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Seed vigour better to be assessed by physiological markers rather than expression of antioxidant enzymes in the common bean (*Phaseolus vulgaris* L.).

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Abstract

Seed vigour assessment based upon seedling development and physiology is time consuming, whilst estimations based upon enzyme activity measurements alone can be performed quickly but may be misleading. The aim of this work was to compare the relationship between the physiological markers of germination and initial seedling growth in common bean with the activities of the reactive oxygen species scavenging enzymes: peroxidase (PRX), catalase (CAT) and superoxide dismutase (SOD), in embryonic axes and cotyledons maintained under both low and high stress storage conditions. Changes in seed physiology were measured by germination after five and eight days, seedling vigour, potassium leakage, root and shoot length and dry weight. The potassium leakage test, vigour classification and 5th day germination were reliable predictors of seed vigour when there were differences among highly stressed lots. Tests based on the length and dry weight of the roots and shoots were efficient for all conditions. SOD and CAT determinations showed a high level of correlation with vigour tests for all three cultivars, but only under high levels of stress, indicating the reliability of those enzymes as markers of stress in poorly stored seeds. SDS-PAGE of soluble protein fractions exhibited degradation of banding patterns in highly stressed seeds from 48h of ageing, whilst no degradation was observed in controls and low stress conditions.

Keywords: CAT activity, germination, physiological potential, PRX activity, SOD activity.
Abbreviations: CAT-Catalase, PRX-Peroxidase, SOD-Superoxide Dismutase, SDS-PAGE-Sodium Dodecil Sulphate Polyacrylamide Gel Electrophoresis, ROS-Reactive Oxygen Species, G germination, G5-germination on fifth day, SDM-Shoot dry mass, RDM-root dry mass; SL-Shoot Length; RL-Root length; TL-Total length; VC-Vigour Classification; KL-potassium leakage.

Introduction

Globally agriculture is a vital activity supporting an increasing population and one enhancing national economies, especially in the developing countries. Most crops are produced from seeds, and although they are individually a small and cheap component, their quality is paramount to successful production (Marcos Filho, 2005). Therefore, for seed companies a major concern is to ensure reproducible seed performance that will guarantee the productivity of high quality crops. Methodology to assess the quality of commercial seed lots has used such parameters as changes in rate observed in the germination progress curves (Matthews et al., 2012), and have been used to evaluate changes in seed behaviour following exposure to different conditions, including storage, thus enabling the analysis of seed performance across a wide range of variables. The development of highly-standardized methodologies is needed to ensure high quality assurance, with tests producing accurate and reproducible results. Where possible, mechanization of those techniques will enable companies to make significant labour and financial savings (Marcos Filho, 2005). Research over the last 60 years has contributed to the development of standardised tests to measure seed vigour, involving artificial ageing, cold tests, electrical conductivity, cell leakage of organic or inorganic molecules, and the determination of enzymatic activities (Matthews et al., 2012). Development of these methods has facilitated automation, image capture and subsequent computer-aided analysis (McDonald et al.; 2001; Dell'Aquila, 2007, Hosomi et al., 2011). Degenerative processes can begin to occur in seeds immediately after shedding, when physiologically the seeds have attainined their maximum regenerative potential. High temperatures and humidity during seed storage contribute to further deterioration by promoting degenerative changes, such as destabilization of enzyme activities and the disruption and loss of cell membrane integrity (Nagel and Börner; 2010) caused mainly by lipid peroxidation due to increased presence of reactive oxygen species (ROS) (McDonald, 2004). Seeds stored under inappropriate conditions may retain functional traits over the short-term, whereas loss of these will be realised during long-term periods of storage (Aragão et al., 2002). The biochemical and physiological deterioration that occurs have been studied mainly under accelerated ageing conditions using elevated temperatures and seeds with higher water content than normal storage conditions (McDonald, 1999). Under these circumstances, the seeds will lose their viability within a few days or weeks, because the temperatures used are high enough to denature protein/enzyme complexes, and to leave the ROS free to react

