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ADAPTED MOLECULAR METHODS TO UNRAVEL THE RECALCITRANT MYCORRHIZAL ASSOCIATIONS OF *AUCOUMEA KLAINEANA* PIERRE

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Abstract

Understanding the role of root microbiota is crucial in sustainable forest management but remains challenging, especially for tropical trees. We developed an efficient and low-toxicity method to extract and amplify the fungal DNA associated with *Aucoumea klaineana* Pierre fine roots. To improve DNA quality, we optimized a commercial extraction kit by incorporating activated charcoal and modifying incubation periods. This enhanced protocol, combined with bovine serum albumin during PCR, effectively mitigated inhibitors present in *A. klaineana* tree root samples. This approach opens new perspectives for studying the microbiota of tropical trees.

The conservation of tropical forests is a crucial issue (Hansen et al., 2013). The role of soil and root microbiota, a major component of these ecosystems, is still poorly understood (Díaz-Vallejo et al., 2021). Mycorrhizal fungi, that form symbiosis with plant roots, play an important role in water and nutrient mobilization and plant protection against pathogens (Smith and Read, 2008). However, information on the mycorrhizal status of tropical trees is often missing, especially for African forests, while understanding their interactions could facilitate better forest management. Methods to characterize the mycorrhizal associations have evolved in the past few years, thanks to the use of Next Generation Sequencing (NGS) on root samples, but still rely on a high-quality DNA and amplification success to target fungal barcodes. The extraction of target DNA, here the fungal DNA associated with roots and mycorrhizae, remains challenging (Peršoh, 2015). The use of commercial kit is strongly advised, but the yield might be reduced, and only few options, for example using cetyltrimethylammonium bromide (CTAB), are mentioned in the literature to improve extractions and target microbial DNA (Simmons et al., 2018). Moreover, tropical tree roots, rhizoplane, rhizosphere and surrounding soil often contain various secondary metabolites such as alkaloids, flavonoids, phenols, terpenoids, lignans, etc. (Becerra, 2015; Richards et al., 2015) that inhibit DNA extractions and PCR reactions. This is particularly true for trees belonging to the Burseraceae family, which secrete substances that contain many inhibiting molecules

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(De Nicolai and Rodrigues, 2022). In this context and considering the frequent lack of fungal amplification on tropical roots, adapted molecular protocols are needed.

Through a research project on okoumé (*Aucoumea klaineana* Pierre, Burseraceae) mycorrhizal associations, we tested and improved our molecular methods. Endemic to the western part of Central Africa, *A. klaineana* is the most harvested and economically important timber species in the region (Guidosse et al., 2022). A single study has reported the occurrence but not abundance of Glomeraceae, which are arbuscular mycorrhizal fungi, on *A. klaineana* roots through staining and microscopic observations (Onguene et al., 2002). However, NGS studies never targeted its root associations before.

To test our protocols, rootlets were collected from 60 random trees across three *A. klaineana* plantations located within a radius of two kilometers in the Luki Biosphere Reserve, Democratic Republic of Congo. The two first monospecific 48 years old plantations were very similar with *A. klaineana* measuring on average 26.1 ± 12.5 cm in diameter and 21.5 ± 7.8 m in height. The last monodominant plantation was 75 years old with *A. klaineana* reaching on average 35.5 ± 14.3 cm in diameter and 28.2 ± 7.3 m in height. For each tree, three different roots were tracked and excavated from the stem to the rootlets (< 2 mm diameter) as shown in Guidosse et al. (2022). Rootlets from each tree were collected, rinsed using a wash bottle and a 500 μM sieve, and gathered to form an about 20 g sample. They were stored in a porous paper bag and then in a sealable bag filled with silica beads for drying. In the lab, samples were gently brushed to remove soil particles and shredded with liquid nitrogen, mortar and pestle. The sixty processed samples were randomly picked for each extraction protocol (see Supplementary Materials 1).

To improve the quality of the DNA extract, we first used the widely used DNeasy® Plant Mini Kit by Qiagen (Hilden, Germany) for DNA extraction as commonly used in temperate regions for root-associated fungi research and by Born et al. (2006) on *A. klaineana* fresh leaves. We compared it to the CTAB method (Doyle and Doyle, 1987) and briefly tested other "classic" methods such as sodium dodecyl sulfate + polyvinylpyrrolidone (SDS + PVP) (Demeke and Jenkins, 2010) and phenol-chloroform protocols. However, none yielded satisfactory results, consistently producing low yields and poor-quality DNA (see below). Second, we optimized the Qiagen extraction kit protocol and improved it by testing the effect of polyvinylpolypyrrolidone (PVPP), a common choice for removing polyphenolic compounds, and of activated charcoal (AC), known to be less expensive and less toxic. We tested protocols to remove the remaining inhibitors using bovine serum albumin (BSA) for the amplification step. During the PCR thermal cycles, the BSA could stabilize the already degraded DNA. Detailed information on the reagents used is available in Supplementary Material 2.

