

A comparison of visualization techniques for recording arbuscular mycorrhizal colonization

ALAN C. GANGE*, ERICA BOWER, PENNY G. STAGG,
DAVID M. APLIN†, ALEXANDRA E. GILLAM
AND MICHAEL BRACKEN

School of Biological Sciences, Royal Holloway University of London, Egham Hill, Egham, Surrey TW20 0EX, UK

Received 3 August 1998; accepted 4 December 1998

SUMMARY

The extent of arbuscular mycorrhizal colonization was assessed in 10 field-collected plant species, representing three annual forbs, three perennial forbs, three perennial grasses and one annual grass. Each root system of each plant was split into four portions, and for each portion, mycorrhizal structures were revealed with epifluorescence microscopy (under which only arbuscules are generally visible) and three commonly used stains (Chlorazol Black E, Acid Fuchsin and Trypan Blue). The aim of the study was not to evaluate the efficacy of each method, but to compare results obtained by each under standard laboratory conditions. The recorded colonization levels of arbuscules, total arbuscular mycorrhizal fungal material and total fungal (arbuscular mycorrhizal + non-arbuscular mycorrhizal) material differed significantly between visualization methods in a number of species. However, there were also interactions between stain and plant species, indicating that the performance of a stain is dependent on the plant species being examined. In some cases (e.g. *Plantago lanceolata*), each visualization method produced the same colonization level, while in others (e.g. *Dactylis glomerata*), each method gave a different result. These data therefore suggest that the level of mycorrhizal colonization recorded in any particular plant species at a particular time is dependent on the technique employed.

Key words: autofluorescence, stains, Chlorazol Black, Trypan Blue, Acid Fuchsin, arbuscular mycorrhiza.

INTRODUCTION

The preparation of plant roots for quantification of the extent of arbuscular mycorrhizal (AM) colonization is probably the most frequently performed procedure in AM research. Biological stains have been selected which bind to the fungal structures without too much background staining of the plant tissue. Over the years, various techniques have been published which document methods for clearing and subsequent staining of roots to reveal the mycorrhiza. The first of these, described by Phillips & Hayman (1970), used Trypan Blue (TB) and this method has been widely adopted. A modification was described by Koske & Gemma (1989), in which some of the toxic reagents were eliminated from the process, although TB remained.

Other authors have proposed the use of Acid Fuchsin (AF) (Kormanik & McGraw, 1982) or Chlorazol Black E (Brundrett *et al.*, 1984). However, a major problem of most stains is that they are known or suspected carcinogens (Coombes & Haveland-Smith, 1982) and in order to solve this, Grace & Stribley (1991) proposed replacing TB with aniline blue or methyl blue, although Brundrett *et al.* (1996) suggest that there is insufficient evidence that these latter two stains are not also toxic. In addition, effective clearing of roots involves the use of KOH, which is caustic.

Grace & Stribley (1991) reviewed the use of stains in the literature for 1989 and 1990 and found that 68% of authors used TB, 18% CBE, 9% AF and 5% some other procedure. We have repeated this review, surveying 1396 manuscripts published 1992–1998 (July). The results in Table 1 bear a remarkable resemblance to those of the previous survey, and although the proposal was made to replace TB with less toxic stains, few workers appear to have heeded this advice.

*Author for correspondence (fax 01784 470756; e-mail a.gange@rhnc.ac.uk).

†Present address: School of Biological Sciences University of Sussex, Falmer, Brighton, E. Sussex BN1 9QG, UK.

A further technique is listed in Table 1, autofluorescence (fluorescence microscopy). This was first described by Ames *et al.* (1982) and involves subjecting roots to ultraviolet illumination, under which the arbuscules autofluoresce. The method was found to work well, and no significant differences were found between the extents of colonization detected by this method and by that of Phillips & Hayman (1970). Subsequent workers have also reported autofluorescence of fungal structures other than arbuscules (Jabaji-Hare *et al.*, 1984). In addition, many plant roots contain yellow pigments which also fluoresce under UV light, but this is not a problem when attempting to differentiate between this material and arbuscules (Klingner *et al.*, 1995). Merryweather & Fitter (1991) subjected roots stained with AF to epifluorescence and found that the quality of the preparation was much improved. However, this method retains the toxic stain element.

Fluorescence microscopy would therefore appear to offer an alternative to the staining methods. It eliminates the use of toxic chemicals and can produce a clear result, unlike many stains in which the preparation often contains much stained non-fungal material. In addition, it predominantly reveals the definitive structure of the arbuscular mycorrhiza. We have encountered many stained preparations in which arbuscules could not be seen, possibly because they are transient structures, with a rapid turnover (Smith & Read, 1997). In such preparations, recording 'arbuscular mycorrhiza' might depend on a completely subjective decision, based on the presence of aseptate hyphae or vesicles. However, neither of these characters, alone or together, is a reliable indicator of AM colonization. Mycorrhizal hyphae cannot easily be distinguished from those of saprotrophic fungi or root pathogens (Smith & Read, 1997), while many other fungi can produce vesicles (Jennings & Lysek, 1996).

Another reason why arbuscules are often not seen is that their detection may depend on the stain used, or the plant life history group (annual or perennial forb or grass) or both. Given that a variety of staining techniques are in regular use (Table 1) it is important to establish whether they perform in a similar fashion. There appears to have been no attempt to compare the performance on a range of plant species, of the most widely used stains with that of autofluorescence. This is a critical aspect of arbuscular mycorrhizal (AM) research, since Smith & Read (1997) comment that a problem with stains is their differential tissue penetration and Clapp *et al.*, (1996) state that AF preparation quality varies according to the root tissue used. It is the aim of this paper to document this comparison, using a variety of plant species. Our aim was not to provide evidence of the efficacy of each method; photographic evidence of each has been provided before in the methodology papers already cited. However, we do

Table 1. The frequency of use of different detection methods in the arbuscular mycorrhizal literature, 1992–1998

Visualization method	% of papers where used
Trypan Blue	66.3
Acid Fuchsin	14.5
Chlorazol Black E	13.2
Autofluorescence	3.1
Aniline Blue	0.8
Other methods ^a	0.7
Methods not given	1.4

^aOther methods include molecular techniques, vital staining of enzyme activity, electrophoresis and laser scanning confocal microscopy.