with other molecules, especially macromolecules. Many causes of seed ageing have been proposed, such as lipid peroxidation mediated by ROS, enzyme or protein inactivation, cell membrane disintegration and genetic damage (McDonald, 2004; El-Maarouf-Bouteau et al., 2011), as described by a damage-repair hypothesis (Matthews et al., 2012). The activity of antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), as well as enzymes in the ascorbate:glutathione cycle, may be components in the mechanisms involved in resistance to many types of stress (Fig. 1), and high activity of these enzymes has been detected in response to biotic and abiotic stress (Foyer and Noctor, 2005; Kranner et al., 2010). In storage experiments, changes in these enzymes have been observed. Cakmak et al. (2010) studied *Medicago sativa* seeds stored for 42 years and observed that over this time a significant increase in lipid peroxidation and the phenolic content of aged dry seeds occurred. The seeds also had low hydrogen peroxide content and low CAT, PRX and SOD activities. The decrease in germination in these aged seeds (54% below the non-aged seeds) correlated strongly with increased levels of lipid peroxidation and decreased activity of the antioxidant enzymes studied. Similarly during seed storage of *Gossypium hirsutum* (Goel and Sheoran, 2003) and *Melanoxylon brauna* (Corte et al., 2010), SOD and CAT activities were progressively reduced, whereas others have described a decrease in SOD activity alone, with unchanged CAT activity during artificial ageing, and the absence of SOD activity (Sung and Jeng, 1994). These contradictions and inconsistencies in the seed ageing studies may be attributed to differences in the ageing method used (Balesevic-Tubic et al., 2011). Studies have been performed investigating either vigour differences in bean seeds in relation to protein degradation and physiological parameters (Machado Neto et al. 2001), or biochemical changes such as enzyme activities and lipid content in *Melanoxylon brauna* seeds (Corte et al. 2010), and Balesevic-Tubic et al. (2011) in soybean seeds to study SOD and PRX activity and lipid peroxidation, but comparative studies remained unresolved. The objective of this study was to investigate the possible relationship between ROS scavenging enzymes (PRX, CAT, SOD) and commonly used physiological markers of germination and initial seedling growth in common beans (*Phaseolus vulgaris* L.) under both low (seed room) and high (artificial ageing) stress storage conditions.

Results

Seed weight, for each cultivar, varied in seed lots from year to year, reflecting their annual cultural conditions. Moisture determinations showed that in the low stressed seeds this remained relatively stable and was at equilibrium with the relative humidity in the storage room, while seeds subjected to the high stress treatments showed a higher moisture content as exposure time increased (Table 1). However, following drying under seed room conditions these seeds showed a uniform MC across all treatments, which was slightly lower than in the low stressed seeds (Table 1). Following storage under low stress conditions, germination and vigour were consistently high and showed minimal variation in all stored seed lots from each of the cultivars sampled, and although differences were observed in root and shoot length and dry weight, no consistent trends were observed in relation to duration of storage. Equally, despite the large variation in potassium leakage in two cultivars, no consistent trend was observed, however the highly levels do show some alignment to higher seed MCs (Tables 1 and 2). On the other hand, the measurement of germination, vigour, and shoot and root length and dry weight all showed a significant reduction and potassium leakage showed a significant increase, with exposure time in all three cultivars under high stress (Table 3). The analysis of linear correlations between the physiological tests results and enzyme activities in low stress conditions for IAC Carioca Akytã indicated significant correlations between the PRX activity in the embryonic axis and the length and dry weight measurements (Table 4). However, this was not repeated in the other two cultivars, and the F test was not significant between the lots from different years. SOD activity in the axis was significantly different among the lots submitted to low stress, but was not correlated with the physiological tests. In IAC Carioca 80SH, significant correlation was only observed between CAT activity and potassium leaching in the embryonic axis. SOD activity in the axis between different annual lots showed a significant F value, but it does not correlate in the same way with all cultivars (Table 4). In Rosinha G2, the F value for SOD determination in the axis was significant but only correlated with germination measurements. A significant F value for enzymatic activity suggests that at least one lot was different from the others, however, no other significant correlations were observed. Following artificial ageing, the embryonic axes of IAC Carioca 80SH seeds showed significant correlations between PRX activity and germination, vigour classification, 5^{th} day germination and potassium leaching, and there were differences among the time treatments. SOD activity was correlated with germination, 5^{th} day germination and the lengths and dry weights of the roots and shoots. While CAT activity showed correlations with measurements of root length and root and shoot dry weight; but these correlations for both SOD and CAT activity were not significant according to the F test indicating no significant difference between ageing treatments (Table 5). In the cotyledons, PRX activity was correlated to most of the physiological parameters, but the F value was not significant (Table 5). SOD activity was also correlated with the most physiological measurements and the F test indicated a significant effect of ageing under high stress. While CAT activity only showed a significant correlation with vigour classification. In Rosinha G2, PRX showed no correlation with any parameter in either the embryonic axis or cotyledon, while the F test indicated that significant differences occurred in axes between time treatments. SOD and CAT activities presented significant correlations with all the physiological tests, except for shoot length and shoot dry weight tests for CAT activity. The activity of both enzymes was highly correlated with the physiological measurements and showed variations that differed significantly between the treatments (Table 5). In Rosinha G2 cotyledons, there were significant correlations between all the physiological parameters and SOD and CAT activities (Table 5). In this study, the tests demonstrated promise only for application within the low and high stress treatment (Table 6). CAT activity decreased as high stress increased; thus, a non-stressed lot was identified as different from the stressed ones, but there was no activity difference between the ageing periods (Table 6). For IAC Carioca 80SH, PRX activity in the axis and SOD activity in the cotyledons showed a decrease in activity with increased exposure to stress conditions. Analysis of PRX activities in the axis split the lots into two groups, where non- aged seeds retained high activity, while 96 and 144 h had the lowest activity. 48 h did not differ from either group and showed intermediate activity (Table 6). SOD activity also split the

