

## Standard Paper

# Quinone reductase activity is widespread in lichens

Calvin Eddington Moyo<sup>1</sup> , Farida Minibayeva<sup>2</sup> , Christiane Liers<sup>3</sup> and Richard Peter Beckett<sup>1,4</sup> 

<sup>1</sup>School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa; <sup>2</sup>Kazan Institute of Biochemistry and Biophysics, Federal Research Center 'Kazan Scientific Center of RAS', PO Box 261 Kazan 420111, Russia; <sup>3</sup>Unit of Environmental Biotechnology, Dresden University of Technology, International Institute Zittau, Markt 23, 02763 Zittau, Germany and <sup>4</sup>Open Lab 'Biomarker', Kazan (Volga Region) Federal University, Kremlevskaya str. 18, Kazan 420008, Russia

## Abstract

In our earlier work, we demonstrated that the oxidases tyrosinase (TYR), laccase (LAC), and a heme peroxidase (POX) occur widely in lichens. Here we report on the occurrence of another oxidoreductase enzyme, quinone reductase (QR) (EC 1.6.5.5). While QR has been reported to occur widely in other organisms, there is currently no information on QR activities in lichens. Here we present a survey of QR activity in 14 species of lichens. Results demonstrate that QR activity is readily detectable in all lichen species tested. However, activities vary greatly, with 'jelly' lichens in the genera *Collema* and *Leptogium* having the highest activities. QR, LAC and POX are all believed to have a role in extracellular hydroxyl radical production. However, in this study no correlation was found between the activities of these enzymes and the rates at which hydroxyl radicals were produced. Possible roles for QR in lichen biology are discussed.

**Key words:** detoxification, extracellular redox cycling, hydroxyl radical, survey

(Accepted 15 March 2021)

## Introduction

Recent studies have demonstrated that lichens contain a wide variety of hydrolases and oxidoreductase enzymes such as cellulases, tyrosinases (TYR), laccase (LAC), and heme peroxidases (POX) (Beckett *et al.* 2013). While these enzymes probably play a great variety of lichen biology roles, in free-living fungi their main function is to participate in soil lignocellulose transformations (Andlar *et al.* 2018). These transformations, particularly for refractive residues, require the production of reactive oxygen species (ROS), with perhaps the most important being the hydroxyl radical (Bissaro *et al.* 2018). We recently showed that given a quinone and chelated ferric ions, many lichens can readily produce hydroxyl radicals (Moyo *et al.* 2017). While the precise mechanism of hydroxyl radical formation remains unclear, it appears to involve a form of extracellular quinone cycling. It has been proposed that chelated Fe<sup>3+</sup> reacts with hydroquinones producing Fe<sup>2+</sup> and semiquinone radicals. These radicals then spontaneously form quinones, reducing O<sub>2</sub> to ·OOH in the process; the ·OOH radical dismutates to H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup> react together to give hydroxyl radicals, while hydroquinones are regenerated from the quinones using a reductase on the surface of the hyphae (Arantes & Goodell 2014). The whole process has been called 'hydroquinone-redox cycling', and an essential aspect of this mechanism is that potentially harmful hydroxyl radical production can occur at

a distance from the fungal hyphae. In white-rot fungi, evidence has been presented that enzymes such as laccases and peroxidases assist in the conversion of hydroquinones to quinone radicals (Gómez-Toribio *et al.* 2009a, b). The combined presence of cellulases, redox enzymes and extracellular redox cycling may enable lichens to obtain carbon from lignocellulosic residues in their substratum, supplementing carbon obtained by photosynthesis, and thus facilitating a partly saprotrophic existence (Beckett *et al.* 2015).

A requirement in all models of extracellular redox in free-living fungi is that hydroquinones need to be regenerated from quinones by the enzyme quinone reductase (QR, EC 1.6.5.5) (Martinez *et al.* 2009). QR is a widely distributed flavin adenine dinucleotide (FAD)-containing enzyme that can reduce a range of quinones (e.g. menadione) but also azo dyes and nitro groups. In addition to a role in extracellular redox cycling, it has been suggested that QR could protect cells by reducing quinones to less harmful hydroquinones, reducing the risk of oxidative stress (Rao *et al.* 1992), and more generally play a role in stress tolerance (Cohen *et al.* 2004). However, the occurrence and properties of QR in lichens have not yet been studied.

