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Modifications to clearing methods used in combination with vital staining of roots colonized with vesicular-arbuscular mycorrhizal fungi

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Abstract. Leek, maize, and pigmented soybean roots colonized by vesicular-arbuscular mycorrhizal (VAM) fungi were assessed for succinate dehydrogenase (SDH) activity using the nitro blue tetrazolium chloride (NBT)-succinate method. NBT-succinate-reacted roots, cleared in a 55° C drying oven in 5% (w/v) KOH for 24 h or longer and observed as whole mounts, revealed signs of intraradical VAM fungus colonization more clearly than roots cleared by the standard 20% (w/v) boiling chloral hydrate method. Combined clearing of NBT-succinate-reacted roots using boiling chloral hydrate followed by clearing in 5% KOH at 55° C for prolonged periods also improved the visualization of intraradical fungal structures. Bleaching of NBT-succinate-reacted roots using the standard NH_3-H_2O_2 method removed pigmentation from roots and did not alter the viability indicator, formazan. Pigmented, field-collected soybean roots were successfully cleared and bleached to reveal signs of viable and nonviable intraradical fungal structures. Counterstaining of NBT-succinate-reacted roots with acid fuchsin clearly revealed both viable and nonviable intraradical fungal structures. The NBT-succinate solution infiltrated all intraradical fungal structures after 24 h; formazan products were observed at similar concentrations in viable structures after 24, 36, and 48 h.

Key words: Vesicular-arbuscular mycorrhizae – Viability – Nitro blue tetrazolium chloride – Succinate dehydrogenase – Formazan

Introduction

Quantification of intraradical vesicular-arbuscular mycorrhizal (VAM) fungal colonization has been based primarily on the assessment of total VAM fungal colonization. However, total colonization does not distinguish between the viable and nonviable VAM fungal fractions present in roots. This distinction is important since physiological processes occur only in metabolically active fungal structures (Hamel et al. 1990). Typically, assessment of total colonization has involved staining mycorrhizal roots with acid fuchsin (Kormanik and McGraw 1982), trypan blue (Phillips and Hayman 1970), or chlorazol black-E (Brundrett et al. 1984). The ability to quantify only the viable fraction (or viable:nonviable ratio) of VAM colonization in roots has gained recent attention (Kough and Gianinazzi-Pearson 1986; Kough et al. 1987; Hamel et al. 1990; McGee and Smith 1990; Smith and Gianinazzi-Pearson 1990; Smith and Dickson 1991; Vierheileg and Ocampo 1991). Researchers have also attempted to determine the proportion of viable VAM spores in soil samples (An and Hendrix 1988) and extraradical VAM mycelium (Schubert et al. 1987; Sylvia 1988; Hamel et al. 1990).

The tricarboxylic acid cycle enzyme, succinate dehydrogenase (SDH), present in viable fungal hyphae, reacts with nitro blue tetrazolium chloride (NBT) and is readily reduced to a dark blue-purple formazan compound (Pearse 1972). MacDonald and Lewis (1978) used NBT on freeze-sectioned VAM-colonized roots and found SDH activity in the fungal hyphae but not in the cytoplasm of the root cells. The enzyme specificity of NBT (Pearse 1972; Hamel et al. 1990) and low level of background reactivity in root cells (MacDonald and Lewis 1978) warrants its use as an indicator of mycorrhizal viability (Smith and Dickson 1991). Smith and Gianinazzi-Pearson (1990), Smith and Dickson (1991), and Pearson et al. (1991), however, have reported difficulties in assessing whole root squashes and root sections due to background reactivity in the stele and dark coloration in the hypodermal and epidermal layers. In addition, pigmented roots rarely clear adequately when processed by the standard boiling chloral hydrate technique used in conjunction with NBT vital staining. Reduced visualization of intraradical fungal structures in whole root squashes can compromise the accuracy of the usual grid-line quantification methods.

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The reliability of estimations of the viable fraction of intraradical VAM colonization depends on accurate determination of the total colonization or nonviable colonization fraction. VAM-colonized root pieces stained with NBT can clearly reveal evidence of viable fungal structures, whereas nonviable fungal structures lacking colored formazan deposits can be overlooked during the grid-line intersect step. Hamel et al. (1990), Smith and Gianinazzi-Pearson (1990), and Smith and Dickson (1991) stained separate root subsamples, once for viable colonization and once for total colonization, to obtain viable colonization fractions. The practice of staining samples separately can be time-consuming, laborious, and in some cases costly. Counterstaining NBT-succinate-reacted root pieces for total colonization offers an advantage over the previously used methods by improving visual clarity of both viable and nonviable intraradical fungal structures. Data on viable and nonviable VAM colonization can thus be obtained.