Total number of manuscripts scanned = 1396.

believe that it is important to establish whether the level of colonization recorded in a plant species at a particular time is dependent on the visualization method used.

MATERIALS AND METHODS

Ten plant species, representing a range of life history strategies (Grime *et al.*, 1988) and mycorrhizal affinity (Harley & Harley, 1987) were chosen for this study. There were three annual forbs (*Capsella bursa-pastoris* (L.) Medikus, *Senecio vulgaris* L. and *Veronica persica* Poiret), one annual grass (*Poa annua* L.), three perennial forbs (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm., *Plantago lanceolata* L. and *Senecio jacobaea* L.) and three perennial grasses (*Brachypodium pinnatum* (L.) P. Beauv., *Dactylis glomerata* L. and *Holcus lanatus* L.). All plant samples were from various sites on the campus of Royal Holloway, University of London, Egham, Surrey, with the exception of *B. pinnatum* and *D. glomerata* which were from Castle Hill National Nature Reserve, Brighton, E. Sussex.

Ten plants of each species were chosen at random from the population, dug up and their roots washed free of soil. The root system of each plant was then chopped into 1-cm portions, mixed in a beaker of water, and four portions randomly selected. One portion was subjected to fluorescence microscopy, one to staining with Chlorazol Black E (CBE) (Colour Index (CI) no. 30235), one to staining with Acid Fuchsin (AF) (CI no. 42685) and one to staining with Trypan Blue (TB) (CI no. 23850). For autofluorescence (Ames *et al.*, 1982) 1-cm root segments were mounted in water on a slide. These were examined at $\times 200$ with a Zeiss[®] Axiophott epifluorescence microscope fitted with a UV lamp. The filters used were as described by Merryweather & Fitter (1991), giving a transmission of 455–490 nm blue. The remaining three samples were cleared in 2.5% KOH. The CBE samples were stained in a solution of 0.03% CBE in lactoglycerol (Brundrett *et*

Table 2. Incubation times (min) for stages in staining procedures for the 10 study plants

Plant species	Clearing		Staining		
	2.5 % KOH	1 % HCl	CBE	AF	TB
<i>Brachypodium pinnatum</i>	40	30	60	40	15
<i>Dactylis glomerata</i>	10	20	60	20	20
<i>Holcus lanatus</i>	15	20	60	20	20
<i>Hyacinthoides non-scripta</i>	30	20	60	15	40
<i>Plantago lanceolata</i>	30	20	60	15	40
<i>Senecio jacobaea</i>	40	20	60	30	40
<i>Senecio vulgaris</i>	15	20	60	30	30
<i>Veronica persica</i>	15	20	60	20	20
<i>Capsella bursa-pastoris</i>	15	20	60	20	20
<i>Poa annua</i>	15	20	60	40	30

All stages took place at 80 °C in a water bath. Stains: Chlorazol Black E (CBE); Trypan Blue (TB); Acid Fuchsin (AF).

Table 3. Summary of ANOVA results testing for differences between visualization methods and the interactions between method and plant species

	Species		Visualization method		Interaction	
	df	<i>P</i>	df	<i>F</i>	df	<i>F</i>
Arbuscules	7,72	36.1	3,216	157.9	21,216	10.9
Vesicles	7,72	17.8	2,144	3.04*	14,144	4.05
Total AM fungus	8,81	60.7	2,162	107.6	16,162	11.9
Total non-AM fungus	9,90	42.4	2,180	9.2	18,180	14.9
Total fungal material	9,90	59.1	2,180	89.8	18,180	13.8

**P* < 0.05.

All *F*-ratios were highly significant (*P* < 0.001) except where noted. Note: Degrees of freedom differ between analyses, because *Capsella bursa-pastoris* and *Poa annua* were omitted from the analyses for arbuscules and vesicles. Autofluorescence was only included in the analysis for arbuscules.

al., 1984). To prepare AF and TB samples, roots were thoroughly washed in water after clearing and acidified in a solution of 1 % HCl before immersion in the staining solution. For AF samples, a solution of 875 ml lactic acid, 63 ml glycerin and 63 ml water containing 0.1 g Acid Fuchsin was prepared following Kormanik & McGraw (1982). For TB samples, a solution containing 500 ml glycerol, 450 ml water, 50 ml 1 % HCl and 0.005 % Trypan Blue was used (Koske & Gemma, 1989). Stained roots were rinsed in water and mounted in water or glycerol. All clearing, acidification and staining took place in a water bath at 80 °C. As noted by Brundrett *et al.* (1996), optimal duration of clearing, acidification and staining can vary greatly from one plant species to another. We therefore performed a series of preliminary experiments in which time in each stage was varied. For each species, we placed roots in KOH for either 5, 10, 15, 30 or 40 min, then in HCl for either 15, 20 or 30 min. Each of these combinations was then subject to either 15, 20, 30, 40 or

60 min in stain. This process enabled us to arrive at a set of time combinations which produced the clearest preparation (i.e. best definition of fungus, with least background staining of plant tissue) for each species and each stain. The optimum timings for the 10 species are given in Table 2.

