

**SUPPLEMENTARY MATERIAL**

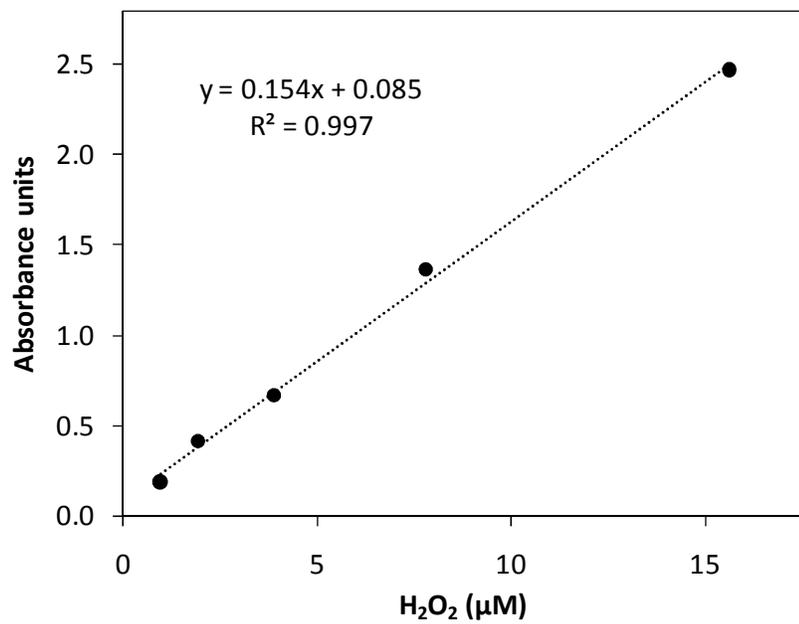
*Supplementary material to the article “A response of the imbibed dormant red rice caryopsis to biotic challenges involves extracellular pH increase to elicit superoxide production” by Ghotbzadeh & Gianinetti.*

**Supplementary Table S1.** XTT assay: absorbance of incubation solutions of dormant seeds after 0, 8 and 20 hours at 30 °C (mean ± se; the number of replications for each treatment is given in parentheses). XTT, when present, was added either at the start of experiment (0 h) or at 12 h, as indicated. Values with the same letter (and colour) are not significantly different ( $P \leq 0.05$ ; Tukey test).

Test	Absorbance ( $A_{470}$ )		
	XTT added at 0 h		XTT added at 12 h
	0 h	8 h	20 h
Seeds	0.043 ±0.024 (n=3) <b>c</b>	0.070 ±0.038 (n=3) <b>c</b>	0.092 ±0.053 (n=3) <b>c</b>
Seeds+pronase+Ca	0.030 ±0.002 (n=3) <b>c</b>	0.159 ±0.038 (n=3) <b>c</b>	0.495 ±0.027 (n=3) <b>b</b>
Seeds+XTT	0.033 ±0.008 (n=5) <b>c</b>	0.091 ±0.009 (n=5) <b>c</b>	0.065 ±0.006 (n=5) <b>c</b>
Pronase+Ca+XTT	0.037 ±0.001 (n=5) <b>c</b>	0.075 ±0.009 (n=5) <b>c</b>	0.084 ±0.011 (n=5) <b>c</b>
Seeds+pronase+Ca+XTT	0.060 ±0.009 (n=5) <b>c</b>	0.674 ±0.107 (n=5) <b>b</b>	1.330 ±0.114 (n=5) <b>a</b>

**Supplementary Table S2.** Xylenol orange assay: absorbance of incubation solutions of dormant seeds after 0, 8 and 20 h at 30 °C (mean ± se; 3-5 replications for each treatment). Values with the same letter (and colour) are not significantly different ( $P \leq 0.05$ ; Tukey test).

