ACCESS MICROBIOLOGY
an open research platform

SHORT COMMUNICATION
Wilkes, *Access Microbiology* 2023;5:000618.v4
DOI 10.1099/acmi.0.000618.v4

MICROBIOLOGY SOCIETY
OPEN DATA OPEN ACCESS

Alternative inks for arbuscular mycorrhizal root staining

Thomas I. Wilkes*

Abstract:

Alternative methods for arbuscular mycorrhizal (AM) fungal colonized root staining have recently gained more attention for the reduction of hazard exposure to the user. Sheaffer blue ink has been employed for such an identification and quantification, having shown an increased degree of image clarity. However, sourcing Sheaffer blue ink is becoming problematic, leading to the need to find alternative inks that are readily available. Parker ink is a well-known brand, providing comparable colour options to Sheaffer. Two Parker inks, blue and washable blue, were employed alongside Sheaffer blue for comparative AM fungal colonized root staining. From quantified AM fungal vesicles and arbuscles, along with the degree of stained image clarity under microscopy, none of the inks utilized for this comparison produce a significantly (P=0.97) different AM fungal quantification or change in image clarity. Therefore, the results of the present communication suggest that Parker blue and washable blue inks are alternative ink stains for the viewing and quantification of AM fungi in host cortical root tissues.

DATA SUMMARY

All data pertaining to the present findings are contained within the manuscript.

INTRODUCTION

There have been many developments in host root staining for the identification and quantification of arbuscular mycorrhizal (AM) fungi [1–4]. Sheaffer blue ink has been shown to be capable of staining AM fungi, with structures easily identifiable [5]. However, the commercial availability of Sheaffer blue ink is becoming limited, with the ink difficult to source. Therefore, a need arises for the identification of a potential substitute ink that has a comparable ability to stain AM fungal structures for easy identification. Blue inks are typically better suited for AM fungal root staining protocols for the ease of identifying differences between structures as well as atypical structures that could easily be misidentified under other staining procedures [3, 4]. Therefore, the present short communication aims to identify a potential substitute for Sheaffer blue ink in AM fungal colonized plant root staining, further utilizing commercially available blue inks.

METHODS

Zulu variety winter wheat (*Triticum aestivum*) (n=15) was grown under controlled conditions for 4 weeks (15 °C, 37% relative humidity, 15 260 lux). Root staining was performed in accordance with Wilkes *et al.* [4] with Sheaffer blue ink substituted for Parker washable blue and Parker standard blue inks. Furthermore, the inclusion of formaldehyde in the plant fixative solution in Wilkes *et al.* [4] was not included in any solutions for the present samples.

Statistical analysis was performed using a single factor analysis of variance (ANOVA) and post-hoc t-testing utilizing R statistical software version 4.2.1. (Hamilton, ON, Canada).

RESULTS

Single-factor ANOVA was able to show no significant difference between any blue inks used [P=0.97, degrees of freedom (df): 104, 2, f value: 0.03, f critical: 3.09] for both arbuscular (Fig. 1) and vesicular counts (Fig. 2). Post-hoc t-testing was not required, as no further significance could be determined.

Received 04 May 2023; Accepted 15 August 2023; Published 31 August 2023
Author affiliations: [1]School of Water, Energy and Environment, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK.
***Correspondence:** Thomas I. Wilkes, tom.wilkes@cranfield.ac.uk
Keywords: arbuscular mycorrhizal fungi; ink staining; Parker ink; root tissue; Sheaffer ink.
Abbreviation: AM, arbuscular mycorrhizal.
000618.v4 © 2023 The Authors

This is an open-access article distributed under the terms of the Creative Commons Attribution License.

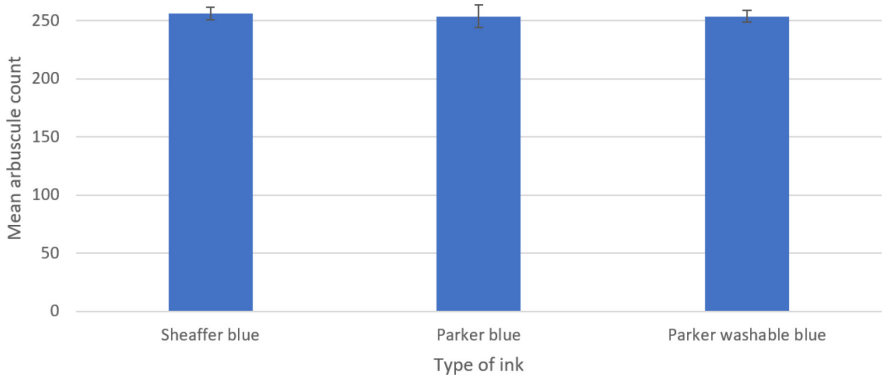

Fig. 1. Mean (*n*=105 overall) arbuscular count of stained Zulu variety wheat 1 cm root sections stained with three different blue inks. Error bars constructed from standard error of the mean (SEM).