Colonization levels were assessed at ×200 by the cross-hair eyepiece intersection method of McGonigle *et al.* (1990). Approximately 150 intersections per slide were recorded, to give a measure of percentage root length colonized (% RLC). The time taken to assess each slide preparation was recorded, and converted to the time required to count 100 intersections, termed 'recording time'. This gave a measure of the clarity of fungal structures for each method, since categorization was easier and therefore quicker owing to greater definition and contrast between root and fungal material. As the arbuscule is the only definitive structure produced by arbuscular mycorrhizas, we recorded arbuscules, vesicles (in the presence of arbuscules) and hyphae

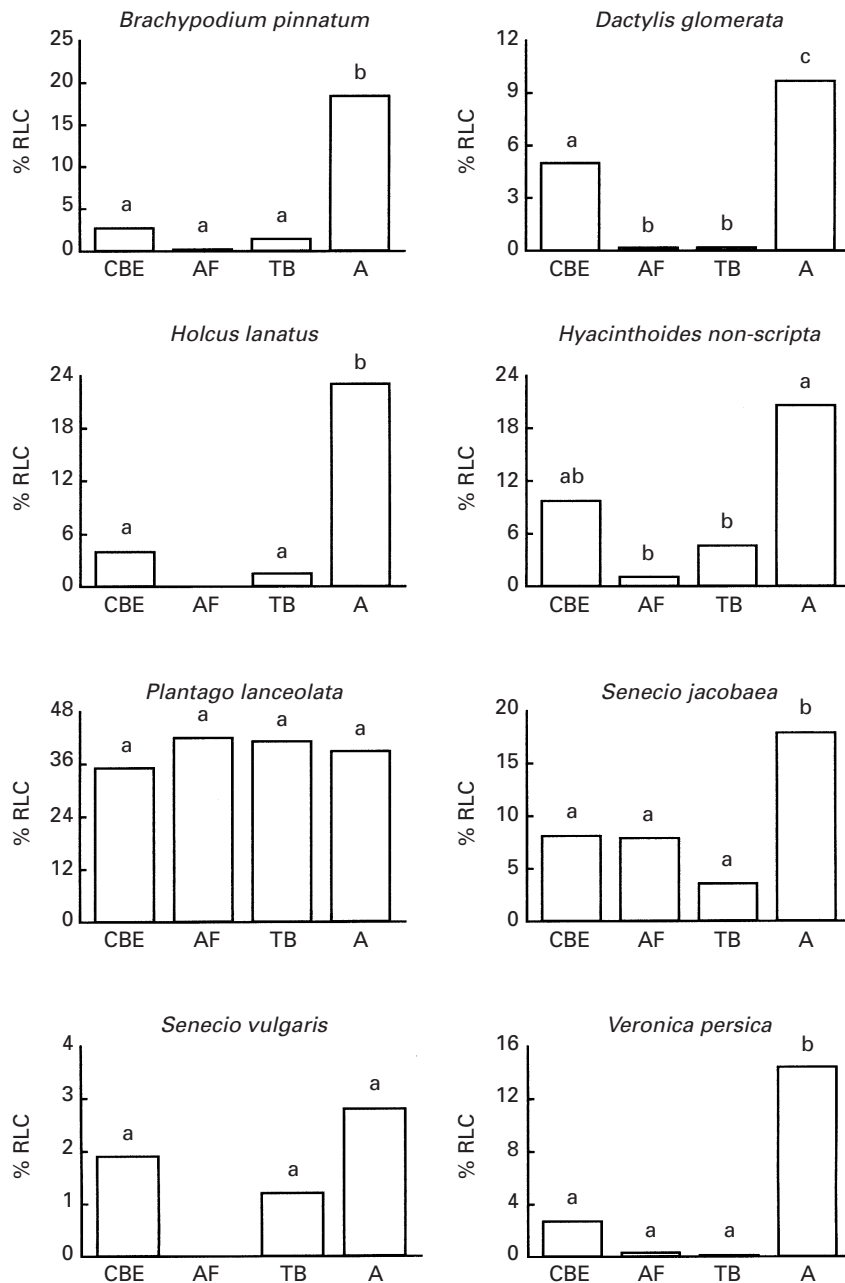


Figure 1. Levels of arbuscule colonization, measured as mean percentage of root length colonized (% RLC). Visualization methods: Chlorazol Black E (CBE); Acid Fuchsin (AF); Trypan Blue (TB); Autofluorescence (A). Different letters above columns indicate significant differences between means (Tukey test). Note: *Capsella bursa-pastoris* and *Poa annua* are omitted from this figure as no arbuscules were detected in either species by any method.

(also in the presence of arbuscules), these three categories representing definite mycorrhizal fungal material. We also recorded other fungal material, which was composed of definitely non-mycorrhizal structures (such as septate hyphae or spores) or dubious material, which consisted of vesicles or hyphae, but with no arbuscules. All assessments of colonization were performed by one person (ACG) to eliminate any possibility of variability between assessors. The data were summarized to give counts of arbuscules, total AM fungal material and total fungal material, and since they were in percentage form, they were subjected to the angular trans-

formation before analysis (Zar, 1996). Because multiple samples were taken from the same root systems, main effects of stain and species and the interactions between them were examined using a two-way ANOVA, employing a mixed within-subjects factorial design model (Keppell *et al.*, 1980). Subsequent separation of means was performed by the Tukey HSD test.