The main objectives of this present investigation were to: (1) improve the clearing technique used in association with NBT-vital staining; (2) assess the effects of the standard NH_3-H_2O_2 bleaching solution on NBT-succinate-reacted root pieces; and (3) develop a counterstaining protocol to enable the quick and efficient determination of the viable:nonviable ratio of intraradical VAM fungal colonization.

Materials and methods

Plant materials

Roots of maize (*Zea mays* L.) colonized by a currently undescribed *Glomus* species referred to as Elora 500 (isolated by T. McGonigle, University of Guelph) and leek (*Allied porrum* L.) colonized by *Glomus versiforme* (Daniels and Trappe) Berch or *Glomus intraradices* Schenck and Smith were obtained from established pot cultures maintained under controlled growth-room conditions. Roots were obtained fresh from pot cultures when necessary, washed thoroughly, and used in the various experiments detailed below. In addition, soybean (*Glycine max* L.) roots, colonized by indigenous VAM fungal species under natural conditions, were obtained from a local field (August, 1992) near the University of Guelph. Soybean roots served as the material for a combined clearing and NH_3-H_2O_2 bleaching experiment.

Staining

VAM-colonized root pieces were incubated at room temperature overnight in the dark in a solution containing 25% (v/v) 0.2 M Tris buffer (pH 7.4), 25% (v/v) (4 mg/ml) nitro blue tetrazolium chloride (NBT; 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene] ditetrazolium chloride), 10% (v/v) 0.05 M $MgCl_2$, 30% (v/v) distilled water and 10% (v/v) 2.5 M sodium succinate (diNa succinate·$6H_2O$) (Troyer 1980; Kough et al. 1987). KCN was not included in the NBT-succinate incubation medium (Smith and Gianinazzi-Pearson 1990). Following NBT-succinate incubation, root pieces were rinsed in three changes of distilled water and fixed in formol-saline [10% (v/v) formaldehyde, 37–40% (v/v); 90% (v/v) distilled water; and 0.9% (w/v) NaCl] (Pearse 1972) for a minimum of 1 h in the dark. Roots were again rinsed in three changes of distilled water prior to the various clearing treatments.

Root piece assessment

Root pieces were mounted whole on glass microscope slides in glycerine under a cover glass and photographed on a Leitz Orthomat photomicroscope.

Experiment 1.0 (clearing)

Clearing of roots removes cytoplasm and some pigmentation (Phillips and Hayman 1970). The standard clearing technique used subsequent to reaction of root pieces with NBT-succinate was 10–15 min in boiling 20% (w/v) chloral hydrate (Kough and Gianinazzi-Pearson 1986; Kough et al. 1987; McGee and Smith 1990; Smith and Gianinazzi-Pearson 1990). The other clearing methods tested were compared to this. Root pieces were cleared with either 5% (w/v) KOH, 20% (w/v) chloral hydrate, or 85% (v/v) lactic acid. Clearing was carried out either in an autoclave (121° C, 1.3 kg cm^{-2}) for 5–15 min, at 55° C in a drying oven for 2–26 h, or boiling on a hot plate for 5–20 min. Root pieces were mounted on microscope slides and assigned codes so that clearing results could be compared without bias. Qualitative results were expressed as poor, fair, satisfactory, good, or excellent.

Experiment 1.1

The effect of KOH clearing on formazan deposition following incubation of root pieces in the NBT-succinate reaction media was tested. Fresh and heat-killed (15 min boiling water) root pieces were separately incubated overnight at room temperature in the dark in four different incubating solutions with or without NBT and/or the enzyme substrate succinate. Appropriate volumes of distilled water were used to replace the solutions omitted from the complete NBT reaction medium. Root pieces were incubated overnight in the dark at room temperature. Following a thorough rinse in distilled water and fixation in formol-saline, root pieces were cleared overnight in 5% (w/v) KOH at 55° C in a drying oven, thoroughly rinsed in distilled water, mounted, and viewed for signs of formazan deposition in intraradical fungal structures.