Table 1. Hundred seeds weight (W) and moisture content (MC) under low stress conditions, and seeds moisture content under high stress conditions (MCa) and after drying prior to vigour tests (MCb) of 'IAC Carioca Akytã', 'IAC Carioca 80SH' and 'Rosinha G2' cultivars after various storage times (T).

	Low stress*			High stress**		
	T (years)	W (g)	MC (%)	T (h)	MCa (%)	MCb (%)
'IAC Carioca Akytã'	0	21.73	11.52	0	11.52	10.53
	1	23.07	11.11	48	22.85	10.42
	2	19.35	11.01	96	23.05	10.18
	3	19.85	10.84	144	22.20	10.64
'IAC Carioca 80SH'	0	19.67	11.04	0	11.04	10.75
	1	22.36	11.16	48	17.2	10.49
	2	23.59	11.10	96	23.5	10.87
	3	23.29	10.57	144	28.66	11.11
'Rosinha G2'	0	21.30	12.44	0	12.44	10.35
	1	24.34	11.33	48	23.35	10.15
	2	22.38	11.37	96	19.87	10.24
	3	22.71	10.71	144	24.76	10.60

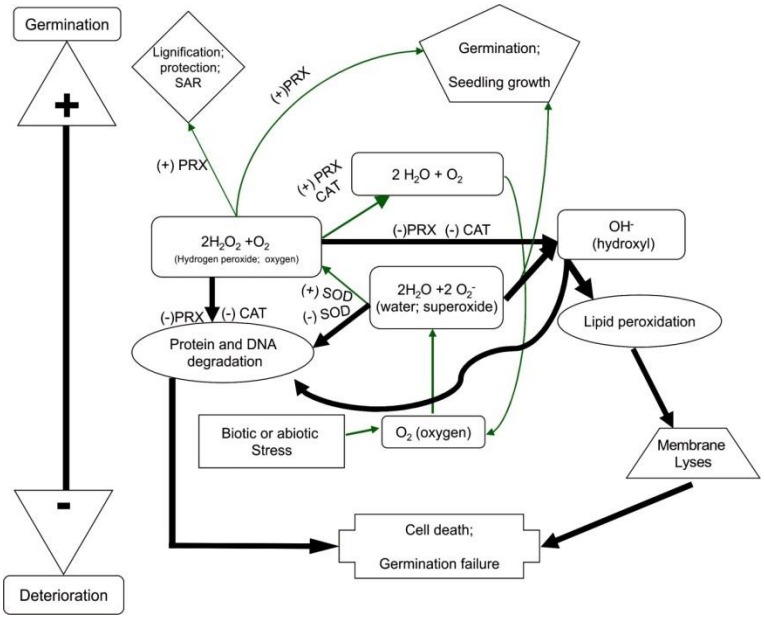

Fig 1. Scheme of reactive oxygen species and their effect over molecules. Large Arrows indicate a negative pathway (distress), thin arrows a positive pathway (eustress). (Adapted from Tian et al., 2008; Bailly et al., 2008; Kranner et al., 2010).

ageing treatments into two groups, where again non-aged seeds retained high activity and 144 h expressed the lowest activity (Table 6). The intermediate values at 48 and 96 h did not differ from one another, nor from respectively from the 0h and 144h treatment. Within axes and cotyledons of Rosinha G2 seeds, SOD and CAT activity generally decreased over the 144h ageing period (Table 6), and these had previously shown a strong correlation to physiological tests (Table 5). The exception was SOD activity in the cotyledons, where values for 48-144h were similar, and were much lower than the non-aged seeds.