RESULTS

Although autofluorescence of hyphae and vesicles has been reported (Jabaji-Hare *et al.*, 1984), we saw little evidence of this, and consider that this method

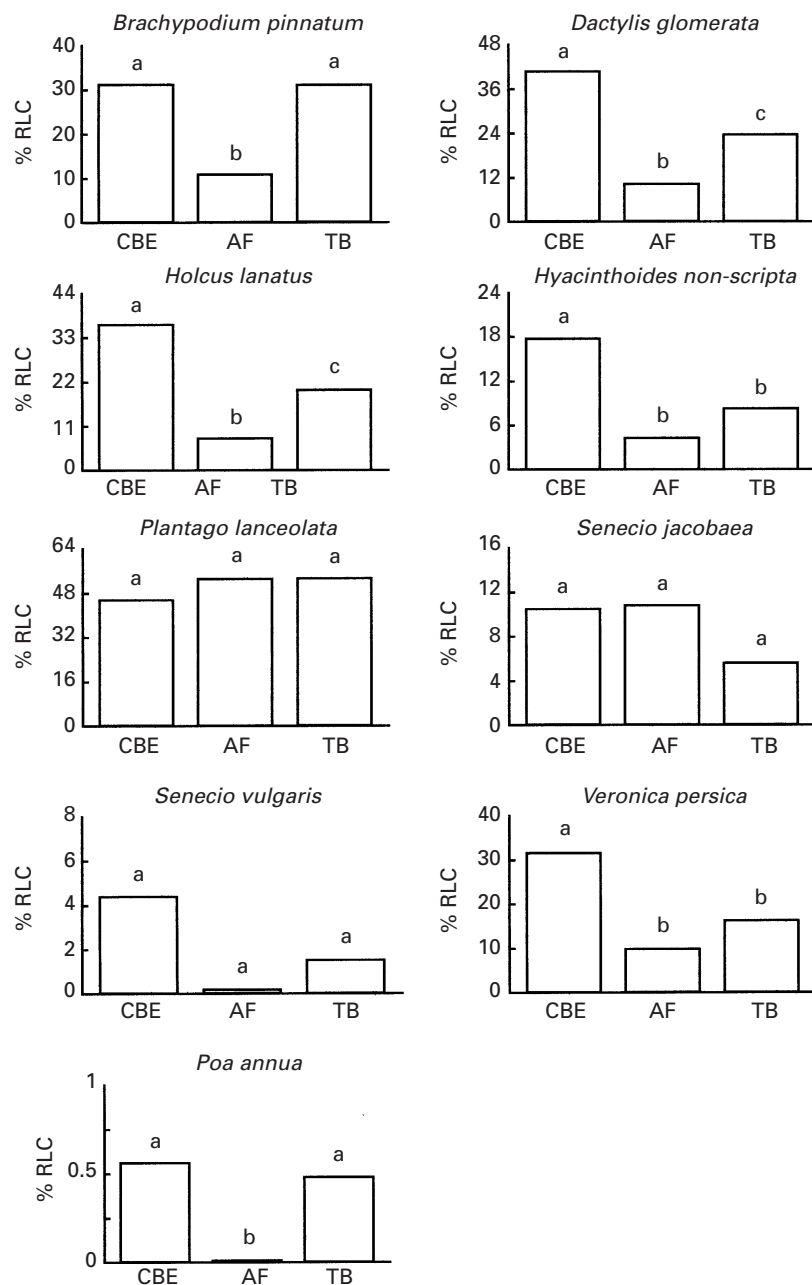


Figure 2. Levels of total arbuscular mycorrhizal colonization (arbuscules + vesicles + hyphae), measured as mean percentage of root length colonized (% RLC). Visualization methods: Chlorazol Black E (CBE); Acid Fuchsin (AF); Trypan Blue (TB); Autofluorescence (A). Different letters above columns indicate significant differences between means (Tukey test). Note: *Capsella bursa-pastoris* is omitted from this figure, as virtually no AM fungal material was detected in this species.

is capable of accurate recording only of arbuscules; hence measurements of other fungal parameters were not obtained by this method. For all the fungal parameters measured, there was a highly significant effect of visualization method (Table 3), indicating that the stains perform differently. The interactions between stain and plant species were also highly significant, indicating that the performance of the visualization method depended on the plant species examined.

The differences between the visualization methods become clear if arbuscule counts for each species are examined (Fig. 1). No arbuscules were seen in

Capsella bursa-pastoris and *Poa annua* roots using any of the methods; these species were omitted from the analysis. In six species, the number of arbuscules observed depended on the method used. The exceptions were *Plantago lanceolata*, (which produced a high count by all methods) and *Senecio vulgaris*. In the six species, autofluorescence consistently produced a higher arbuscule count than did the stains, especially in the grasses *Brachypodium pinnatum* and *Holcus lanatus*. In the latter species and *S. vulgaris*, AF failed to reveal arbuscules when the autofluorescence count was 23% (percentage of root length colonized, % RLC) in the former and