Experiment 1.2

The potential of clearing root pieces subsequent to incubation in NBT-succinate in chloral hydrate solution followed by overnight clearing in 55° C KOH was tested. Fixed root samples were cleared in boiling 20% (w/v) chloral hydrate for 5, 10, or 15 min, rinsed, and cleared again in 5% (w/v) KOH at 55° C in a drying oven overnight. Root pieces were thoroughly rinsed, mounted, and viewed.

Experiment 2.0 (bleaching)

Bleaching of pigmented VAM-colonized roots with NH_3-H_2O_2 is a standard procedure used to remove pigmentation to improve the visual clarity of subsequently stained intraradical fungal structures (Kormanik and McGraw 1982). The potential of bleaching fixed NBT-succinate-reacted roots in NH_3-H_2O_2 bleaching solution was tested. Root pieces were cleared overnight in 5% (w/v)

KOH at 55° C in a drying oven, rinsed thoroughly, and incubated in the NH_3-H_2O_2 bleaching solution for 10, 15, 25 or 45 min or 3 h. Root pieces were thoroughly rinsed, mounted, and viewed for signs of reduced formazan deposition or formazan degradation.

Experiment 3.0 (clearing and bleaching pigmented roots)

The assessment of VAM-colonized roots is often hindered by natural root pigmentation. The potential of viewing viable and nonviable VAM fungal structures in soybean roots colonized by indigenous VAM fungal species in soil was tested. Roots that had been incubated in NBT-succinate and fixed were cleared and bleached with NH_3-H_2O_2 by a variety of methods, as detailed in Table 3. Root pieces were mounted and viewed for signs of reduced formazan deposition, formazan degradation, and presence/absence of natural root pigmentation.

Experiment 4.0 (counterstaining)

The ability to assess whole root squashes for both viable and nonviable VAM fungal structures is preferable to using two separate staining procedures (i.e. separate treatments for total colonization and viable colonization). The potential of counterstaining root pieces incubated in NBT-succinate in acid fuchsin/lactic acid staining solution was tested. Acid fuchsin counterstaining of NBT-succinate-incubated and fixed root pieces followed acidification of roots in 1% (v/v) HCl for 3–5 min (Kormanik and McGraw 1982). Fresh and heat-killed root pieces were counterstained for 1 h at 55° C (oven) in acid fuchsin/lactic acid staining solution [87.4% (v/v) 85% lactic acid, 6.3% (v/v) glycerine, 6.3% (v/v) distilled water, and 0.01% (w/v) acid fuchsin] (Kormanik and McGraw 1982). Root pieces were mounted and viewed for signs of viable and nonviable intraradical fungal structures.

Experiment 5.0 (vesicles and spores)

The ability of NBT and succinate to infiltrate and react with SDH in thick-walled intraradical spores and vesicles over a period of 48 h was tested. Samples of leek root pieces colonized by *G. intraradices* were incubated in NBT-succinate for 24, 36, or 48 h. Root pieces were rinsed, fixed in formol saline, rinsed again, and cleared overnight in 5% (w/v) KOH at 55° C in a drying oven. Root pieces were mounted and viewed for signs of formazan deposition in intraradical spores and vesicles.

Results and discussion

General

Control root pieces killed in boiling water before incubation in the NBT reaction medium contained no evidence of formazan deposits in fungal structures or root cells (Fig. 1). Fresh VAM-colonized root pieces incubated in the NBT reaction medium always exhibited evidence of both viable and nonviable intraradical fungal structures. Formazan deposits were also observed in root cells, especially in stelar parenchyma cells, but this rarely hindered visualization of formazan deposits in intraradical fungal structures. Portions of roots often contained high concentrations of dark blue-purple formazan deposits; this was due to many viable arbuscules (Fig. 8). In addition, viable root meristems always possessed high concentrations of formazan.