The aim of the present study was to determine QR activity in a range of lichen species and test factors that might affect quinone reductase activity, such as the presence of exogenous quinones and desiccation. To elucidate the role of QR and other redox enzymes in hydroxyl radical formation, QR, LAC and POX activities were measured in all species surveyed and correlated with hydroxyl radical production rates. Results suggest that while high QR is common in many lichens, no simple correlation exists between the rates of hydroxyl radical production and QR, LAC or POX activities.

**Author for correspondence:** Richard Peter Beckett. Email: [rpbeckett@gmail.com](mailto:rpbeckett@gmail.com)

**Cite this article:** Moyo CE, Minibayeva F, Liers C and Beckett RP (2021) Quinone reductase activity is widespread in lichens. *Lichenologist* 53, 265–269. <https://doi.org/10.1017/S0024282921000190>

**Table 1.** Collection localities, quinone reductase (QR) activity, rates of hydroxyl radical production and the activities of laccase (LAC), and heme peroxidase (POX) activity in a range of lichen species. Values are given  $\pm 1$  SD,  $n=4$ . ND = Not determined.

Species	Collection locality	QR activity (units $g^{-1}$ dry mass)	Hydroxyl radical production ( $\mu\text{mol } g^{-1}$ dry mass $h^{-1}$ )	LAC activity (units $g^{-1}$ dry mass)	POX activity (units $g^{-1}$ dry mass)
<i>Cetrelia cetrarioides</i> (Delise ex Duby) W.L. Culb. & C.F. Culb.	Fort Nottingham, RSA	2.27 $\pm$ 0.21	0.34 $\pm$ 0.07	0.86 $\pm$ 0.07	3.09 $\pm$ 0.32
<i>Collema lactuca</i> (Weber) F.H. Wigg.	Fort Nottingham, RSA	73.87 $\pm$ 3.95	0.04 $\pm$ 0.01	4.38 $\pm$ 0.75	18.46 $\pm$ 3.32
<i>Crocodia aurata</i> (Ach.) Link	Monks Cowl, RSA	4.57 $\pm$ 0.51	0.60 $\pm$ 0.23	3.58 $\pm$ 0.31	2.89 $\pm$ 0.22
<i>Heterodermia leucomela</i> (L.) Poelt	Fort Nottingham, RSA	4.68 $\pm$ 0.54	0.54 $\pm$ 0.22	ND	ND
<i>H. speciosa</i> (Wulfen) Trevis.	Monks Cowl, RSA	5.83 $\pm$ 0.90	0.90 $\pm$ 0.23	0.12 $\pm$ 0.09	4.23 $\pm$ 1.65
<i>Lasallia pustulata</i> (L.) M�erat	Rock, outskirts of Colmar, France	0.60 $\pm$ 0.07	0.42 $\pm$ 0.04	0.2 $\pm$ 0.02	0.02 $\pm$ 0.01
<i>Leptogium furfuraceum</i> (Harm.) Sierk	Fort Nottingham, RSA	39.22 $\pm$ 4.13	0.71 $\pm$ 0.04	0.1 $\pm$ 0.02	4.3 $\pm$ 0.52
<i>Lobaria retigera</i> (Bory) Trevis.	Monks Cowl, RSA	8.70 $\pm$ 0.57	0.23 $\pm$ 0.19	1.01 $\pm$ 0.21	2.09 $\pm$ 0.16
<i>Nephroma helveticum</i> Ach.	Monks Cowl, RSA	8.53 $\pm$ 0.19	0.19 $\pm$ 0.02	0.82 $\pm$ 0.34	2.14 $\pm$ 0.12
<i>Peltigera membranacea</i> (Ach.) Nyl.	Outskirts of Kazan, Russia	21.10 $\pm$ 0.51	0.01 $\pm$ 0.00	8.7 $\pm$ 0.38	0.00 $\pm$ 0.00
<i>Ramalina celastri</i> (Spreng.) Krog. & Swinsc.	Fort Nottingham, RSA	6.64 $\pm$ 0.35	0.86 $\pm$ 0.12	0.54 $\pm$ 0.04	0.74 $\pm$ 0.12
<i>Rocella fuciformis</i> (L.) DC.	Beachwood Nature Reserve, Durban, RSA	4.77 $\pm$ 0.20	0.37 $\pm$ 0.04	0.12 $\pm$ 0.02	0.05 $\pm$ 0.01
<i>Sticta limbata</i> (Sm.) Ach.	Fort Nottingham, RSA	5.73 $\pm$ 0.56	0.76 $\pm$ 0.18	0.76 $\pm$ 0.25	0.01 $\pm$ 0.00
<i>Usnea undulata</i> Stirton	Fort Nottingham, RSA	7.95 $\pm$ 0.59	0.76 $\pm$ 0.07	3.24 $\pm$ 0.84	1.67 $\pm$ 0.54