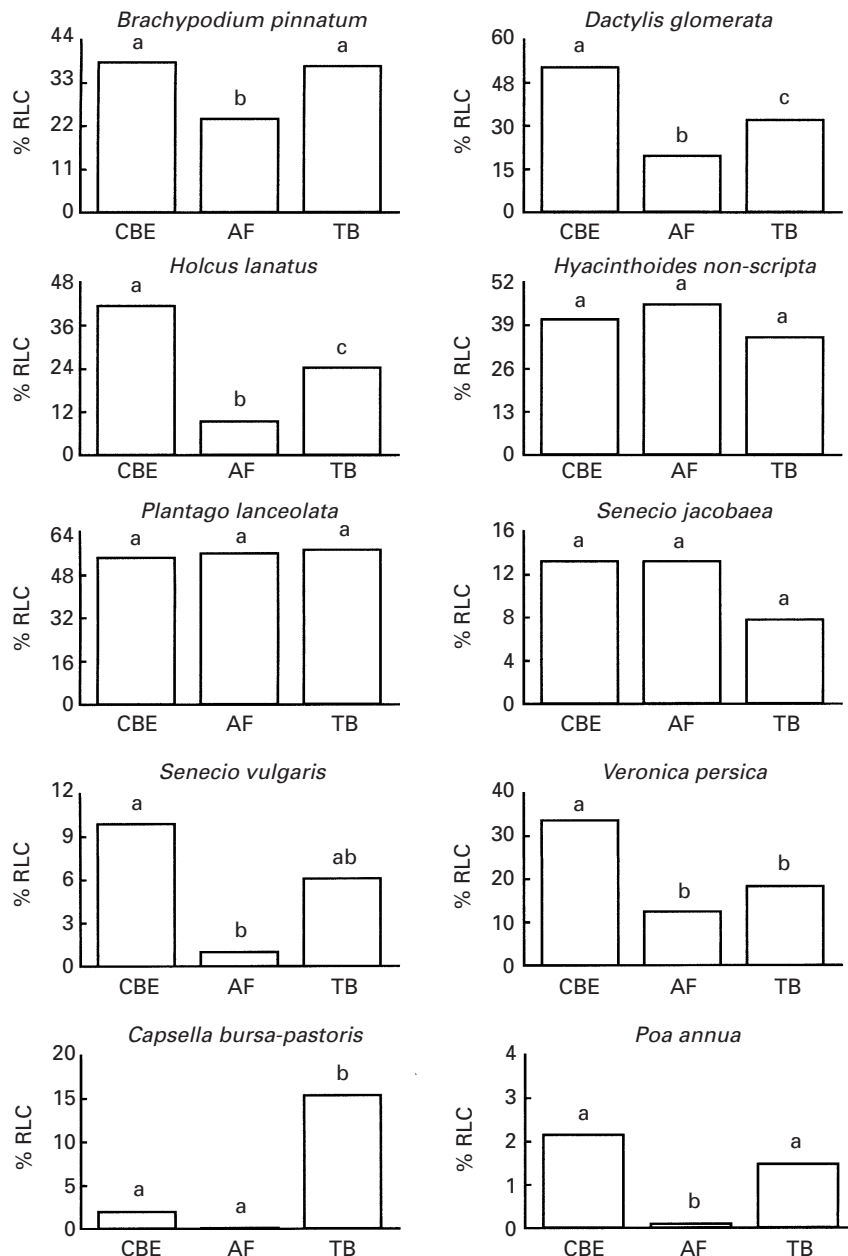


Figure 3. Levels of total fungal material (arbuscular mycorrhizal+all other fungi), measured as mean percentage of root length colonized (% RLC). Visualization methods: Chlorazol Black E (CBE); Acid Fuchsin (AF); Trypan Blue (TB); Autofluorescence (A). Different letters above columns indicate significant differences between means (Tukey test).

3% in the latter. In the annual *Veronica persica*, which is widely considered only weakly if at all mycorrhizal, the three stains resulted in low arbuscule counts, whereas autofluorescence revealed a level of over 14% RLC.

Fig. 2 depicts the total mycorrhizal colonization recorded by each stain, except for *C. bursa-pastoris*, in which counts were zero in all treatments apart from in two plants subjected to TB, where small amounts of aseptate hyphae were seen. In six species there was a significant difference between the counts produced by the three stains, but the pattern was not consistent, leading to the significant interaction term (Table 3). In all grasses, AF gave lower counts

than the other two stains. In the perennial forbs, only in *Hyacinthoides non-scripta* was a difference seen between stains, but in *H. non-scripta* and *Senecio jacobaea*, the total AM fungal count (arbuscules+vesicles+hyphae) revealed by any stain was lower than the arbuscule count alone recorded by autofluorescence (Fig. 1). Staining with CBE also revealed the most fungal material in the annual forbs.

Fig. 3 depicts the total fungal material recorded in the roots (i.e. mycorrhizal+non-mycorrhizal fungi). In seven of the 10 plant species there was a significant difference between the stains (Table 3). Once again, the pattern was not consistent, but CBE

