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Short Communication

Are colorimetric assays appropriate for measuring phenol oxidase activity in peat soils?

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ABSTRACT

The activity of extracellular phenol oxidases is believed to play a critical role in decomposition processes in peatlands. The water logged, acidic conditions, and recalcitrant litter from the peatland vegetation, lead to exceptionally high phenolics in the peat. In order to quantify the activity of oxidative enzymes involved in the modification and break down of phenolic compounds two types of assays are primarily utilized: L-DOPA and ABTS. This note focuses on the strengths and weaknesses of both approaches. Both assays involve a redox reaction and the resulting oxidized chromophore is measured spectrophotometrically. However, in the presence of reducing agents such as the phenolics commonly found in peat the colorimetric reaction is reversed and cannot be used to quantify phenol oxidase activity.

This note is motivated by the question, do we have sufficiently good assays to draw major conclusions about phenol oxidase activity in peatlands? Extracellular phenol oxidases (enzymes that oxidize phenols and consume oxygen; as defined by Sinsabaugh (2010)) are believed to play a key role for decomposition processes in peat (Fenner and Freeman, 2011; Freeman et al., 2001) yet methods for measuring their activity in peatlands have weaknesses, not all of which have been clearly articulated. Recent reviews on assays used for measuring extracellular oxidative enzyme activity list a long array of possible interferences and shortcomings of the most common approaches (Bach et al., 2013; German et al., 2011; Sinsabaugh, 2010). Phenol oxidases have low substrate specificity and a very wide range of reactions that they can catalyze (e.g. Baldrian, 2006; Thurston, 1994; Zaidi et al., 2014), which adds to the challenges in search for a specific substrate for measuring oxidative enzyme activity in soils and peat. The following note focuses on: whether either the substrate 2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) or the commonly used 3,4-Dihydroxy-L-phenylalanine (L-DOPA) assay can be used as a method to quantify phenol oxidase activity in acid peatlands.

There are several reasons why ABTS should be better suited than L-DOPA to assay phenol oxidases in peat soils. First, the ABTS assay operates at a pH that is representative of acid peatlands. German et al. (2011) recommend an assay pH representing field conditions for *in situ* enzyme activity studies, which suggests that ABTS, with a pH optimum around 4 (Bach et al., 2013), is a better choice than the frequently used L-DOPA assay, with a pH optimum ranging between 8.0 and 9.6 (Pind et al., 1994). Also, the activity of laccases (EC 1.10.3.2) is inhibited as pH increases (Eichlerova et al., 2012) because of an inhibitory effect of hydroxyl ions on the enzyme's T2/T3 center (Xu, 1997). Yet, numerous studies using L-DOPA report increased phenol oxidase activity with increased pH (e.g. Pind et al., 1994; Sinsabaugh et al., 2008; Williams et al., 2000), which is likely due to the fact that the redox potential of the substrate itself declines with increasing pH (Bach et al., 2013) and potential auto-oxidation of L-DOPA increases (Tahvanainen and Haraguchi,

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2013). In contrast, the redox potential of ABTS does not vary with pH (Xu, 1997). Second, (Eichlerova et al., 2012) report ABTS exhibits 40 times more sensitivity to laccase activity than the L-DOPA assay, despite its comparatively higher redox potential (Bourbonnais et al., 1998). Third, the produced chromophore $ABTS^{\bullet+}$, the preferred cation radical (Xu, 1997), was reported to be chemically very stable over time (Cano et al., 1998) when the reaction system reaches a steady state, whereas the L-DOPA chromophore produced by oxidation is not stable over time (German et al., 2011). Lastly, ABTS is not thought to be oxidized by abiotic constituents of soil, which allows for adequate controls, whereas no such controls exist for the L-DOPA assay (Bach et al., 2013).