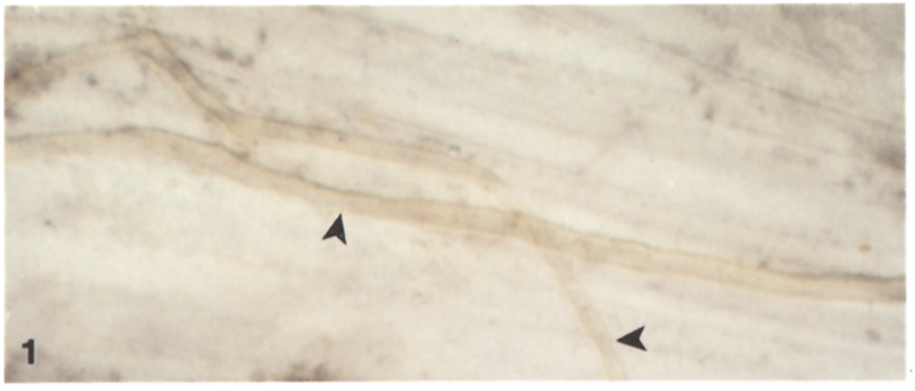
In agreement with Smith and Gianinazzi-Pearson (1990), the exclusion of KCN from the NBT incubating solution had minimal effect on formazan deposition. The clearing methods tested did not degrade or destroy formazan deposits. Storage of root pieces subsequent to incubation in NBT-succinate in glycerine under absolute dark conditions was necessary; formazan is photoreactive and quickly breaks down under normal light conditions (Pearse 1972).

Experiment 1.0 (clearing)

Although all clearing methods, with the exception of roots cleared in 55° C chloral hydrate for 24 h or prolonged periods (i.e. 4 days), resulted in adequate clearing, clearing of fixed NBT-succinate-reacted root pieces in 5% (w/v) KOH at 55° C for a minimum of 24–26 h proved to be the best method (Table 1, Figs. 2, 3, 5). Clearing roots in 5% KOH for 5–10 min in the autoclave or on the boiling plate also produced good results (Fig. 4). Clearing in KOH improved the visualization of NBT-stained hyphae, vesicles, appressoria, and arbuscules as compared to the same structures cleared by the standard 10–15 min 20% (w/v) boiling

Table 1. Qualitative assessment of clearing methods subsequent to nitro blue tetrazolium chloride (NBT)-succinate incubation of vesicular-arbuscular mycorrhizal (VAM)-colonized leek and maize root pieces