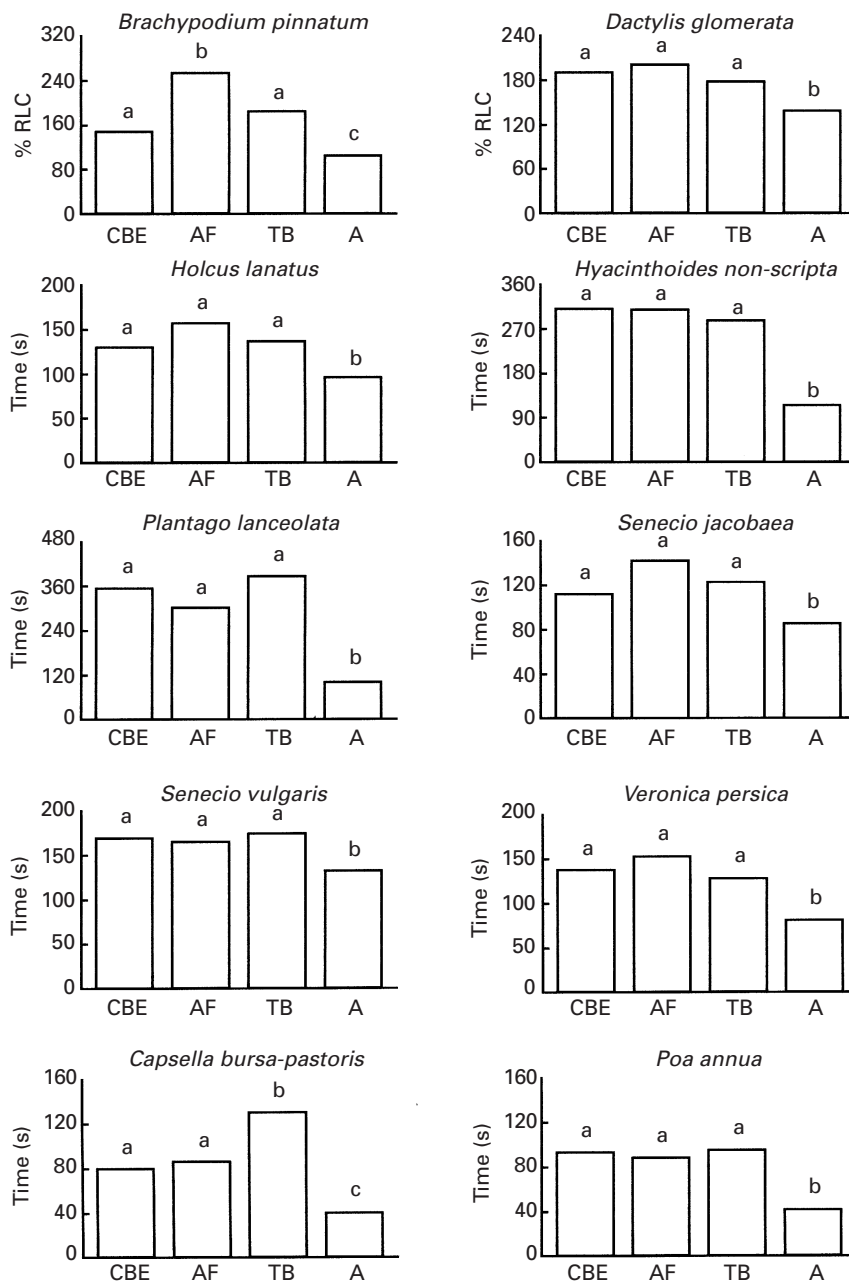


Figure 4. The recording time for arbuscules (time taken to assess 100 observations per slide) for each visualization method: Chlorazol Black E (CBE); Acid Fuchsin (AF); Trypan Blue (TB); Autofluorescence (A). Different letters above columns indicate significant differences between means (Tukey test). Note: although arbuscule counts for *Capsella bursa-pastoris* and *Poa annua* were zero, time taken to establish this is recorded.

generally revealed more fungal structures than did AF or TB. In *S. jacobaea*, the total fungal count was still lower than the arbuscule count achieved by using autofluorescence.

Some plant species clearly had much non-mycorrhizal or not definitely mycorrhizal fungi in the roots (e.g. *Dactylis glomerata* and *H. non-scripta*, shown by the difference in the y axes in Figs 2, 3). In contrast, the majority of the fungal material in *P. lanceolata* roots appears to have been mycorrhizal, as there was very little difference between the AM fungal and total fungal counts.

In all cases, time taken for recording using

autofluorescence was less than that for any of the stains (Fig. 4). This was particularly noticeable in those species for which it was necessary to decide whether fungal material was mycorrhizal or non-mycorrhizal (e.g. *H. non-scripta*). Autofluorescence was significantly quicker in *P. lanceolata* and *S. vulgaris*, which is important because the arbuscule counts obtained by the different methods did not differ in these species (Fig. 1). Furthermore, these data do not take into account the time required to clear and stain the roots (Table 2) of each species, which can be up to 2 h, whereas only 1 or 2 min was required to prepare roots for autofluorescence.

DISCUSSION

In the majority of the literature in our survey (85%), authors do not distinguish between recordings for arbuscules, vesicles and hyphae, but instead give a composite value for 'percentage of root length colonized by AM fungi'. The data depicted in Fig. 2 are therefore the typical form of presentation. It is clear from this figure that the extent of AM colonization recorded could depend on the stain being used. For example, for *H. lanatus*, each of the three stains results in a different % RLC, with CBE highest and AF lowest. By contrast, for a few species (e.g. *P. lanceolata*) all stains produce a similar result. The latter situation is the most desirable, but only occurred in three of the nine species colonized by AM fungi.

The differences in the ability of stains to reveal AM fungi is likely to be due to differential stain penetration of the roots of different plant species, and the extent to which the stain is taken up by the fungus (Morton, 1985). We attempted to optimize the procedure for each plant species, giving each stain as fair a chance as possible. However, the ability of a stain to penetrate a piece of plant tissue is affected by many factors, including stain type, the vehicle in which it is dissolved, the concentration and pH of the staining solution, the temperature and duration of staining and the thickness of the plant material (Hayat, 1993). In addition, the nature and location of the target material also has an effect, and it is possible that the depth at which arbuscules are located in the roots differs between plant species. This is because AM hyphae grow through intercellular air spaces, and suberin in the cell walls regulates the growth direction of the fungus (Brundrett *et al.*, 1996), two factors which could vary between plant species. To these criteria, we would add that the identity of both the plant and possibly the fungus is also important, because it appears that CBE, AF and TB have different staining abilities in different plant species. CBE is generally best, followed by TB and AF. This could be the reason for the clarity of preparations obtained with CBE being commented upon (Brundrett *et al.*, 1996), although Clapp *et al.* (1996) state that CBE often stains all fungal tissue intensely, giving very dark preparations. Therefore, before treatment of a hitherto unexamined plant species, a careful comparison of stain performance is necessary.

Data on mycorrhizal colonization obtained by staining are very variable (Smith & Read, 1997), particularly for field-collected specimens, and can result in data sets containing many zero or near-zero values which are so difficult to normalize by conventional transformations, that logistic regression has been proposed for their analysis (Alvarez Santiago *et al.*, 1996). It is therefore possible that the large numbers of plants in the field

recorded as non-mycorrhizal or with low colonization are not so at all; the data simply reflect the inability of the stain used to detect the fungus. We also found that stained preparations gave variable results, but that autofluorescence gave much more consistent data; the overall coefficients of variation for the data set on arbuscules were CBE, 86%, AF 149%, TB 115% and autofluorescence 46%. Autofluorescence therefore produced fewer of these low values, and data sets which were nearer to normal.