Hence, ABTS would appear to be the ideal substrate to measure extracellular oxidative enzyme activity in peat, were it not for one flaw that it shares with other commonly used substrates. The assay involves a redox reaction and the oxidized chromophore is subject to the reverse chemical reaction. Thus in the presence of reducing agents the colorimetric reaction is reversed, and therefore under such conditions it is impossible to quantify the absolute amount of the enzymatically oxidized chromophore. Humic and fulvic acids as well as some phenolics themselves are strong enough electron donors to reduce the oxidized chromophore $ABTS^{\bullet+}$ back into its colorless, reduced state ABTS (Collins et al., 1998; Eichlerova et al., 2012; Terron et al., 2004). In fact the oxidized ABTS chromophore ($ABTS^{\bullet+}$) is frequently used as reagent to measure the antioxidant activity of carotenoids, phenolics, and some plasma antioxidants in fruit juices (e.g. Arnao et al., 1999; Cano et al., 1998; Re et al., 1999). We had concerns when testing this assay in peatland soils in the PEATcosm experiment (Potvin et al., 2015), because some of our preliminary results were indicating negative oxidation. To test the reversibility of the reaction in peat, we oxidized ABTS (2.5 mM) to $ABTS^{\bullet+}$ using refined laccase from *Coriolus versicolor* (Sigma 38837), then purified it using ultrafiltration as described in Floch et al. 2007. Under stirred, oxidizing conditions we added the $ABTS^{\bullet+}$ to naturally reduced pore water samples taken at 60–70 cm depth from *Sphagnum* peat. To 60 ml of this pore water we added 35 ml of a 2.5 mM $ABTS^{\bullet+}$ solution and stirred the mixture gently under oxidizing conditions. All of the added $ABTS^{\bullet+}$ solution was completely and rapidly reduced, leaving no stable color in solution, while increasing the redox potential. The peat pore water started at a redox potential (Eh7) of 265 mV, and the addition of the $ABTS^{\bullet+}$ oxidized the solution to about 550 mV for a short while, but the reducing compounds in the pore water continued to react with the $ABTS^{\bullet+}$, and within 5 min of the addition, the solution redox potential had dropped below 505 mV (Eh7), after which no $ABTS^{\bullet+}$ color was visible. This clearly illustrates a principal difficulty with the approach of using $ABTS^{\bullet+}$, or indeed any other compound which is reversibly redox-active, as an indicator of the presence of oxidizing enzymes in a reducing environment, or even in the presence of reduced phenolics in an oxidizing environment (cf. Farnet et al., 2009). While not all peat or pore water is this reducing, it is clear that the presence of reduced organic molecules in the peat could lead to artifacts (cf., Klüpfel et al., 2014). These organics are a prominent component of any organic soil and are found in particularly high concentrations in peat. An additional weakness of ABTS is that it has been commonly used as a mediator of phenoloxidase (mainly laccase) oxidation. Therefore it is not an adequate substrate since the product of the oxidative reaction, quantified to assess enzyme activity, interacts with other molecules (Calcaterra et al., 2008; Baiocco et al., 2003).

Therefore, given the weaknesses of both approaches, neither can be recommended for work in acid peatlands unless these artifacts can be controlled for. To make comparisons of extracellular oxidative enzyme activity among ecosystems it is crucial to use an appropriate assay that has a pH invariant redox potential, yields stable oxidation products, and permits control for abiotic interferences. It would certainly be worth evaluating other assays for phenol oxidase activity, e.g., those reviewed in Sinsabaugh (2010), to determine if any might perform better in peat. Furthermore, alternate sample processing techniques, including titrating to an Eh endpoint (Bauer et al., 2007), or reducing phenolic quenching via addition of sequestering agents such as PVP(P) (e.g. McMurrough et al., 1995; Kleiner et al., 1999) might overcome some of the issues of reversibility. The complexity and variability of the group of extracellular enzymes capable of oxidizing phenols (Baldrian, 2006) leads to an ongoing challenge for inventing and testing new approaches. Perhaps with emerging molecular techniques such as metagenomics, metatranscriptomic and metaproteomic methods (Burns et al., 2013) we will be able to achieve a more accurate understanding of the complexity of extracellular enzymes in peatland soils (e.g. Baldrian and Stursova, 2011; Talbot et al., 2015). Of course these methods come with their own challenges (Keiblinger et al., 2016), and will only complement and not replace enzyme assays.

In the meantime our results support a change in terminology from “potential oxidative enzyme activity” to “oxidative potential.” It has been previously suggested that instead of referring to extracellular oxidative enzyme activity in soils measured by those assays, we are more likely characterizing the oxidative potential of the soils, meaning the oxidative potential of the entire soil solution instead of specifically referring to enzyme activity only (Bach et al., 2013; Sinsabaugh, 2010), which we consider to be a crucial distinction.

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