Clearing agent	Temperature	Clearing times	Results
KOH	55° C oven	2.75 h	Good
		5 h	Good
		26 h	Excellent
	121° C, 1.3 kg cm^{-2} autoclave	5 min	Good–excellent
		10 min	Good–excellent
		15 min	Good
	Boiling plate	5 min	Satisfactory
		10 min	Good
		15 min	Good
		20 min	Good
Chloral hydrate	55° C oven	24 h	Poor
		2 days	Satisfactory
		4 days	Fair
	121° C, 1.3 kg cm^{-2} autoclave	10 min	Satisfactory
		15 min	Satisfactory
	Boiling plate	5 min	Satisfactory
		10 min	Satisfactory
		15 min	Satisfactory
		20 min	Satisfactory
Lactic acid	121° C, 1.3 kg cm^{-2} autoclave	10 min	Good
		15 min	Good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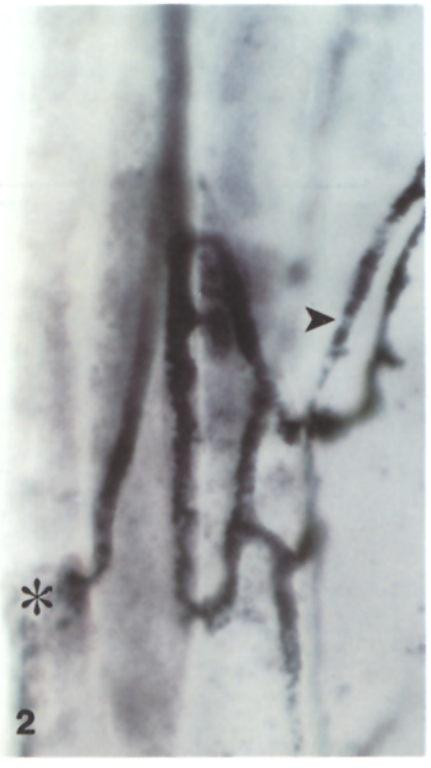
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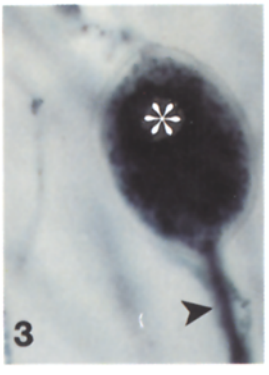
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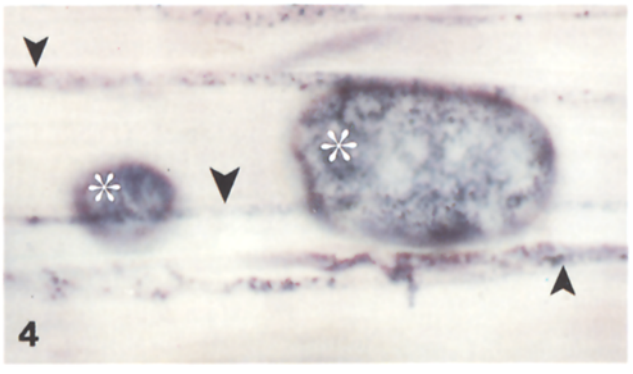
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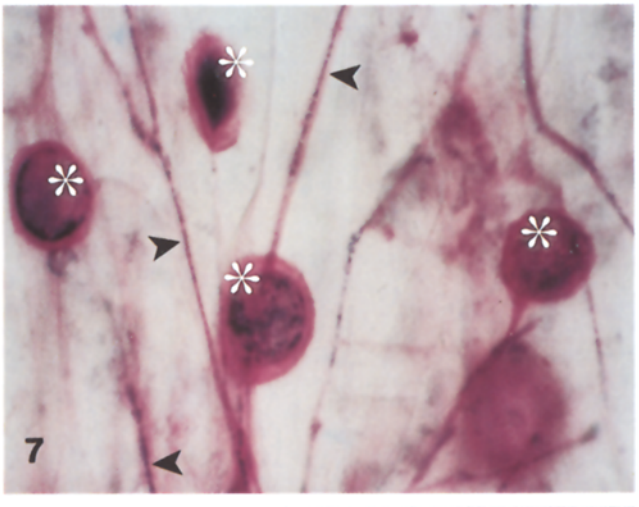
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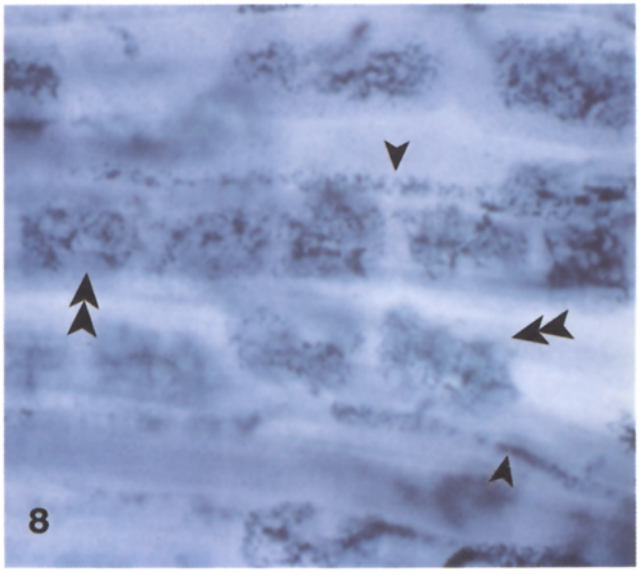
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chloral hydrate treatment. NBT-succinate-reacted roots cleared in KOH were softened and easily squashed, whereas roots cleared in chloral hydrate remained firm. Fixed root pieces cleared by the standard chloral hydrate treatment retained a hazy opaqueness which reduced the visual clarity of viable and nonviable intraradical fungal structures. However, clearing by the standard chloral hydrate technique was more effective than KOH alone in removing the dark coloration from root cell walls.

Experiment 1.1

Formazan deposition did not occur in heat-killed root pieces immersed in the four NBT incubating solution combinations, but did occur when living root pieces were incubated (Table 2) in the NBT and succinate-containing solution and then cleared with KOH. Low levels of formazan deposition were also observed in living root pieces incubated in NBT solution lacking the enzyme substrate, succinate. Formazan deposits in this case were attributed to the reduction of endogenous succinate by succinate dehydrogenase, and the probable reduction of other substrates by dehydrogenases present in the fungal mitochondria (Pearse 1972).