The inability to detect arbuscules with some stains was particularly acute in some plant species. For example, AF failed to reveal arbuscules in any of the 10 *H. lanatus* plants examined, while autofluorescence detected arbuscules in all the plants, with a mean of 23% RLC. TB-stained roots appeared to have only 4% RLC by arbuscules in *S. jacobaea*, while autofluorescence revealed 18% RLC (Fig. 1). It is impossible to believe that such differences were due to random variations in the root systems, as root pieces from each plant were well mixed before subsampling. We performed multiple paired comparisons in order to provide a realistic test of the different techniques, which should eliminate the possibility that variation from plant to plant could affect the outcome of the test. The conclusion therefore remains that in six of the 10 plant species studied, autofluorescence revealed a higher arbuscule count than did the stains, in direct contrast with the study accompanying the original description of mycorrhizal recording by autofluorescence (Ames *et al.*, 1982), in which no differences were found. The most likely explanation for this discrepancy is the differential ability of stains to penetrate the tissue of different plant species.

In our samples, there was much non-AM fungal material in the roots of some species, hampering identification of the AM fungus, and leading to a long recording time. An example is *H. non-scripta*, though this might not be a universal phenomenon, since in other sites this species has been found to have little non-AM fungus in the roots (J. W. Merryweather, pers. comm.). It is quite possible that differences in fungal colonization between sites can be another confounding variable. Autofluorescence did not appear to be hampered by the non-AM fungus in *H. non-scripta* leading to a relatively short recording time. Therefore, where roots might be colonized by many fungi, such as perennial plants, autofluorescence might be a quicker and more accurate means of mycorrhizal recording. Perhaps more importantly, autofluorescence was also quicker when there was much mycorrhizal fungal material, as in *P. lanceolata*, where all roots were heavily colonized and recording time was on average > 5 min, but just under 2 min with autofluorescence.

As the arbuscule is the only definitive structure produced by AM fungi, it seems rather odd that many workers persist in recording structures which

might not be mycorrhizal. In situations where known mycorrhizal inoculum is added to pot cultures and the danger of cross-contamination by other fungi eliminated, the recording of hyphae and vesicles cannot be criticized, but in situations where plants are collected from field soil, it is untenable. Even if authors identify mycorrhizal material correctly, without arbuscules there is no way of telling if the symbiotic relationship is functioning. Many workers have attempted to overcome the problem of establishing whether fungal material is alive or dead by using vital stains which detect different enzyme activity (e.g. Hamel *et al.*, 1990, Tisserant *et al.*, 1993). However, these techniques are still hampered by the necessity of use of toxic chemicals, poor penetration of stain and background staining of plant tissues. We originally planned to use the vital stain Nitro Blue Tetrazolium in the current study, but despite following recommended clearing procedures (Schaffer & Peterson, 1993) we could not produce clear preparations from which data could be obtained. These results have therefore been omitted from this paper.

We feel that the differential ability of stains to detect AM fungi calls into question much of the mycorrhizal literature. For example, non-mycorrhizal species might have been recorded as mycorrhizal because the fungal material in the roots is non-mycorrhizal. An example is *Lamium album* L., which was listed as forming AM associations by Harley & Harley (1987) and Grime *et al.* (1988); however in the examination under autofluorescence of dozens of plants of varying age from six different localities, we have yet to see an arbuscule. Meanwhile, many plants could be listed as non-mycorrhizal because the stain failed to detect the fungus. An example is *Stellaria media* (L.) Villars, listed as generally not forming an association by Harley & Harley (1987) and Grime *et al.* (1988), but in which we have detected arbuscule colonization of over 10% with autofluorescence. Finally, studies in which fungicide has been applied to field plots, reducing mycorrhizal colonization in some species but not in others (e.g. Gange *et al.*, 1990) could reflect differential staining, rather than failure of the experimental method (Morton, 1985).

It is not the intention of this paper to state that any one method is better than the others, but merely to highlight the fact that the different methods can produce different results. While autofluorescence might be better for arbuscule recording in some species, this technique also has its drawbacks. For example, the fact that other structures in a root, such as lignin-like compounds sometimes fluoresce, (Stockwell & Hanchey, 1987) or yellow pigments (Klingner *et al.*, 1995) might hamper the recognition of arbuscules. We noticed some such fluorescence in a few species (particularly *S. jacobaea*), but the characteristic 'texture' and position of arbuscules

meant that in no case did this interfere with arbuscule recording. Ames *et al.* (1982) comment that both live and dead arbuscules fluoresce, but conventional strains cannot differentiate them either. Jabaji-Hare *et al.* (1984) reported that arbuscules, vesicles and hyphae of an unidentified *Glomus* species (herb DAOM 181602) in leek roots fluoresced. If this were a universal phenomenon, measurements could easily be made of all parts of the mycorrhizal fungus. However, we rarely saw any fluorescence of structures other than arbuscules in our 10 species, although spores of *Glomus intraradices* can easily be seen in preparations from pot cultures (Bower, unpublished). Clearing with KOH might be required in the case of roots which are exceptionally dark, but this is likely to be very rare. Furthermore, if a plant is sampled when it is in the early or late stages of mycorrhizal colonization, arbuscules might not be present and hence the recording from autofluorescence would be zero. However, mycorrhizal material (e.g. vesicles and hyphae) would still be present, but not recorded. To an extent, one could argue that if arbuscules are not present the association is non-functional and therefore no problem is presented. However, other parameters such as entry points and hyphal coiling are also useful measurements and these would be missed. It is possible that the *C. bursa-pastoris* and *P. annua* plants in this study were either too young or too old, but we believe their very low AM levels were due to the nature of the site sampled, rather than the plants themselves. This is because all sampled plants were mature, but not senescent, and *P. annua* sampled at the same time in an adjacent locality had arbuscule counts with autofluorescence as high as 11% RLC (Gange, 1998).