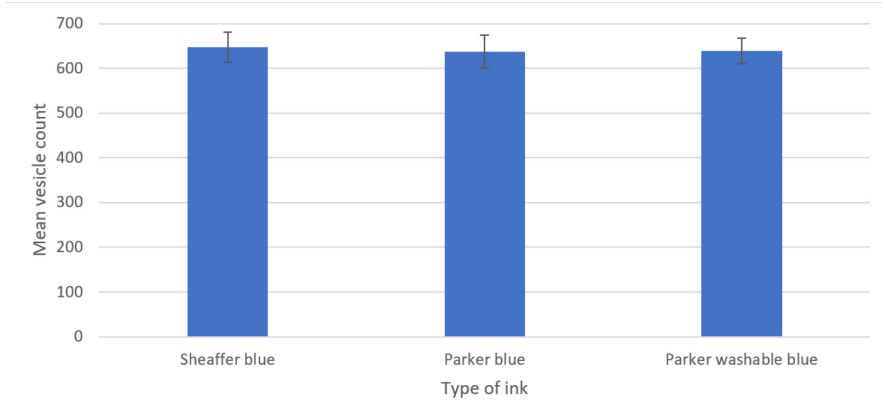

Fig. 2. Mean (*n*=105 overall) vesicle count of stained Zulu variety wheat 1 cm root sections stained with three different blue inks. Error bars constructed from SEM.

DISCUSSION

The present short communication has been able to provide indications that there is no discernible difference in AM fungal quantification between Sheaffer blue, Parker blue and Parker washable blue inks. Furthermore, as presented in Figs 3–5, there is little difference in the clarity of viewing and overall ability to quantify AM fungal root cortical structures between the three inks, allowing easy interpretation of stained tissues following the ink staining protocols developed by Hewitt *et al.* [6], Wilkes *et al.* [4] and Kowal *et al.* [5]. Yon *et al.* [7], however, did not follow the sample preparation protocols as described by Hewitt *et al.* [6], Wilkes *et al.* [4], or Kowal *et al.* [5], whilst using Parker blue and Parker washable blue inks. Micrograph images presented by Yon *et al.* [7] do not present discernible identified AM fungal structures. This is likely due to the drying of root tissues before staining, damaging the delicate AM fungal structures [4, 8]. As the present communication has been able to demonstrate, Parker blue and Parker washable blue inks are able to stain AM fungal structures. This highlights the importance of sample preparation. It is worth noting that the interpretation of Sheaffer blue-stained root tissues has been mistakenly assumed to be stained plant cell components [9]. As shown by Wilkes [8] and Wilkes and Warner [10], Sheaffer blue was not able to stain any cellular components in wheat samples grown under aseptic conditions, i.e. in the absence of AM fungi. This was further shown by micrographs presented by Kowal *et al.* [5].

CONCLUSION

From AM fungal structures quantified and the clarity of micrograph images, no difference between ink brands was detectable. Therefore, the present communication can conclude Parker blue and washable blue inks are equally effective for quantifying and viewing root cortical AM fungal structures for the assessment of AM fungal–host symbiosis.