Discussion

To differentiate seed lots based solely on traditional physiological tests has been considered time-consuming, whilst the use of ROS scavenging enzymes could be more specific and potentially faster utilising more economic automated process. This study assessed the relationship between these two approaches to seed quality assessment by comparing *P.vulgaris* seeds under low and high stress conditions. The potential of particular enzyme activity to act as a marker of seed vigour depends on its ability to detect a pattern resulting from seed deterioration. This was verified by analysis of simple linear correlations between different enzyme activities and physiological assessments, as well as the enzymes' ability to discriminate between seed lots (different ages) (Tables 3 and 5). Only SOD activity exhibited potential to discriminate between seed lots from low stress conditions (based on significant F values), although this did not correlate with germination or vigour tests (Table 4). For IAC Carioca Akytã, only CAT activity determination in the axis appeared to consistently distinguish between lots using the germination and vigour tests as indicators. According to Kranner et al. (2010), a normal distribution curve might represent the SOD activity during ageing, where at both ends of the curve the enzyme may have the same activity. However, this would reflect limited response to no or very low stress, but under high stress enzyme activity would be inhibited. Seed moisture should be considered when attempting to standardize methodologies to ensure uniform results, as it enables the diagnosis of different levels of vigour in different seed lots (TeKrony, 2003; Torres and Marcos Filho, 2003). Moisture content is a key factor

Table 2. Germination (G), vigour classification (VC), 5th day germination (G5), potassium leakage (KL), root (RL) and shoot length (SL), root (RDW) and shoot dry weight (SDW) of 'IAC Carioca Akytã', 'IAC Carioca 80 SH' and 'Rosinha G2' cultivars following low stress treatment.

		G	VC	G5	KL	RL	SL	RDW	SDW
		%			$ppmK^{+}.g^{-1}$	cm		g	
'IAC Carioca Akytã'									
Years	0	96 a	93 a	96 a	138.55 a	6.02 b	3.15 b	0.070 b	0.146 b
	1	99 a	96 a	99 a	114.18 a	9.90 a	4.75 a	0.090 a	0.227 a
	2	99 a	98 a	99 a	128.17 a	7.37 b	3.67 b	0.073 ab	0.146 b
	3	100 a	98 a	100 a	103.76 a	6.92 b	3.47 b	0.067 b	0.141 b
'IAC Carioca 80 SH'									
Years	0	99 a	99 a	99 a	121.98 b	6.00 b	2.85 b	0.058 b	0.134 b
	1	98 a	97 a	98 a	124.15 b	10.70 a	4.60 a	0.106 a	0.224 a
	2	99 a	97 a	99 a	271.12 c	10.42 a	4.85 a	0.100 a	0.219 a
	3	99 a	98 a	99 a	45.72 a	10.05 a	4.00 ab	0.094 a	0.190 ab
'Rosinha G2'									
Years	0	98 a	98 a	97 a	214.43 c	10.02 ab	4.57 a	0.095 a	0.226 a
	1	97 a	97 a	97 a	40.73 a	8.10 b	3.62 b	0.084 a	0.192 b
	2	98 a	98 a	98 a	136.39 b	10.67 a	4.57 a	0.106 a	0.266 a
	3	100 a	100 a	100 a	79.14 a	10.00 ab	4.15 ab	0.097 a	0.216 ab

Mean values in columns followed by the same letters do not differ following Tukey's test at 5% significance.