## Materials and Methods

### Plant material

Collection localities for the 14 lichen species tested are given in Table 1. In addition to the species used in the survey, some preliminary experiments to determine the pH optima of QR were carried out on *Peltigera didactyla* collected from the outskirts of Zittau, Germany. If collected wet, material was air dried between layers of filter paper. All material was kept refrigerated for a maximum of four weeks before use. Unless the effect of desiccation on enzyme activity was being studied, before each experiment air-dried material was weighed into replicates, and then rehydrated for 24 h on wet filter paper at 13 °C and a light intensity of 30  $\mu\text{mol } m^{-2} s^{-1}$ . A small correction for the difference between air-dry and oven-dry weight was applied when expressing enzyme activities on a dry mass basis.

### Measurement of enzyme activities

The activities of QR, LAC and POX were measured using three biological replicates, each comprising the equivalent of *c.* 0.5 g dry mass. Material was ground in 10 ml of 50 mM phosphate buffer pH 7, centrifuged at 5000 g for 20 min, and the supernatant stored at  $-24$  °C until required. All chemicals were purchased from Sigma-Aldrich (St Louis, USA). LAC activity was estimated by following the oxidation of 0.3 mM 2, 2-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS) ( $\epsilon_{420} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$ ) in 50 mM sodium acetate buffer pH 4.5 (Eggert *et al.* 1996). POX activity was estimated as the stimulation of the rate of ABTS oxidation following the addition of 0.1 mM  $\text{H}_2\text{O}_2$  (Liers *et al.* 2011). QR activity was assayed by following the rate of oxidation of 62.5  $\mu\text{M}$  NADH ( $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ) in a mixture of 20  $\mu\text{M}$  flavin mononucleotide (FMN) and 30  $\mu\text{M}$  2,

6-dimethoxy-1, 4-benzoquinone (DMBQ) in phosphate buffer pH 7 (Bongard *et al.* 2011). This pH was chosen based on initial experiments using repeat analytical replicates from a large volume of crude extract derived from *c.* 10 g dry mass of *Peltigera didactyla*. Results showed that the activity of QR from the lichen has a broad pH optimum, with activity changing little from pH 5 to 8 (Fig. 1), similar to QR activity in free-living fungi (Brock *et al.* 1995; Brock & Gold 1996).

### Measurement of hydroxyl radical formation

Hydroxyl radical formation was estimated by measuring the oxidation of deoxyribose (Moyo *et al.* 2017). Briefly, typically four replicates comprising the equivalent of 0.2 g dry mass were gently shaken in 20 ml of 20 mM phosphate buffer pH 5 containing 0.5 mM 6-dimethoxy-1, 4-benzoquinone (DMBQ), 0.1 mM  $\text{FeCl}_3$ , 0.6 mM oxalic acid and 2.8 mM deoxyribose. Samples (990  $\mu\text{l}$ ) were taken at the start, and after 1, 2, 3 and 4 h, 10  $\mu\text{l}$  50%  $\text{H}_3\text{PO}_4$  was added as a stop solution and then the samples were frozen. Later, samples were thawed and 50  $\mu\text{l}$  was mixed with 250  $\mu\text{l}$  of 2.5% trichloroacetic acid and 250  $\mu\text{l}$  of 1% thiobarbituric acid in 50 mM NaOH, and the volume made up to 1 ml. Samples were heated in water at 90 °C for 10 min. Blanks were solutions lacking lichen material but otherwise treated in the same way. Readings were converted to MDA equivalents ( $\epsilon_{532} = 0.156 \mu\text{M } \text{cm}^{-1}$ ; Devasagayam *et al.* 2003), and rates of hydroxyl radical production estimated by linear regression over 4 h.