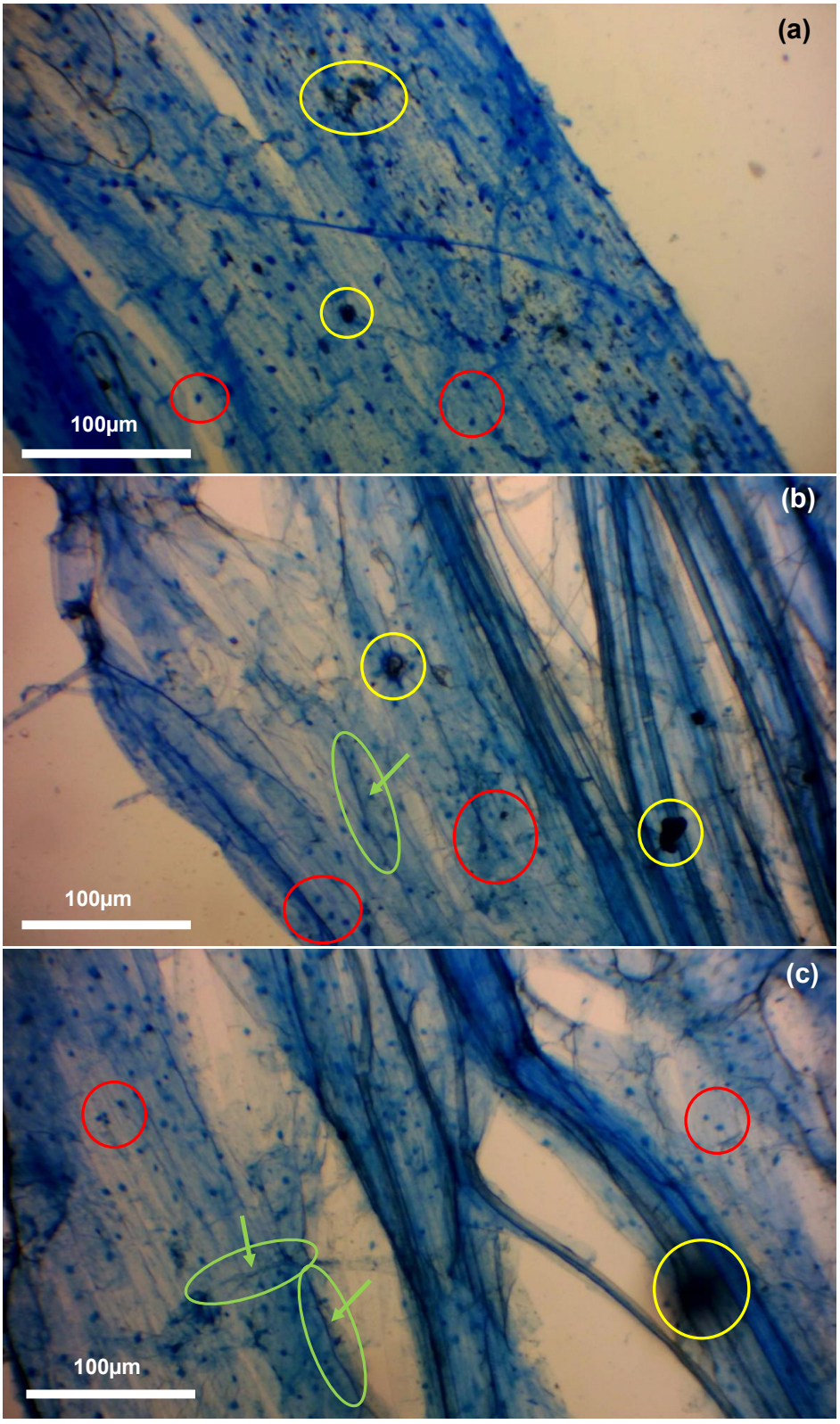

Fig. 3. Stained Zulu variety winter wheat with (a) Sheaffer blue, (b) Parker blue and (c) Parker washable blue ink at 40× magnification under an Apex microscope taken with a Bresser HD microscope camera. Yellow circle, debris; red circle, vesicles; green circle, intraradical hyphae.