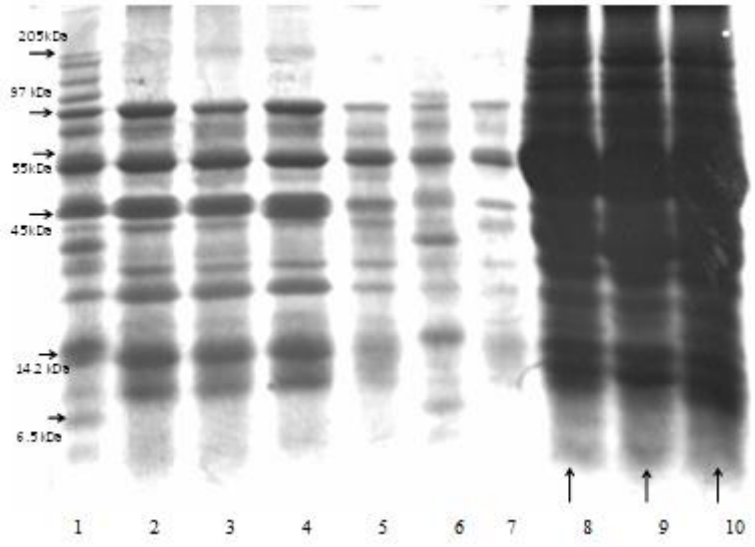

Fig 2. SDS-PAGE protein banding of 'Rosinha G2'. From left to right, lines 1 and 6 molecular markers (Sigma Wide range; 205 up to 6,5 kDa); 2 to 4 seeds under low stress and three, two and one year following harvest respectively; 5 and 7 freshly harvested seeds (zero year and zero time of artificial ageing); 8-10 seeds under high stress after 48, 96 and 144h respectively, showing of the beginning of protein degradation.

affecting seed preservation, as hydrated seeds degrade more quickly due to elevated water availability maintaining metabolic activity over the optimum for seed storage, allowing subsequent respiration and macromolecule degradation (Ellis and Hong, 2007; Matthews et al., 2012). There were differences in water content among the years studied, which may have given rise to observd elevated levels of potassium leakage (Table 1 and 2). The moisture acquired during the artificial ageing treatment showed the similar gradual increase described by Binotti et al. (2008) working with the cv. Pérola. Whereas the lower water content in IAC Carioca Akytã compared to IAC Carioca 80SH under artificial ageing could be attributed to varying tegument permeability between cultivars (Table 1), which was also seen in *Glycine max* (Ranathunge et al., 2010) and *Vigna umbelatta* (Isemura et al., 2010), where seed coat structures are known to influence water absorption due to differences in permeability caused by differences in cutin or phenolic compounds. This variability was minimal in a common seed lot, such as those used for the high stress treatment (Table 1), however, this variation could be attributed to the hysteresis (Bewley ar al 2013). Both classifications of germination, along with vigour (Tables 2 and 3) illustrate the differences in germination velocity and seedling development without abnormal morphology under different levels of stress. As noted by Matthews et al. (2012), the velocity is one of the best forms of vigour evaluation, as when a seed lot begins to age, the molecular structure starts to incur damage that requires time to repair. This in turn decreases first the germination velocity, then seedling growth, and finally increases the morphology defects. For seeds experiencing high stress, this is seen as a decrease in all physiological parameters with increased exposure time (Table 3). . Root and shoot length and dry weight tests were sensitive to loss of physiological seed vigour, distinguishing treatments according to the intensity of degradation caused by low and high stress treatments (Tables 2 and 3). The development of seed quality is affected by field growing conditions: from