Experiment 1.2

The combination of boiling chloral hydrate and 55° C KOH effectively cleared root pieces. No indication of significant distinctions between 5, 10, and 15 min of chloral hydrate–KOH combined clearing was observed (Table 1). The hazy opaqueness observed in roots cleared by only chloral hydrate was removed by the additional KOH clearing. The dark discoloration observed in roots cleared only with KOH was removed by the combined clearing technique. Visual clarity of roots cleared by the combined technique surpassed that of roots cleared by either of the single clearing techniques.

Table 2. Effect of 5% (w/v) KOH clearing agent after 24 h at 55° C on formazan deposits in fungal structures

NBT incubating solution[a]		Root piece type[b]	Presence of formazan in fungal structures
NBT	Succinate		
+	+	Live	Yes
		Heat-killed	No
−	+	Live	No
		Heat-killed	No
−	−	Live	No
		Heat-killed	No
+	−	Live	Yes[c]
		Heat-killed	No

[a] Inclusion of NBT or succinate in the incubation solution is denoted by +; exclusion of NBT or succinate is denoted by −
[b] Live roots are leak and maize roots obtained freshly from pot cultures; heat-killed roots were boiled in water for 10 min
[c] Minimal deposits of formazan were observed

Experiment 2.0 (bleaching)

NH_3-H_2O_2 bleaching of NBT-succinate-reacted roots for as long as 3 h (Fig. 6) resulted in no observable decline in formazan deposition present in the stele, meristems, or fungal structures within roots. The majority of dark coloration was removed from the root cell walls, enhancing the visualization of fungal structures containing formazan deposits.

Experiment 3.0 (clearing and bleaching pigmented roots)

The combined clearing of soybean root pieces using boiling chloral hydrate and 55° C KOH (Fig. 8) was significantly better than clearing by chloral hydrate or KOH separately (Table 3); pigmentation was effectively eliminated to clearly reveal viable and nonviable intraradical fungal structures. Clearing in combination with bleaching also improved the visual clarity of viable and nonviable intraradical fungal structures. Roots bleached after the combined clearing technique, however, exhibited greater structural damage than roots bleached between the two clearing steps. Formazan deposition in fungal structures was not affected by any of the seven techniques tested; only the visual quality of the roots was affected.

Figs. 1–8. Vesicular-arbuscular mycorrhizal (VAM)-colonized roots stained in nitro blue tetrazolium chloride (NBT) reaction media, rinsed in water, fixed in formol-saline solution, rinsed, and cleared by a variety of techniques. Blue-purple formazan deposits in fungal structures signify viability; lack of formazan deposits signify lack of viability. **Fig. 1.** Heat-killed intraradical hyphae (▲) of *Glomus versiforme* in leek roots. Roots were cleared in boiling KOH for 10 min. **Fig. 2.** Intraradical hyphae (▲) and appressorium (✳) of *G. intraradices* in leek. Roots were cleared in a 55° C oven overnight in KOH. **Fig. 3.** Intraradical hyphae (▲) and vesicle (✳) of *G. intraradices* in leek. Roots were cleared in a 55° C oven overnight in KOH. **Fig. 4.** Intraradical hyphae (▲) and vesicles (✳) of the *Glomus* Elora 500 isolate in maize. Roots were cleared in boiling KOH for 10 min. **Fig. 5.** Intraradical hyphae (▲) and arbuscules (⁂) of *G. intraradices* in leek. Roots were cleared in a 55° C oven overnight in KOH. **Fig. 6.** Intraradical hyphae (▲) and vesicles (✳) of the *Glomus* Elora 500 isolate in maize. Roots were cleared in a 55° C oven overnight in KOH and bleached in NH_3-H_2O_2 for 3 h. **Fig. 7.** Intraradical hyphae (▲) and vesicles (✳) of the *Glomus* Elora 500 isolate in maize. Roots were cleared in a 55° C oven overnight in KOH and counterstained in acid fuchsin staining solution. **Fig. 8.** Intraradical hyphae (▲) and arbuscules (⁂) of VAM-colonized soybean roots obtained fresh from the field. Only two arbuscules are labeled. Roots were cleared in boiling chloral hydrate for 10 min, rinsed, and further cleared in a 55° C oven overnight in KOH