### Effect of desiccation and hydration on QR activity

To determine the effect of hydration and desiccation on QR activity in *Leptogium furfuraceum*, lichens were subjected to

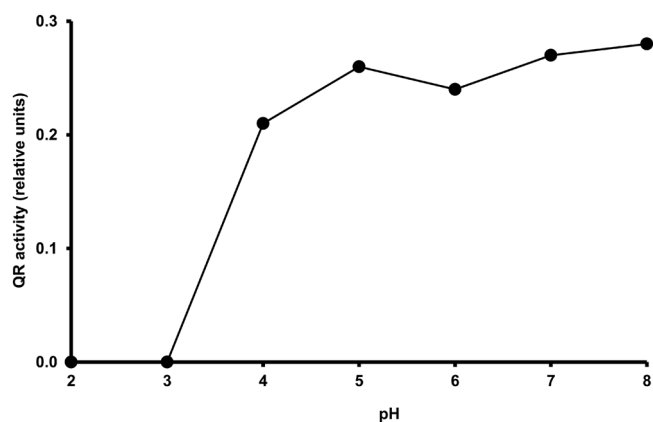


Fig. 1. Effect of pH on quinone reductase (QR) activity in *Peltigera didactyla*. Error bars are not included as they were smaller than the symbols,  $n=4$ .

continuous hydration and desiccation treatments. For QR activity, each replicate comprised 1 g dry mass of the lichen material, and there were three biological replicates in each treatment. For measurement of changes in relative water content (RWC), four replicates of *c.* 200 mg dry mass were used, and to determine turgid weights, material was incubated in distilled water for 1 h and weighed just before the start of the experiment as recommended by Beckett (1995). In the continuous hydration treatment, dry material was placed hydrated on wet filter paper in a growth cabinet at 13 °C and a light intensity of  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Samples were measured for RWC and QR activity after 0, 1, 6, 24, 72 and 120 h. In the desiccation treatment, the material was first hydrated for 24 h and then placed in Petri dishes and suspended above a saturated solution of  $\text{CaCl}_2$  in a desiccator, which was placed in the growth cabinet. At 13 °C, the  $\text{CaCl}_2$  solution gave a relative humidity of *c.* 35% and allowed for a reasonably slow drying (Fig. 2A). Samples were taken for RWC and QR activity measurements after 0, 12, 24, 48, 72 and 120 h. At each point RWC was estimated in replicate samples of material as (fresh mass – dry mass) / (turgid mass – dry mass), with dry mass measured after drying replicate material for 48 h at 80 °C.

### Effect of DMBQ on QR activity

Approximately 0.5 g (three biological replicates) of fresh mass of *Leptogium furfuraceum* was gently shaken in flasks containing 0, 0.12, 1.2 mM and 12 mM DMBQ for 1 h, then placed on wet filter paper at 13 °C,  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 24 h. QR activity was then assayed as above.

### Data analysis

Correlations between the parameters measured were calculated using SPSS version 25.

## Results and Discussion

### QR activity and correlation with other parameters

All lichens tested possessed at least some QR activity, although activity varied significantly between species (Table 1). The highest activity occurred in the 'jelly' lichens *Collema lactuca* and *Leptogium furfuraceum*, with 74 and 39 units  $\text{g}^{-1}$  dry mass respectively. By contrast, the lowest activity was found in