flower fertilization to harvest, fluctuations in the environment directly affect the seed quality. So, despite minimal deterioration, it is not the case that freshly harvested seeds (zero year of storage, Table 2) are the better quality. Alternative physiological parameters have been considered to assess quality, such that Corbineau (2012) used seedling dry weight in soybean and maize to evaluate growth potential, Which was based upon the vigorous seeds of one lot producing plants with high growth due to their ability to mobilise seed reserves (e.g. in cotyledons) and transfer these to the embryonic axis (Mohammadi et al., 2011). The vigour tests exhibited differences among the lots, but significant trends were only observed under artificial ageing, while loss of vigour was not detected under low stress, except in the shoot and root length and dry weight measurements. This reflects the suitability of storage conditions (15ºC and 10 to 11% of seed moisture content), which can maintain seeds in a reduced metabolic state that favours seed preservation (Ellis and Hong, 2007). Deterioration becomes irreversible and inevitable as seeds age (McDonald, 2004). The vigour tests' inability to detect differences between seed lots under low stress (Table 2) does not mean that deterioration did not occur, but that the tests were not sufficiently sensitive to detect any minor loss of vigour, because the time to respond to low stress-induced damage is longer than for seeds submitted to high stress (Machado Neto et al., 2001). As stated by Kranner et al. (2010), stress marker responses are not linear, but bell shaped and vigour loss is sigmoidal, indicating that at the beginning of the ageing process stress in insufficient to decrease the vigour. As the stress rises, the enzyme activities also increase, but then with a corresponding reduction in the vigour markers, but and as stress increases further, the disorganization of the cells and tissues also decreases enzyme activities. Matthews et al. (2012) stated that under these conditions self repair could occur, but would have two consequences: i) energy would be lost from the seed for the repair *per se* and ii), there would be less time to transfer energy from the reserves to the growing structures. The results of this work with seeds under high stress is closely related to that of Binotti et al. (2008), in which germination decreased considerably and electrolytic leakage increased significantly with increasing seed exposure time to artificial ageing conditions; the results were highly related to germination and vigour loss (Table 3). Vigour and seed viability loss are consequences of deep damage originating from molecular deterioration, caused by ROS and leading to lipid, protein and, consequently, membrane integrity loss; metabolic failure; DNA and RNA degradation and programmed cell death (El-Maarouf-Bouteau et al., 2011; Kranner et al., 2011; Corbineau, 2012). Plants have a number of antioxidants in their tissues, which protect them against ROS, such as superoxide (O_2^-), peroxide (H_2O_2) and hydroxyl (OH^-) which may be produced in non-photosynthetic organs, such as seeds by mitochondrial activity when the seed moisture is suitable for respiration (Bailly et al., 2002, Kibinza et al., 2006; Tian et al., 2008) through an apparent coordination of scavenging enzymes (Bailly et al., 2001, Bailly, 2004). These ROS are involved in the biosynthesis of several classes of compounds (Siegel, 1993) and in molecular signalling (Oracz et al., 2007; Bailly et al., 2008). ROS scavenging enzymes' activity can vary temporally, through zygote formation, seed shed and during storage or germination (Bailly et al., 2000, 2001, 2004). Some studies have shown that antioxidant enzyme activities are very low in dry seeds (c. 13% moisture content), such as maize (Chiu et al., 2002), and high in germinating seeds (Cakmak et al., 2010). Priming studies on sunflower seeds have detected increases in SOD and CAT activities at three and six hours after hydration, which were attributed to increased metabolic activity (respiration) with the generation of secondary products (ROS) (Bailly et al., 2000). In this work, activities were measured after soaking for 24 h at 25°C, meaning that some repair occurred and the enzyme activity decayed as part of the stress was removed. Some studies, most of them conducted with artificially aged seeds, have suggested that a relationship exists between vigour and ROS scavenging systems (Bailly et al., 1996, 2002, 2004, 2008). The defence enzymes response is not constant among species. Responses corresponding to vigour loss have been found as declining activity (Jeng and Sung, 1994; Sung, 1996; Bailly et al., 2002, Santos et al., 2004, Demirkaya et al., 2010), maintenance at the same level (Bailly et al., 1998) or increased activity (Kalpana and Madhava Rao, 1994). In the current study, the activity of antioxidant enzymes in the embryo axis was greater than that found in cotyledons (Table 6): in agreement with Jeng and Sung (1994) and Scialabba et al. (2002). These are the actively growing parts of germinating seeds and thus have a higher metabolic activity, which includes the antioxidant enzyme system. The enzyme localization within tissues is very important. PRX activity occurred mainly in the embryo axis of *Raphanus sativus*, and the PRX activity was different between fresh and extremely aged seeds (Scialabba et al., 2002). While in *Glycine max*, the SOD, PRX, ascorbate, CAT and glutathione reductase activities of embryo axes under high stress conditions decreased (Tian et al., 2008). In this work, there were variations in enzymatic activities under low stress conditions, but these variations in enzyme activity were not significantly correlated with seed vigour tests, even in lots stored for up to three years (Tables 4 and 6). In *Medicago sativa* seeds stored over long periods, the deterioration was similar for naturally and artificially aged seeds, but the germination velocity was different and enzymatic activity decreased with ageing (Cakmak et al., 2010). Corte et al. (2010) observed a similar pattern in *Melanoxylon brauna* seeds under natural and artificial ageing conditions. In naturally aged soybeans, decreases in SOD, CAT and PRX activities were observed with increasing time and appear to have a direct relationship with germination loss (Sung, 1996). Balesevic-Tubic et al. (2011) found similar results in soybeans, in which SOD and PRX activity declined with the length of the ageing period for both naturally (4ºC at 80-85%RH for six or 12 months) and artificially stressed seeds. The deterioration was also related to seed composition: for example, in oil seeds, deterioration occurs more rapidly than in other types of reserves (Nagel and Börner, 2010). The drop observed in SOD and CAT activity (Table 6) was similar to that cited by Bailly et al. (2001, 2002) and Santos et al. (2004). Bailly et al. (1998) observed that artificial ageing in sunflower seeds resulted in a sharp decrease in both germination and CAT activity, while SOD activity was much less affected. Whereas Demirkaya et al. (2010) reported SOD and CAT activities in onion seeds both decreased with artificial ageing and were correlated with viability and vigour loss. In both cases, PRX activity was not detected. Similarly in the current study both SOD (in cotyledons) and CAT (in the axis), showed significantly decreased activity with artificial ageing and also correlated with vigour tests (Tables 5 and 6). Chiu et al. (1995) observed a decrease in melon seed germination and antioxidant enzyme activities. When these seeds were hydrated, an increase in the activities of enzymes in the axis and cotyledons was concomitant with protein synthesis. Accelerated ageing and subsequent priming yielded the same pattern in peanut seeds (Jeng and Sung, 1994; Sung and Jeng,