Table 3. Effect of various clearing and bleaching methods on pigmented NBT-succinate-incubated soybean roots

Method[a]		Visual clarity
(1)	Cleared overnight in 5% (w/v) KOH at 55° C in a drying oven	Poor
(2)	Cleared overnight in 5% (w/v) KOH at 55° C in a drying oven, rinsed, and NH_3-H_2O_2 bleached for 20 min	Poor
(3)	Cleared for 15 min in 20% (w/v) boiling chloral hydrate	Poor
(4)	Cleared for 15 min in 20% (w/v) boiling chloral hydrate, rinsed, and NH_3-H_2O_2 bleached for 20 min	Poor
(5)	Cleared for 15 min in 20% (w/v) boiling chloral hydrate, rinsed, NH_3-H_2O_2 bleached for 20 min, rinsed, and again cleared overnight in 5% (w/v) KOH at 55° C in a drying oven	Good
(6)	Cleared for 15 min in 20% (w/v) boiling chloral hydrate, rinsed, and again cleared overnight in 5% (w/v) KOH at 55° C in a drying oven	Excellent
(7)	Cleared for 15 min in 20% (w/v) boiling chloral hydrate, rinsed, again cleared overnight in 5% (w/v) KOH at 55° C in a drying oven, and NH_3-H_2O_2 bleached for 20 min	Satisfactory

[a] Rinsing in three changes of distilled water was performed after each step within each technique

Experiment 4.0 (counterstaining)

Root pieces incubated in NBT-succinate were readily counterstained with acid fuchsin (Fig. 7), revealing *both* metabolically active and inactive fungal structures. The pink-stained fungal walls did not interfere in the observation of the dark blue-purple formazan deposits.

Experiment 5.0 (vesicles and spores)

Leek roots colonized with *G. intraradices*, incubated in NBT-succinate reaction media for 24 h, and cleared in 55° C KOH overnight in a drying oven contained formazan deposits throughout all types of fungal structures, including intraradical spores/vesicles. Similar results were observed after 24 h NBT-succinate incubation in the other experiments, and in intraradical fungal structures of roots incubated for 36 and 48 h.

Conclusions and recommendations

The ability to use whole root mounts to efficiently quantify viable and nonviable VAM fungal colonization offers great potential. The majority of studies conducted to date on VAM colonization using the NBT-succinate method for viability have used sectioned root material (MacDonald and Lewis 1978; Smith and Gianinazzi-Pearson 1990; Smith and Dickson 1991), a time-consuming and potentially costly (Smith and Dickson 1991) approach to assessing viable and nonviable VAM colonization. Assessment of NBT-succinate-reacted whole root mounts is simple, and the data have potentially greater statistical relevance.

Whole-root VAM colonization assessment depends upon accurately distinguishing between viable and nonviable intraradical fungal structures. This paper shows the potential of using two alternative clearing techniques, 55° C KOH clearing and combined clearing in boiling chloral hydrate followed by 55° C KOH clearing, and an acid fuchsin counterstaining procedure. Acid fuchsin counterstaining in combination with either of the two alternative clearing techniques significantly improved visualization of viable and nonviable fungal structures. However, acid fuchsin counterstaining has the disadvantage that the stained roots fade rapidly when stored in glycerine (Brundrett et al. 1984). It is recommended, because of this fading phenomenon, that NBT-succinate/acid fuchsin-counterstained roots be assessed immediately after preparation. In addition to the alternative clearing techniques and counterstaining method, the standard NH_3-H_2O_2 bleaching technique was observed to effectively remove root pigmentation in NBT-succinate-reacted roots without degradation of formazan deposits.

We recommend that NBT-succinate-reacted roots with little or no pigmentation be cleared in 5% (w/v) KOH at 55° C for 24–48 h. Pigmented roots can be first cleared in boiling 20% (w/v) chloral hydrate, rinsed thoroughly, and then cleared again in 5% KOH at 55° C for 24 h or longer. NH_3-H_2O_2 bleaching of NBT-succinate-reacted roots is recommended only in cases where double clearing does not sufficiently remove pigmentation.

When combined with a highly objective grid-line quantification method such as that reported in McGonigle et al. (1990), the methods reported here can conceivably aid in the assessment of viable and nonviable VAM colonization in both unpigmented and pigmented roots.

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