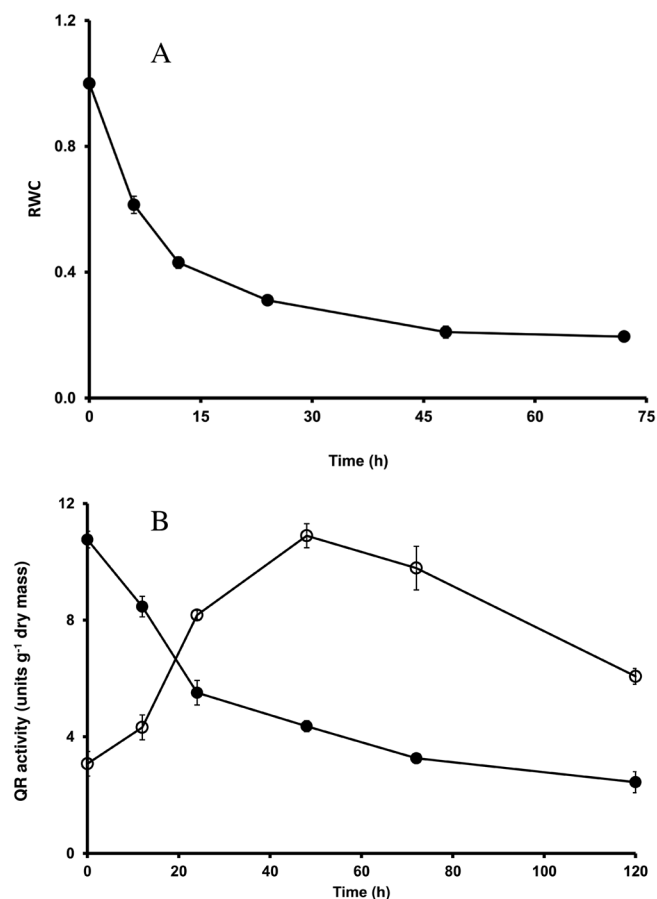


Fig. 2. A, effect of desiccation over a saturated solution of  $\text{CaCl}_2$  (corresponding to a relative humidity of 35%) on the relative water content (RWC) of *Leptogium furfuraceum*. B, effect of hydration from the desiccated state (open circles), and slow desiccation (closed circles, above saturated  $\text{CaCl}_2$ ) from the hydrated state on quinone reductase (QR) activity in *L. furfuraceum*. Values are given  $\pm 1$  SD,  $n=4$ .

*Lasallia pustulata* with only 0.6 units  $\text{g}^{-1}$  dry mass. With the exception of *C. lactuca* and *Peltigera membranacea*, all species tested could produce hydroxyl radicals, and rates were similar to those reported by Moyo *et al.* (2017). However, there were no significant correlations between rates of hydroxyl formation and QR, LAC or POX activity (Table 2). Similarly, QR activity was not correlated with LAC activity, although there was a strong positive correlation between POX and QR activity. As discussed in the Introduction, all models of extracellular quinone redox cycling in free-living fungi require the regeneration of hydroquinones from quinones (Gómez-Toribio *et al.* 2009a, b; Arantes & Goodell 2014). Intuitively, it could be predicted that possessing higher QR activities would enable lichens to produce hydroxyl radicals at higher rates. However, the absence of a correlation between QR activity and hydroxyl radical formation rates found here does not necessarily mean that the enzyme is not involved in redox cycling. Possibly, rates of hydroxyl formation might be limited by factors other than QR activity. Furthermore, our extraction procedure and QR enzyme activity assay did not allow us to distinguish between different QR isoforms. Free-living fungi contain multiple isoforms and while some drive a bio-degradative quinone redox cycle, others appear to be more important in general stress tolerance (Cohen *et al.* 2004). Future studies, probably at the molecular level, are needed to determine the number and cellular location of different lichen

**Table 2.** Pearson correlations between quinone reductase (QR) activity, rates of hydroxyl radical formation, and the activities of laccase (LAC) and heme peroxidase (POX) in the lichens surveyed.

Activity		Hydroxyl radical production	LAC	POX
QR	Pearson Correlation	-0.403	0.362	0.876**
	Sig. (2-tailed)	0.173	0.224	0.000
	<i>n</i>	13	13	13
Hydroxyl radical production	Pearson Correlation		-0.537	-0.328
	Sig. (2-tailed)		0.072	0.298
	<i>n</i>		13	13
LAC	Pearson Correlation			0.203
	Sig. (2-tailed)			0.505
	<i>n</i>			13

\*\* = correlation is significant at  $P \leq 0.05$  (2-tailed).

QR isoforms. The absence of correlations between rates of hydroxyl production and the activities of LAC and POX is consistent with our earlier findings in *Usnea undulata* (Beckett *et al.* 2015) and later surveys of a range of lichen species (Moyo *et al.* 2017), and suggests that hydroxyl radical production in lichens does not require redox enzymes to facilitate the oxidation of hydroquinones. Extracellular redox cycling in lichens therefore more closely resembles that in brown- rather than white-rot fungi (Gómez-Toribio *et al.* 2009a, b; Arantes & Goodell 2014).