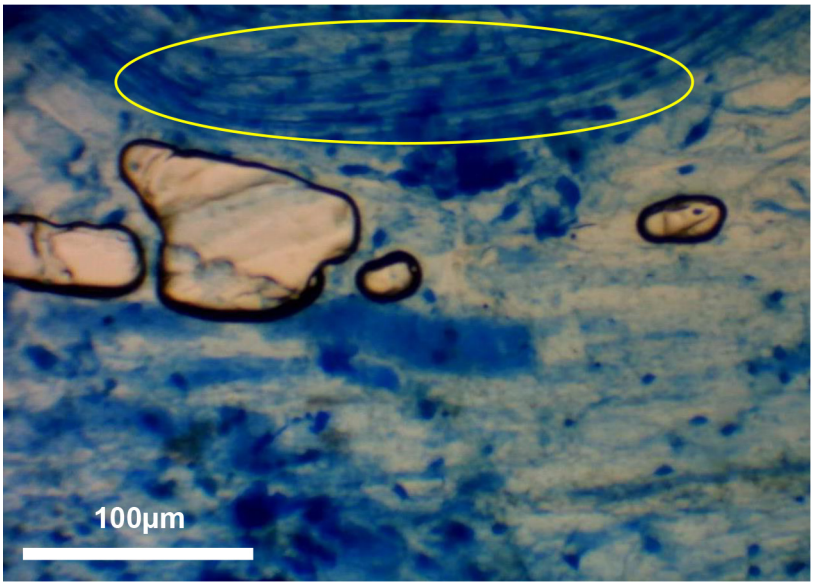

Fig. 4. Clusters of arbuscules (yellow circle) in Zulu variety wheat as seen under an Apex microscope at 40× magnification stained with Parker washable blue ink. Image taken using a Bresser HD microscope camera.

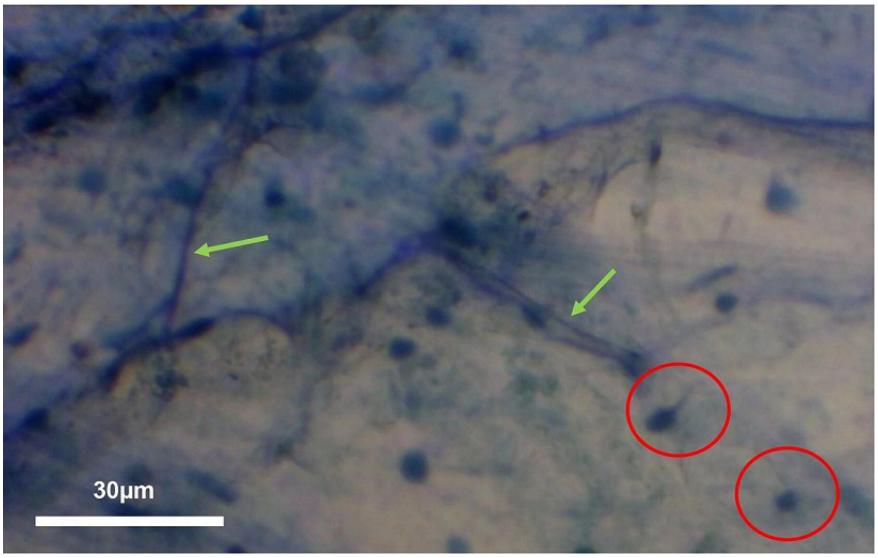

Fig. 5. Intraradical hyphae connected to a root cortical vesicle (red circle) and stained intraradical hyphae (green arrow) in Zulu variety wheat observed under an Apex microscope at 100× magnification using Parker blue ink. Imaged taken using a Bresser HD microscope camera.

Funding information
This work received no specific grant from any funding agency.

Author contributions
T.I.W., conceptualization, method development, data generation, data analysis, manuscript writing, manuscript editing.

Conflicts of interest
The author declares that there are no conflicts of interest.

References

1. **Kiheri H**, **Heinonsalo J**, **Timonen S**. Staining and microscopy of mycorrhizal fungal colonization in preserved ericoid plant roots. *JBR* 2017;7:231–237.
2. **Moukarzel R**, **Ridgway HJ**, **Guerin-Laguette A**, **Jones EE**. An improved clearing and staining protocol for evaluation of arbuscular

mycorrhizal colonisation in darkly pigmented woody roots. *NZPP* 2020;73:33–39.