Table 3. Germination (G), vigour classification (VC), 5th day germination (G5), potassium leakage (KL), root (RL) and shoot length (SL), root (RDW) and shoot dry weight (SDW) of 'IAC Carioca Akytã', 'IAC Carioca 80 SH' and 'Rosinha G2' cultivars following high stress treatment.

		G	VC	G5	KL	RL	SL	RDW	SDW
		%			$ppmK^{+}.g^{-1}$	cm		g	
'IAC Carioca Akytã'									
Hours	0	95 a	92 a	89 a	264.75 a	6.37 a	3.15 a	0.084 a	0.145 a
	48	82 b	80 b	72 b	516.75 b	3.29 b	1.90 b	0.052 ab	0.076 b
	96	61 c	59 c	43 c	861.75 c	1.50 bc	0.89 c	0.026 bc	0.029 b
	144	30 d	28 d	21 d	1173.00 d	0.68 c	0.39 c	0.013 c	0.018 b
'IAC Carioca 80 SH'									
Hours	0	99 a	97 a	98 a	248.50 a	7.60 a	3.31 a	0.076 a	0.148 a
	48	99 a	96 ab	95 a	395.25 ab	6.97 a	3.04 ab	0.068 a	0.142 ab
	96	86 b	84 b	77 b	670.75 bc	4.23 a	1.88 ab	0.053 a	0.085 ab
	144	65 c	62 c	53 c	733.00 c	2.56 b	1.35 b	0.041 b	0.055 b
'Rosinha G2'									
Hours	0	99 a	98 a	98 a	488.00 a	9.26 a	4.55 a	0.095 a	0.235 a
	48	86 b	84 b	83 b	680.75 ab	7.25 ab	3.56 ab	0.077 ab	0.169 ab
	96	70 c	69 c	61 c	870.50 b	3.75 bc	1.89 bc	0.043 bc	0.083 b
	144	55 c	53 d	39 d	880.75 b	1.84 c	1.27 c	0.031 c	0.047 b

Mean values in columns followed by the same letters do not differ following Tukey's test at 5% significance.

Table 4. Single linear correlation coefficient (r) between peroxidise (PRX), superoxide dismutase (SOD) and catalase (CAT), for each physiological variable: germination (G), vigour classification (VC), 5th day germination (G5), potassium leakage (PL), root length (RL), shoot length (SL), root dry mass (RDW) and shoot dry mass (SDW) in three cultivars under low stress conditions.

Variables			r							
	F value	CV(%)								
			G	CV	G5	KL	RL	SL	RDW	SDW
'IAC Carioca Akytã' axis										
PRX	3.49	62.6	-0.03	-0.15	-0.06	0.08	**-0.57***	**-0.54***	**-0.58***	**-0.49***
SOD	**12.37****	26.45	0.35	0.21	0.36	-0.09	-0.22	-0.22	-0.29	-0.40
CAT	3.10	68.9	0.20	0.17	0.18	-0.09	-0.27	-0.31	-0.33	-0.44
'IAC Carioca Akytã' cotyledon										
PRX	0.52	70.5	0.26	0.29	0.24	-0.25	0.12	0.10	0.01	0.12
SOD	0.64	19.5	0.07	0.03	0.05	-0.01	0.35	0.09	0.31	0.18
CAT	1.26	123.6	0.00	0.02	0.02	0.20	-0.04	-0.08	-0.13	-0.20
'IAC Carioca 80SH' axis										
PRX	1.16	63.1	-0.26	-0.35	-0.26	0.32	0.07	0.07	0.09	0.07
SOD	**13.13****	16.7	-0.42	-0.13	-0.42	-0.14	-0.02	-0.04	0.02	0.07
CAT	1.23	158.7	-0.28	-0.27	-0.28	**0.64****	-0.04	0.09	-0.01	0.07
'IAC Carioca 80SH' cotyledon										
PRX	0.52	70.5	0.18	0.14	0.18	-0.05	0.20	0.04	0.17	0.07
SOD	0.64	19.5	-0.01	-0.10	-0.01	-0.43	0.14	-0.18	0.07	-0.08
CAT	1.26	123.6	-0.24	-0.19	-0.24	-0.41	0.33	0.19	0.39	0.27
'Rosinha G2' axis										
PRX	1.27	58.8	0.23	0.18	0.22	-0.11	0.15	0.04	0.01	0.01
SOD	**8.70****	30.35	**0.52***	0.47	**0.50***	-0.07	0.41	0.22	0.23	0.20
CAT	2.20	123.5	0.41	0.49	0.39	-0.12	0.27	-0.02	0.19	-0.05
'Rosinha G2' cotyledon										
PRX	1.83	60.3	-0.12	-0.12	-0.07	0.02	0.33	0.27	0.31	0.27
SOD	0.41	59.3	-0.29	-0.15	-0.34	0.14	0.08	-0.06	0.06	0.01
CAT	1.42	162.7	-0.24	-0.18	-0.19	-0.09	0.07	0.06	0.23	0.12