The best DNA extractions consisted of a modified Qiagen kit protocol, including AC, as recommended by Vroh et al. (1996). Root powder (15–20 mg) was poured in a 2 ml tube with 15–20 mg of AC before adding SDS (AP1 buffer). Other protocol modifications were: (i) a period of incubation of 30 min instead of 10 min after adding SDS (AP1 buffer), (ii) incubation period on ice of 15 min instead of 5 min after adding acetic acid (P3 buffer) and (iii) pre-heating the AE buffer to 65 °C before the elution step. Final incubation was also extended to 15 mins. Then, DNA quantity and quality were assessed with a Quantus™ Fluorometer (Promega, Madison, WI, USA) and a NanoDrop™ 2000 (Thermo fisher, Waltham, MA, USA). The addition of AC and modification of the kit protocol not only increased the quantity of DNA extracted compared to other methods (Fig. 1, A; Kruskal-Wallis test (Kruskal and Wallis, 1952), $\chi^2 = 27.283$, $p = 1.2*10^{-6}$), but also significantly improved its purity (Fig. 1, B-C) according to the 260/280 nm ratio (Kruskal-Wallis test, χ^2

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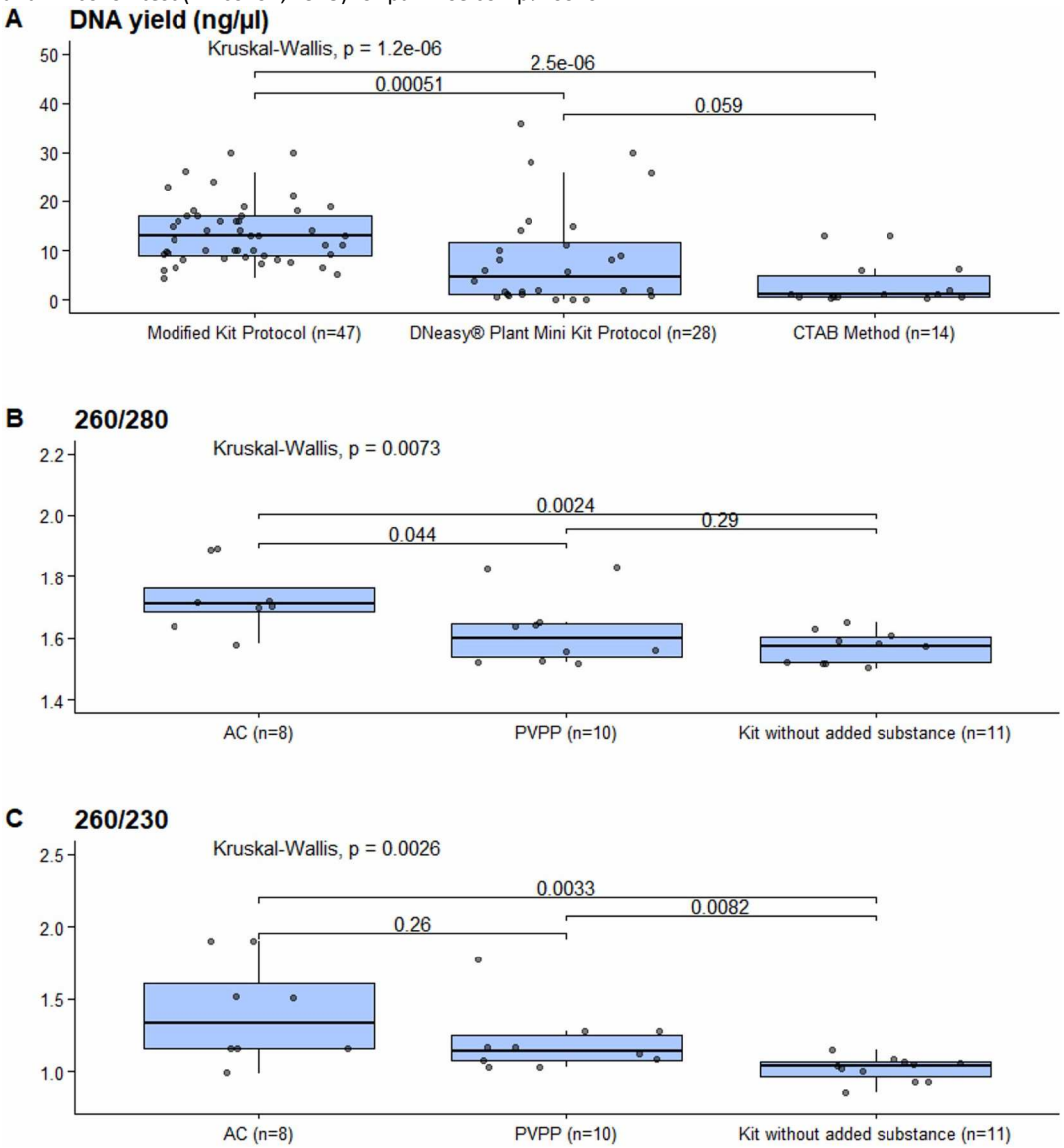
= 9.846, p = 0.0073) and to the 260/230 ratio (Kruskal-Wallis test, χ^2= 11.900, p = 0.0026). When improving the kit protocol, we also compared the addition of AC versus PVPP. AC is slightly more efficient than the PVPP regarding the 260/280 nm ratio (Wilcoxon test (Wilcoxon, 1945), p = 0.044), but not regarding 260/230 nm purity. Finally, the 260/230 nm ratio shows no significant improvement when adding PVPP only compared to the standard kit protocol.