3. Vierheilig H, Coughlan AP, Wyss U, Piche Y. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl Environ Microbiol* 1998;64:5004–5007.
4. Wilkes TI, Warner DJ, Edmonds-Brown V, Davies KG, Denholm I. A comparison of methodologies for the staining and quantification of intracellular components of arbuscular mycorrhizal fungi in the root cortex of two varieties of winter wheat. *Access Microbiol* 2020;2:acmi000083.
5. Kowal J, Arrigoni E, Serra J, Bidartondo M. Prevalence and phenology of fine root endophyte colonization across populations of *Lycopodiella inundata*. *Mycorrhiza* 2020;30:577–587.
6. Hewitt RE, Day NJ, DeVan MR, Taylor DL. Wildfire impacts on root-associated fungi and predicted plant–soil feedbacks in the boreal forest: research progress and recommendations. *Functional Ecology* 2023;37:2110–2125.
7. Yon Y, Perez L, Carmona A, Perez Y, Garcia L, *et al*. Alternative staining technique to determine mycorrhizal colonization. *Cultivos Tropicales* 2015;36.
8. Wilkes TI. *A Comparison of Tillage Techniques on Selected Soil Microbes within the Rhizosphere of Wheat. PhD Thesis*. UK: University of Hertfordshire; 2021.
9. Brundrett MC. Auditing data resolves systemic errors in databases and confirms mycorrhizal trait consistency for most genera and families of flowering plants. *Mycorrhiza* 2021;31:671–683.
10. Wilkes TI, Warner DJ. An evaluation for the medium-term storage and viability of root cortex tissues stained with blue ink in the assessment of arbuscular mycorrhizal fungi. *Access Microbiol* 2021;3:000292.

Five reasons to publish your next article with a Microbiology Society journal

1. When you submit to our journals, you are supporting Society activities for your community.
2. Experience a fair, transparent process and critical, constructive review.
3. If you are at a Publish and Read institution, you'll enjoy the benefits of Open Access across our journal portfolio.
4. Author feedback says our Editors are 'thorough and fair' and 'patient and caring'.
5. Increase your reach and impact and share your research more widely.

Find out more and submit your article at microbiologyresearch.org.

Peer review history

VERSION 3

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v3.1
© 2023 Bandara N. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Nihal Bandara; University of Bristol, Bristol Dental School, Lower Maudlin Street, UNITED KINGDOM, Bristol

Date report received: 15 August 2023
Recommendation: Accept

Comments: This is a study that would be of interest to the field and community. The authors have addressed reviewers' comments sufficiently.

Author response to reviewers to Version 2

Reviewers' comments and responses to custom questions:

Reviewer 1:

Please rate the manuscript for methodological rigour

Reviewer 1: Satisfactory

Please rate the quality of the presentation and structure of the manuscript

Reviewer 1: Satisfactory

To what extent are the conclusions supported by the data?

Reviewer 1: Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 1: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 1: Yes:

Reviewer 1 Comments to Author: I thank the reviewer for their responses, but my main concern regarding the citation of previous work was denied. In my previous review, pointed out the importance of citing similar work which had used the same dye for the similar purpose of staining the fungi in the plant roots. In fact, Yon et al (2015) used Parker QuinK blue ,washable in water, and this work used Parker washable blue and Parker standard blue inks. Proper citations will make readers aware of the similar work and the increase the credibility of the current work.

There are many errors in the work by Yon et al. that prevent the staining and identification of AM fungi – mainly the total lack of any AM fungal structures in their micrograph images. Just because you inoculate roots with AM fungi, doesn't necessarily mean you'll be able to identify their symbiosis. What they are showing is highly unlikely to be AM fungi. However, a brief statement has been added to the present manuscript.

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v2.3
© 2023 Bandara N. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Nihal Bandara; University of Bristol, Bristol Dental School, Lower Maudlin Street, UNITED KINGDOM, Bristol

Date report received: 08 August 2023
Recommendation: Minor Amendment

Comments: The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v2.1
© 2023 Santana M. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Marcos Santana; Universidade Federal do Oeste do Para, Instituto de Ciências e Tecnologia das Águas, Laboratório de Fisiologia Vegetal e Crescimento de Plantas, BRAZIL
https://orcid.org/0000-0002-0421-3420

Date report received: 06 August 2023
Recommendation: Accept

Comments: 1. Methodological rigour, reproducibility and availability of underlying data Satisfactory 2. Presentation of results Satisfactory 3. How the style and organization of the paper communicates and represents key findings Satisfactory 4. Literature analysis or discussion Satisfactory 5. Any other relevant comments

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v2.2
© 2023 Bandara C. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Chaturanga Bandara; University of Bristol, Dental School, Bristol, UNITED KINGDOM

https://orcid.org/0000-0002-0688-4260

Date report received: 02 August 2023
Recommendation: Minor Amendment

Comments: I thank the reviewer for their responses, but my main concern regarding the citation of previous work was denied. In my previous review, pointed out the importance of citing similar work which had used the same dye for the similar purpose of staining the fungi in the plant roots. In fact, Yon et al (2015) used Parker QuinK blue ,washable in water, and this work used Parker washable blue and Parker standard blue inks. Proper citations will make readers aware of the similar work and the increase the credibility of the current work.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Author response to reviewers to Version 1

Reviewers' comments and responses to custom questions:

Reviewer 1:

Please rate the manuscript for methodological rigour

Reviewer 1: Poor

Please rate the quality of the presentation and structure of the manuscript

Reviewer 1: Good

To what extent are the conclusions supported by the data?