Perhaps the best method is a combination of autofluorescence and staining, as described by Merryweather & Fitter (1991) and practised by Allen *et al.*, (1989). By this means, an accurate arbuscule count could be obtained, while still allowing determination of the presence of other mycorrhizal structures, such as entry points, intercellular hyphae and vesicles. We hope that our data have shown that conventional stains alone are not sufficiently reliable to allow comparisons between data sets, and that it is perhaps time for AM researchers to work towards adopting a common method of recording the fungi.

ACKNOWLEDGEMENTS

We are grateful to the Natural Environmental Research Council and the Nuffield Foundation for funding projects of which this study formed a part.

REFERENCES

- Allen MF, Allen EB, Friese CF. 1989. Responses of the non-mycotrophic plant *Salsola kali* to invasion by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **111**: 45–49.

- Alvarez Santiago SA, Garcia Oliva F, Varela L. 1996.** Analysis of vesicular–arbuscular mycorrhizal colonization data with a logistic regression model. *Mycorrhiza* **6**: 197–200.
- Ames RN, Ingham ER, Reid CPP. 1982.** Ultraviolet-induced autofluorescence of arbuscular mycorrhizal root infections: an alternative to clearing and staining methods for assessing infections. *Canadian Journal of Microbiology* **28**: 351–355.
- Brundrett MC, Bougher N, Dell B, Grove T, Malajczuk N. 1996.** *Working with mycorrhizas in forestry and agriculture*. Canberra, Australia: ACIAR Monograph 32.
- Brundrett MC, Piché Y, Peterson RL. 1984.** A new method for observing the morphology of vesicular–arbuscular mycorrhizae. *Canadian Journal of Botany* **62**: 2128–2134.
- Clapp JP, Fitter AH, Merryweather JW. 1996.** Arbuscular mycorrhizas. In: Hall, GS, ed. *Methods for the examination of organismal diversity in soils and sediments*. Wallingford, UK: CAB International, 146–161.
- Coombes RD, Haveland-Smith RB. 1982.** A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes. *Mutation Research* **98**: 101–248.
- Gange AC. 1998.** A potential microbiological method for the reduction of *Poa annua* L. in golf greens. *Journal of Turfgrass Science* **74**: 40–45.
- Gange AC, Brown VK, Farmer LM. 1990.** A test of mycorrhizal benefit in an early successional plant community. *New Phytologist* **115**: 85–91.
- Grace C, Stribley DP. 1991.** A safer procedure for routine staining of vesicular–arbuscular mycorrhizal fungi. *Mycological Research* **95**: 1160–1162.
- Grime JP, Hodgson JG, Hunt R. 1988.** *Comparative plant ecology*. London, UK: Unwin Hyman.
- Hamel C, Fyles H, Smith DL. 1990.** Measurement of development of endomycorrhizal mycelium using three vital stains. *New Phytologist* **115**: 297–302.
- Harley JL, Harley EL. 1987.** A check-list of mycorrhiza in the British flora. *New Phytologist (Suppl.)* **105**: 1–102.
- Hayat MA. 1993.** *Stains and cytochemical methods*. New York, USA: Plenum Press.
- Jabaji-Hare S, Perumalla CJ, Kendrick WB. 1984.** Autofluorescence of vesicles, arbuscules, and intercellular hyphae of a vesicular–arbuscular fungus in leek (*Allium porrum*) roots. *Canadian Journal of Botany* **62**: 2665–2669.
- Jennings DH, Lysek G. 1996.** *Fungal biology: understanding the fungal lifestyle*. Oxford, UK: BIOS Scientific Publishers.
- Keppell G, Saufley WH, Tokunaga H. 1980.** *Introduction to design and analysis*. New York, USA: Freeman & Co.
- Klingner A, Hundeshagen B, Kernebeck H, Bothe H. 1995.** Localization of the yellow pigment formed in roots of graminaceous plants colonized by arbuscular fungi. *Protoplasma* **185**: 50–57.
- Kormanik PP, McGraw A-C. 1982.** Quantification of vesicular–arbuscular mycorrhizae in plant roots. In: Schenk NC, ed. *Methods and principles of mycorrhizal research*. St Paul, MN, USA: American Phytopathological Society, 37–45.
- Koske RE, Gemma JN. 1989.** A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research* **92**: 486–505.
- McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. 1990.** A new method which gives an objective measure of colonization of roots by vesicular–arbuscular mycorrhizal fungi. *New Phytologist* **115**: 495–501.
- Merryweather JW, Fitter AH. 1991.** A modified method for elucidating the structure of the fungal partner in a vesicular–arbuscular mycorrhiza. *Mycological Research* **95**: 1435–1437.
- Morton JB. 1985.** Underestimation of Most Probable Numbers of vesicular arbuscular endophytes because of non-staining mycorrhizas. *Soil Biology and Biochemistry* **17**: 383–384.
- Phillips JM, Hayman DS. 1970.** Improved procedures for clearing and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**: 158–161.
- Schaffer GF, Peterson RL. 1993.** Modification to clearing methods used in combination with vital staining of roots colonized with vesicular–arbuscular mycorrhizal fungi. *Mycorrhiza* **4**: 29–35.
- Smith SE, Read DJ. 1997.** *Mycorrhizal symbiosis*. San Diego, CA, USA: Academic Press.
- Stockwell V, Hanchey P. 1987.** Lignification of lesion borders in *Rhizoctonia*-infected bean hypocotyls. *Phytopathology* **77**: 589–593.
- Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A. 1993.** *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. *Mycological Research* **97**: 245–250.
- Zar JH. 1996.** *Biostatistical analysis*. Upper Saddle River, USA: Prentice Hall Inc.