Test	Absorbance ( $A_{560}$ )		
	0 h	8 h	20 h
Seeds	0.014 ±0.001 <b>d</b>	0.062 ±0.011 <b>abcd</b>	0.106 ±0.003 <b>ab</b>
Pronase+Ca	0.033 ±0.019 <b>cd</b>	0.050 ±0.005 <b>bcd</b>	0.081 ±0.023 <b>abc</b>
Seeds+pronase+Ca	0.049 ±0.007 <b>bcd</b>	0.051 ±0.001 <b>bcd</b>	0.132 ±0.003 <b>a</b>



**Supplementary Figure S1.** Calibration curve for the xylenol orange assay, based on serial two-fold diluted H<sub>2</sub>O<sub>2</sub> solutions.

## **Auxiliary considerations**

### ***Additional features of the red rice caryopsis/pronase system***

Although the red rice dispersal unit is the spikelet (Gianinetti, 2016), the hull is a dead tissue that acts as a first, mechanical barrier, whereas the dehulled caryopsis is a living organism that can proactively respond to biotic challenges. We have hence used, in this system, the dehulled caryopsis to understand how it responds to an exogenous protease that, not having been blocked by the hull, simulates a microbial attack. The presence of a living micro-organism, in fact, complicates the study of the seed response, as the former alters the microenvironment to make it more favourable to its own growth and at the same time it could even try to curb the defensive response eventually actuated by the seed. In addition, *Streptomyces griseus* produces desferrioxamine E (Yamanaka *et al.*, 2005), a siderophore closely similar to desferal and therefore possibly able to interfere with some assay.

### ***No direct effect of extracellular alkalinisation in the seed–microbe interaction***

Regulation of superoxide production by pH might also be of importance in regulating superoxide activity in relation to other cell wall related processes, since many wall-modifying enzymes show pH-regulated activity (Vreeburg and Fry, 2005). Eventually, pH can have a decisive role in the final outcome of the seed–microbe interaction, but this is dependent upon the specific interaction (Prusky and Yakoby, 2003; Alkan *et al.*, 2013). Although, in fact, an increase in extracellular pH is known to be an early defence response in plants (Wu *et al.*, 2014; Moroz *et al.*, 2017; Yu *et al.*, 2017), modulation of ambient pH by the pathogen itself is important too, since it determines the ability of the pathogen to successfully colonize and invade the targeted host (Prusky and Yakoby, 2003). Indeed, several fungal pathogens actively acidify or even alkalinize their ambient pH by secreting organic acids or ammonia, respectively, to increase fungal pathogenicity (Alkan *et al.*, 2013). This adds a further level of complexity to seed–microbe interaction. As *Streptomyces griseus* is an alkaliphilic species (Antony-Babu and Goodfellow, 2008), extracellular alkalinisation, by itself, does not provide a direct, negative effect on microbial growth in this seed–microbe interaction, evidently.

### ***Possible involvement of extracellular enzymes***

In the arabidopsis seed coat, the oxidation of soluble PAs (seed browning) is caused by a laccase, a type of polyphenol oxidase (Pourcel *et al.*, 2007). Accordingly, polyphenol oxidase activity, potentially using flavonols and condensed tannins (PAs) as substrates, was strongly associated with darkening of seed coat in recombinant inbred lines of pinto bean (Marles *et al.*, 2008). A polyphenol oxidase was shown to be induced by *Fusaria* in dormant wild oat caryopses, and it was supposed to be activated and released following fungal proteolysis (Fuerst *et al.*, 2011). If a polyphenol oxidase is involved in the darkening of red rice caryopses as well, an effect of pH on enzyme activity could have a role too. Thus, pronase-induced seed response might promote the mechanical enforcement of the caryopsis, and thereby the darkening of its colour, through multiple, synergic mechanisms. Indeed, plant defence systems consist of both preformed physical and chemical barriers and

induced defence mechanisms, and ROS production in the apoplast in response to pathogens has been proposed to orchestrate the enforcement of defensive barriers (Levine *et al.*, 1994; Lamb and Dixon, 1997; Torres *et al.*, 2006; Dalling *et al.*, 2011).

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