Reviewer 1: Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 1: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 1: Yes:

Reviewer 1 Comments to Author: In this short communication, the author tests suitability of Parker washable blue and Parker Blue as alternatives to stain AM in winter wheat roots due to the scarcity of Sheaffer blue in the market.

Thank you for taking the time to review the short communication. Please see responses below.

The research objectives are clearly stated in the introduction and has justified the underlying reasons for the research. However, the citations are not representing the broad literature available and very similar previous work is not cited. For example, use of Parker Blue dye to stain MA by Yon et al in 2015. Therefore highly recommend to include similar work done previously in this topic.

Yon etal, DOI: 10.13140/RG.2.2.10232.65287

Direct comparison would be very difficult with the suggested article as the staining preparation procedure is different. The acidity of the stain solution is greater in your mentioned paper, this will have an impact in the incorporation of stain into root and fungal tissues. Also, without knowing the exact chemical constituents of the inks, it is difficult to comment on any degree of thermal stability of the inks between employed methods.

Research methodology is not concisely presented to reproduce the experiment. For example, important factors like ink pre-preparation conditions, the staining time is not mentioned. Without this information, this method cannot be reproduced. It is also important to state if the inks are used as received or any dilution or filtration is carried out prior to staining procedure.

The procedure is already reported and published in the cited reference. Due to journal requirements, and potentially higher similarity scores, repetition of the method was removed with only modifications remaining in the short communication. The details you request are in the referenced article.

Though the statistics of counts are presented, microscopic image acquisition parameters, nor the number of images analysed are not revealed in the methods section.

These are provided in the referenced article and n numbers.

For the analysis of dye performance, and comparison between different dyes, intensity of the signal is a valid parameter rather reporting the number of features identified, which is adapted in this experiment. Instead, recommend to include signal intensity which will provide a qualitative measurement, therefore, dye performance can be correctly quantified across samples.

This is not entirely correct. As several references have shown, comparisons between stains for AM fungi are described in relation to stained AM fungal structures quantified. Signal intensity would be a valid parameter when considering different colours of stain. However, the present communication only considers blue inks.

It would also be interesting to discuss reports on toxicity of the inks being used (if available) in this study.

This was searched for. However, as these inks are still actively produced, their constituent compounds are not disclosed, making it difficult to describe any toxicity effects.

Reviewer 2:

Please rate the manuscript for methodological rigour

Reviewer 2: Good

Please rate the quality of the presentation and structure of the manuscript

Reviewer 2: Good

To what extent are the conclusions supported by the data?

Reviewer 2: Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 2: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 2: Yes:

Reviewer 2 Comments to Author: In Title

Suggestion: In the title, please do not use "root staining for arbuscular mycorrhizal". The short communication is interesting and can be a differential access material for teaching activities at higher levels, young scientists at the beginning of their careers, in addition to other functions in science, however the way it is understood suggests that fungi have roots, which does not occurs. It is convention among mycologists to try their best not to associate botanical structures with fungi. Please always use "roots colonized by MA fungi". Do this for all text.

Thank you for taking the time to review the short communication. Please see responses below.

In Abstract

Review previous comment and apply to lines 13;19-20.

The p for significance, on line 22, is lower case.

Amended

In Introduction

Revise previous comment and apply to line 43.

Amended

1. Methodological rigour, reproducibility and availability of underlying data

While using the wheat variety for the text (line 47), please provide the name of the wheat species to increase the replicability of the work.

Amended

Please cite the authorship of the R software package used for this analysis.

Amended

In Results

Please, put the p of significance, on line 58, in lower case. Same thing for the F value and F critical.

Amended

In References

Please add https://doi.org/on line 102.

Some newspaper names are abbreviated and others are not. Please check this in all references.

Amended

2. Any other relevant comments

The coloring really worked, but the photos I had access to in the file for review didn't turn out well. If possible, please replace with better quality images.

I've had a look at the images again. Downloading them from Editorial Manager has seemed to reduce their quality. The original file format is in high definition (1080p). I'll reupload the images again with the resubmission.