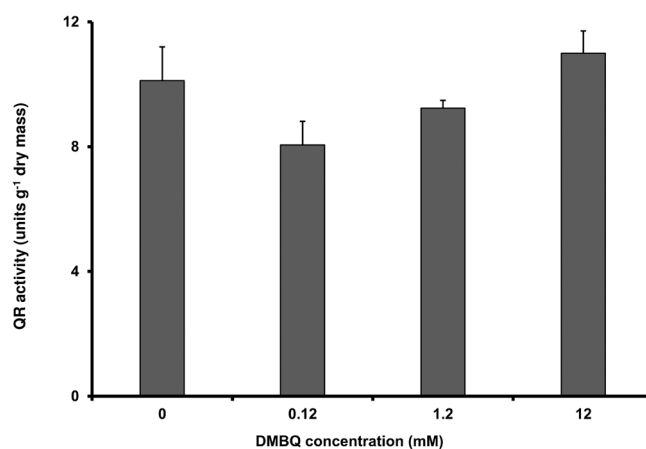
#### Effect of desiccation and pretreatment with DMBQ on QR activity

The effect of hydration and slow desiccation on QR activity is shown in Fig. 2B. During the hydration of dry material, QR activity progressively increased for 48 h, but longer periods of hydration reduced QR activity. In other lichens, prolonged moist storage has been found to cause similar reductions in the activities of other redox enzymes (LAC, TYR and POX) (Beckett *et al.* 2014), probably as a result of the well-known stresses caused by continuous hydration (Farrar 1976). Relatively slow desiccation did not induce QR activity, rather activity progressively decreased (Fig. 2B). It has been suggested that QR activity is involved in the stress tolerance of fungi (Cohen *et al.* 2004). While possibly under conditions of even slower desiccation some increases in activity may occur, drying rates were lower than those that have been observed for lichens in the field (e.g. Leisner *et al.* 1997). Therefore, results from the present study suggest that the enzyme plays little role in desiccation tolerance.

In free-living fungi, QR activity is strongly induced by quinones and vanillic and ferulic acids, by-products of delignification, suggesting that they may be involved in the metabolism of the breakdown of toxic soil compounds (Brock *et al.* 1995; Akileswaran *et al.* 1999). However, unlike free-living fungi, pretreatment of *Leptogium furfuraceum* with DMBQ in the present study had very little effect on QR activity (Fig. 3). The absence of an induction of QR by exogenous quinones may mean that in this lichen QR activity is not involved in quinone detoxification.

#### Conclusions


Taken together, the results presented here show that, as for free-living Ascomycetes (Espagne *et al.* 2008), QR activity is



**Fig. 3.** The effect of incubating *Leptogium furfuraceum* with different concentrations of 6-Dimethoxy-1, 4-benzoquinone (DMBQ) on quinone reductase (QR) activity after 24 h. Values are given  $\pm 1$  SD,  $n = 4$ .

widespread in lichens. Activity varies greatly between species and is notably high in jelly lichens from the *Collemataceae*, but there are no clear reasons for large variations in activity. While all models of extracellular redox cycling proposed for fungi suggest that the enzyme is essential, in the present study QR activity was not correlated with rates of hydroxyl radical production. Presumably, factors other than QR activity limit rates of hydroxyl radical production. However, QR probably plays other roles in lichen biology. In *Leptogium furfuraceum*, QR activity was not induced by desiccation (Fig. 2B) or the exogenous application of a quinone (Fig. 3), suggesting that the enzyme might not be involved in tolerance to these stresses. However, in future it would be desirable to test the effects on activity of treatment with other quinones, and more generally subjecting thalli to other stresses. Such studies will enable us to understand in more detail the regulation and physiological functions of QR activity in lichens.

**Acknowledgements.** CM thanks the University of KwaZulu Natal Research Fund for partial financial support. RB thanks the Russian Government Program of Competitive Growth of Kazan Federal University for partial financial support. FM thanks the Russian Science Foundation (grant no. 18-14-00198, desiccation experiments) and the Russian government assignment of FRC Kazan Scientific Center of RAS for financial support.

**Author ORCIDs.**  Calvin Eddington Moyo, 0000-0001-5292-0536; Richard Peter Beckett, 0000-0002-0530-4244.

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