximate a uniform distribution of hyphae, hyphal fragments and spores. It was wet sieved over a 43 μm mesh screen for 5 min to eliminate the fine colloidal material which interfered with the colorimetric assay or contained non-VAM amino sugar contaminants. The sieving procedure was standardized and consisted of 100 swirls of the slurry under running water. Subsequently, the sieve was tilted, the slurry collected against the rim and redistributed over the sieve surface with a slow shaking motion a dozen times. The slurry was then rinsed under warm water and allowed to drain. Soil samples of 50 cm^3 from VAM and control treatments were dried for 2 days at 90°C. Duplicate soil samples were taken from each of the five replicates and analyzed for chitin. To determine if there was loss of VAM fungal hyphae during wet sieving, soil from additional VAM-colonized plants was wet sieved sequentially through screens of increasingly fine mesh prior to sample preparation. No VAM spores or hyphal fragments were found to pass a screen of 43 μm pore size, though clay, humic material, and spores of non-VAM fungi were found to pass through the sieve.

Chitin assay

The dried soil samples were mixed with 40 ml of concentrated KOH (120 g KOH/100 g H_2O) and autoclaved for 1 h at 120°C to degrade organic materials and to hydrolyze chitin contained in the soil and VAM fungal cell walls to chitosan [poly-beta-(1,4)-glucosamine]. Portions of the supernatant (4 ml), while still warm, were subsequently withdrawn and cooled to 4°C. Smaller portions were diluted to 4 ml with the KOH solution when high levels of VAM fungal chitin were expected in the soil based on a prior histological determination[2] of VAM fungal colonization of the host root. Ice-cold ethanol (70%) was used to precipitate the chitosan. Celite was added and the sample was centrifuged at 6000 rpm at 4°C for 10 min. The pellet was then resuspended and washed sequentially in 40% ethanol, 0.01 *N* HCl (pH 2), and glass-distilled water following centrifugation as described previously[2]. A second water wash was added as it tended to minimize interference with the colorimetric assay by unidentified soil substances. Chromophore development was accomplished according to Ride and Drysdale[17] based on the formation of a complex between the dye MBTH (3-methyl-2-benzo-thiazolinone hydrazone HCl), $FeCl_3$, and 2,5-anhydromannose, the final reaction product of chitin. Absorbance due to the resulting blue chromophore was measured with a Bausch & Lomb spectronic 20 spectrophotometer at 650 nm. Complete chromophore development required 30 min.

For the calculation of chitin in the soil mix, a standard curve was constructed by utilizing purified chitin (Sigma Chemical Co., St. Louis, MO 63178, U.S.A.) as an internal standard. One, 0.5 or 0.25 mg of chitin was added to a suspension of 50 cm^3 of the moist, sieved soil mix in 40 ml of concentrated KOH with vigorous swirling. An additional suspension without chitin was also included to determine the background hexosamine level present in the soil mix. Hydrolysis of added amounts of chitin in this soil was compared to chitin hydrolysis in an inert potting medium composed of sand/perlite (2/1, v/v). The standard curve for the soil mix was used to calculate the amount of chitin in VAM fungi grown in association with soybeans in the moist, sieved soil. The standard curve for the inert potting

mix was used to compare chitin hydrolysis in the soil mix with hydrolysis in a medium containing no interfering substance, and it was used to calculate the level of residual chitin in the unamended soil mix. To account for chitin contamination due to sources other than *G. fasciculatum*, the amount of chitin in non-VAM control soils was also determined. The average chitin values from five control replications was subtracted from the chitin value of each of five replicates of VAM soil. Plants were harvested and soil was prepared for chitin determination at 2 or 3 week intervals.

Results and discussion

Colorimetric assay

The chitin assay, as originally introduced[17] and later adapted to the quantification of intraradical VAM fungal biomass[9] and of the extraradical mycelium in an inert rooting medium[1] needed modification when applied to soil. The number of water washes of the chitosan precipitate depends on the incidence of spurious color reactions due to unidentified contaminants in the soil, and should therefore depend on the type of soil assayed. It should be minimized, however, as with each wash some chitosan may be lost. Reaction of chitosan with HONO must be given sufficient time for the hydrolysis and deamination of the glucosamine polymers. It is essential that any residual HONO be completely reacted with the $NH_4SO_3NH_2$ under vigorous shaking, otherwise MBTH will autoxidize and couple to produce a violet pigment which will obscure measurement of the blue chromophore.