The circles used to present the structures of the AM fungi are too thick, added to the colors, they are points of distraction. It would be interesting to make them less evident by drawing attention to the structures, which are the targets. These structures were barely visible, possibly due to the quality of the image. If you could check that too, that would be great.

Circle have been reduced in thickness and resized to narrow down the focal point in the image.

It would be interesting to standardize the position of the scales in the images.

Amended. Figure 4 had to be rotated to move the scale bar, however, it is still the same image.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v1.5
© 2023 Bandara N. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Nihal Bandara; University of Bristol, Bristol Dental School, Lower Maudlin Street, UNITED KINGDOM, Bristol

Date report received: 14 July 2023
Recommendation: Minor Amendment

Comments: The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v1.3
© 2023 Santana M. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Marcos Santana; Universidade Federal do Oeste do Para, Instituto de Ciências e Tecnologia das Águas, Laboratório de Fisiologia Vegetal e Crescimento de Plantas, BRAZIL
https://orcid.org/0000-0002-0421-3420

Date report received: 13 July 2023
Recommendation: Minor Amendment

Comments: In Title Suggestion: In the title, please do not use "root staining for arbuscular mycorrhizal". The short communication is interesting and can be a differential access material for teaching activities at higher levels, young scientists at the beginning of their careers, in addition to other functions in science, however the way it is understood suggests that fungi have roots, which does not occurs. It is convention among mycologists to try their best not to associate botanical structures with fungi. Please always use "roots colonized by MA fungi". Do this for all text. In Abstract Review previous comment and apply to lines 13;19-20. The p for significance, on line 22, is lower case. In Introduction Revise previous comment and apply to line 43. 1. Methodological rigour, reproducibility and availability of underlying data While using the wheat variety for the text (line 47), please provide the name of the wheat species to increase the replicability of the work. Please cite the authorship of the R software package used for this analysis. In Results Please, put the p of significance, on line 58, in lower case. Same thing for the F value and F critical. In References Please add https://doi.org/ on line 102. Some newspaper names are abbreviated and others are not. Please check this in all references. 2. Any other relevant comments The coloring really worked, but the photos I had access to in the file for review didn't turn out well. If possible, please replace with better quality images. The circles used to present the structures of the AM fungi are too thick, added to the colors, they are points of distraction. It would be interesting to make them less evident by drawing attention to the structures, which are the targets. These structures were barely visible, possibly due to the quality of the image. If you could check that too, that would be great. It would be interesting to standardize the position of the scales in the images.

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000618.v1.4
© 2023 Bandara C. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License.

Chaturanga Bandara; University of Bristol, Dental School, Bristol, UNITED KINGDOM
https://orcid.org/0000-0002-0688-4260

Date report received: 11 July 2023
Recommendation: Minor Amendment

Comments: In this short communication, the author tests suitability of Parker washable blue and Parker Blue as alternatives to stain AM in winter wheat roots due to the scarcity of Sheaffer blue in the market. The research objectives are clearly stated in the introduction and has justified the underlying reasons for the research. However, the citations are not representing the broad literature available and very similar previous work is not cited. For example, use of Parker Blue dye to stain MA by Yon et al in 2015. Therefore highly recommend to include similar work done previously in this topic. Yon etal, DOI: 10.13140/RG.2.2.10232.65287 Research methodology is not concisely presented to reproduce the experiment. For example, important factors like ink pre-preparation conditions, the staining time is not mentioned. Without this information, this method cannot be reproduced. It is also important to state if the inks are used as received or any dilution or filtration is carried out prior to staining procedure. Though the statistics of counts are presented, microscopic image acquisition parameters, nor the number of images analysed are not revealed in the methods section. For the analysis of dye performance, and comparison between different dyes, intensity of the signal is a valid parameter rather reporting the number of features identified, which is adapted in this experiment. Instead, recommend to include signal intensity which will provide a qualitative measurement, therefore, dye performance can be correctly quantified across samples. It would also be interesting to discuss reports on toxicity of the inks being used (if available) in this study.

Please rate the manuscript for methodological rigour
Poor

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000618.v1.1
© 2023 The Authors. This is an open-access article report distributed under the terms of the Creative Commons License.

iThenticate report

https://doi.org/10.1099/acmi.0.000618.v1.2
© 2023 The Authors. This is an open-access article report distributed under the terms of the Creative Commons License.