Fig. 1. Comparison of DNA extraction protocols. (A) DNA yield (ng/µl) according to extraction protocols. (B) Average 260/280 nm purity ratios obtained by Nanodrop per treatment in terms of proteins, phenols and other organic compounds. DNA extracts are considered as pure between c.a. 1.8 to 2.0. (C) Average 260/ 230 nm purity ratios obtained by Nanodrop per treatment such as salts, phenols and other organic compounds. AC: Activated Charcoal; PVPP: polyvinylpolypyrrolidone. DNA purification Kit: DNeasy® Plant Mini Kit (Cat. No. 69106) (Qiagen, Hilden, Germany) without additional reagent. Significant differences are tested using the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) and Wilcoxon test (Wilcoxon, 1945) for pairwise comparisons.



To test the amplification of fungal DNA, the entire ITS rDNA region was first targeted by the popular primers pair ITS1 and ITS4 (White et al., 1990; Gardes and Bruns, 1993; Porter and Golding, 2011). Once the inhibition was not apparent anymore due to the addition of 2% (*w*/*v*) BSA (Fig. 2A), the protocol was repeated to test its efficacity on a larger number of samples and on short fragments dedicated to MiSeq sequencing (Hu et al., 2021), using more specific

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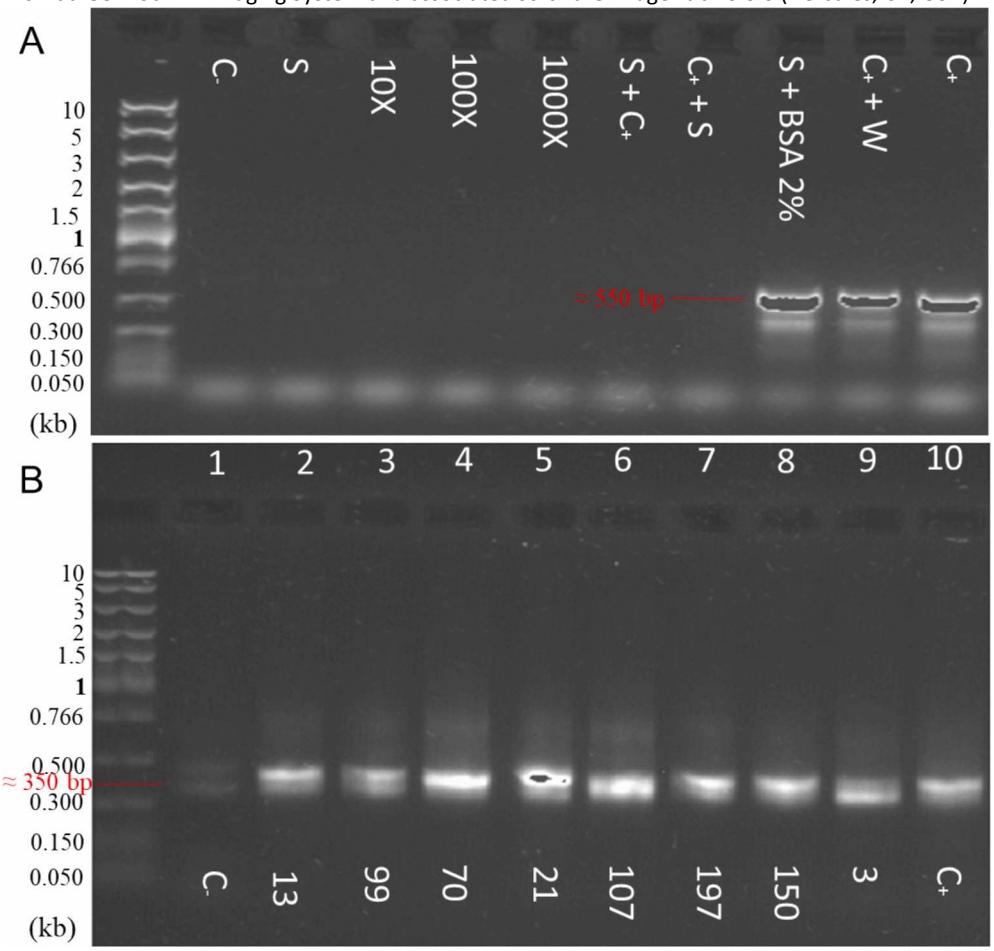
primer pair, ITS86F and ITS4 (Vancov and Keen, 2009; Waud et al., 2014) targeting only the ITS2 region (Fig. 2B). This excludes the ITS1 but also the 5.8S rRNA gene that can cause chimera formation (Haas et al., 2011).