Comparison of standard curves

Absorbance at each level of chitin addition were significantly higher ($P < 0.01$ by two-tailed t-test) in the standard curve based on soil than in the curve based on the inert medium (Fig. 1). The slopes of the curves were also significantly different ($P < 0.01$ by multiple regression analysis). The higher absorbance values for chitin when hydrolysed in the presence of the soil mix may result from the stabilizing effect of soil components[5]. Thus colloidal clay particles and humic acid have been implicated as stabilizing hexosamines from biodegradation[13,14]. A comparison of absorbance values between the inert medium and soil (Fig. 1) suggests that such soil components may protect hexosamines from chemical degradation as well. A possible mechanism for this protection at the high pH used for hydrolysis, may be a linkage of free $-NH_2$ groups to soil phenolic polymers through

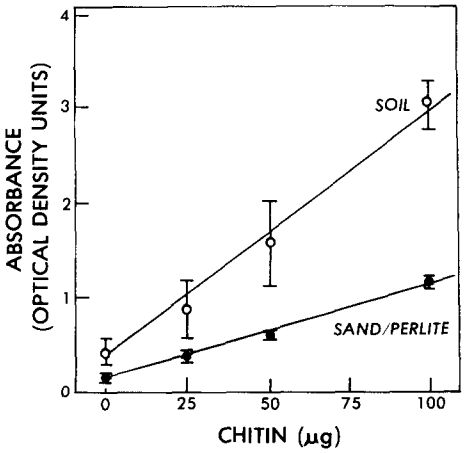

Fig. 1. Comparison of standard curves for chitin hydrolysis in an inert potting medium (sand/perlite, 2/1, v/v) and a soil mix. Absorbance values (in optical density units at 650 nm) represent means and S.D. of at least six replications. Slopes were significantly different ($P < 0.01$) by multiple regression analysis.

autoxidation reactions[13]. Later in the colorimetric assay, acid conditions during the deamination step may favor release of the hexosamines and chitosan oligomers from the protective soil particles[5]. This protection of chitin hydrolysis products from alkaline degradation followed by a release during the colorimetric assay may account for the higher absorbances associated with the soil mix.

The amount of background hexosamine present in the soil mix varied with the soil treatment prior to the chitin assay. The moist sieved soil without added chitin contained 9.8 μg hexosamine/g soil as determined from a glucosamine standard curve[2]. Soil not wet sieved yielded results up to six times those reported here (Table 1). Such values were within the lower range reported for natural soils elsewhere[21,22,23]. Assuming that a large proportion of the hexosamines were derived from the hydrolysis of chitin, a value for the residual or background soil chitin may be calculated as 26.3 μg chitin/g soil from the standard curve for chitin hydrolysis in soil (Fig. 1). The discrepency between the soil concentrations of chitin and hexosamine is due to the incomplete hydrolysis of chitin to glucosamine during autoclaving. Previous work[2] showed that only 37% of the chitin amino sugars contribute to chromophore development, while the remainder of the chitin subunits are either unreacted or degraded by autoclaving.

Mycorrhizal chitin

Chitin content of the soil increased to week 10 and declined slightly thereafter (Table 1). The initial increase is ascribed to the development of the extraradical VAM fungal mycelium. The slow decline in soil chitin content after week 10 may be explained by the cessation of VAM-fungal growth, autolysis of senescent hyphae, and eventual decomposition of dead mycelium by bacteria[6].

Table 1. Change in soil chitin content during the ontogeny of soybean plants colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus fasciculatum* or left uncolonized as controls.

Time after inoculation (weeks)	Chitin content*, mg	
	VAM	Control
3	3.61 ± 1.41	1.51 ± 0.46
5	8.90 ± 3.31	1.79 ± 0.56
7	20.2 ± 2.60	2.09 ± 0.50
10	27.8 ± 8.21	2.56 ± 0.41
13	22.7 ± 4.32	3.24 ± 0.44
16	22.0 ± 4.32	3.53 ± 0.81
19	18.1 ± 2.16	4.06 ± 0.70

* Numbers represent means and standard deviations of five replications.

Conclusions

Estimation of extraradical VAM fungal biomass based on the quantification of chitin is hampered by the native amino sugar component[15] present in soil. Soil may be manipulated, however, to reduce background hexosamine to levels permitting colorimetric chitin determinations based on internal chitin standards. Soil manipulation must be accomplished carefully to minimize loss of VAM fungal materials and uniformly to insure comparability of results. The method described is expected to be readily applicable to soils of lesser clay and organic matter content than used here.

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Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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