Fig. 2. Agarose gel electrophoresis (1.5% w/v agarose) of PCR amplified products performed for 50 min at 15 V/cm on 6 cm. DNA Fast Ladder (NEB, Ipswitch MA, USA) was used as the molecular marker. (A) inhibition of amplification of the entire ITS region (ITS1-ITS4) due to secondary metabolites present in the DNA sample (S), even after dilutions (10-1000×). The inhibitors also affect the amplification of the positive control (S + C+; C+$_+$ S). When BSA 2% (*w*/*v*) is added to the DNA sample (S + BSA 2% w/v), the inhibition was not apparent. For two substances in a single well (marked with a "$_+$"), the first substance is in 3 µl and the second in 2 µl. W is PCR-grade water (Suppl. Mat. 2) free of nucleases and free of nucleic acid contamination. (B) shows amplification of the ITS2 region (ITS86F-ITS4) for multiple samples to which 2 µl of BSA 2% (*w*/*v*) was added. Samples IDs and controls are indicated vertically on bottom of the gel. Lanes are numbered from 1 to 10. Images were acquired with a Bio-Rad Gel Doc XR+ Imaging System and associated software Image Lab v6.0.0 (Hercules, CA, USA).



PCR amplifications were performed on a Bio-Rad T100 thermal cycler (Hercules, CA, USA) by mixing 1 µl of DNA template, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 2 µl of BSA 2% (*w*/*v*), 12.5 µl of Q5 High-Fidelity 2× Master Mix (NEB, Ipswitch, MA, USA) and completed with 8.5 µl PCR-grade water to reach a 25 µl volume. We used the following amplification program: denaturation step 5 mins. at 95 °C, 35 cycles of 30 s. at 95 °C, 30 s. at 55 °C, 20 s. at 72 °C and a final extension phase of 10 mins. at 72 °C. As controls, PCR-grade water (Suppl. Mat. 2) free of nucleases and of nucleic acid contamination was used as negative and a DNA strain of *Aspergillus* sp. was also extracted using the same protocol as for the samples, then amplified following the same conditions to serve as a positive.

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Inhibitors were present in our DNA extracts, as adding a root sample DNA mixed with positive control (C_+, *Aspergillus* sp.*)* hindered the amplification of the positive control, regardless of the proportions of the mixture (3/2 µl or 2/3 µl) (Fig. 2A). When 2% (w/v) BSA was added to the various DNA extracts from *A. klaineana* roots, all samples were amplified, indicating the presence of root-associated fungi (Fig. 2B). We also tested this protocol to detect arbuscular mycorrhizal fungi using the more specific primer pair NS31 and AML2 (Morgan and Egerton Warburton, 2017) targeting the nuclear SSU rRNA gene. This yielded amplification of the gene, confirming at least the presence of arbuscular mycorrhizal fungi associated with *A. klaineana* roots.

Our relatively simple and innovative method enabled the extraction and amplification of DNA from *A. klaineana* root-associated fungi, the production of high-quality DNA for NGS studies. It should now be tested on other recalcitrant tropical tree roots, even though they are slightly lignified and highly pigmented. By using activated charcoal (AC) and bovine serum albumin (BSA), we have successfully addressed the challenges associated with inhibitors and proposed a cost-effective and low-toxicity solution to conventional approaches. It is particularly suitable for tropical species and research on soil biodiversity in developing countries.

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CRediT authorship contribution statement

Quentin Guidosse: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Mélanie Roy:** Methodology, Writing – review & editing. **Ludivine Lassois:** Conceptualization, Supervision. **Jean-Louis Doucet:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Project administration.

Declaration of Generative AI and AI-assisted technologies in the writing process

Occasional utilization of ChatGPT-3.5 to enhance the clarity of English sentence structures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.mimet.2024.107000.

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