*, ** Significant at 5% or 1% of probability by F-test. F values and coefficients of variation (CV) from the ANOVA, with three Degrees of Freedom, of the enzymes activities and four low stressed lots for each cultivar.

Table 5. Single linear correlation coefficient (r) between peroxidise (PRX), superoxide dismutase (SOD) and catalase (CAT), for each physiological variable: germination (G), vigour classification (VC), 5th day germination (G5), potassium leakage (PL), root length (RL), shoot length (SL), root dry mass (RDW) and shoot dry mass (SDW) in three cultivars under high stress conditions.

Variables			r							
	F value	CV(%)								
			G	CV	G5	KL	RL	SL	RDW	SDW
'IAC Carioca Akytã' axis										
PRX	1.49	70.3	-0.38	-0.37	-0.45	0.25	-0.40	-0.38	-0.45	-0.40
SOD	2.60	29.1	-0.02	-0.08	-0.01	0.05	0.10	0.16	0.16	0.18
CAT	**39.7****	17.3	**0.75****	**0.71****	**0.77****	**-0.71****	**0.84****	**0.86****	**0.80****	**0.81****
'IAC Carioca Akytã' cotyledon										
PRX	1.66	67.7	-0.22	-0.21	-0.22	0.31	-0.26	-0.30	-0.31	-0.36
SOD	3.05	44.5	**0.59***	**0.60***	**0.54***	**-0.65****	0.48	**0.61***	0.48	0.45
CAT	0.37	95.0	-0.21	-0.19	-0.22	0.04	-0.37	-0.38	-0.43	-0.42
'IAC Carioca 80SH' axis										
PRX	**3.70***	36.5	**0.58***	**0.62****	**0.66****	**-0.54***	0.30	0.31	0.29	0.28
SOD	3.23	62.5	**0.55***	0.49	**0.53***	-0.15	**0.56***	**0.50***	**0.54***	**0.57***
CAT	1.14	53.6	0.39	0.37	0.43	-0.14	**0.53***	0.47	**0.54***	**0.54***
'IAC Carioca 80SH' cotyledon										
PRX	3.19	51.0	**-0.54***	**-0.68****	**-0.55***	0.37	**-0.61***	**-0.63****	**-0.56***	**-0.57***
SOD	**8.12****	19.1	**0.73****	**0.62***	**0.74****	**-0.84****	**0.51***	**0.61***	0.36	**0.57***
CAT	1.51	19.7	-0.47	**-0.56***	-0.44	0.34	-0.13	-0.15	-0.10	-0.14
'Rosinha G2' axis										
PRX	**4.12***	76.0	-0.24	-0.26	-0.19	0.10	-0.35	-0.32	-0.32	-0.33
SOD	**5.39***	31.9	**0.82****	**0.79****	**0.76****	**-0.76****	**0.51***	**0.52***	**0.50***	**0.55***
CAT	**10.24****	29.9	**0.83****	**0.80****	**0.82****	**-0.79****	**0.59***	0.49	**0.54***	0.49
'Rosinha G2' cotyledon										
PRX	0.29	69.5	-0.01	0.03	0.03	0.05	0.08	-0.02	-0.03	-0.07
SOD	**14.46****	17.9	**0.81****	**0.84****	**0.82****	**-0.67****	**0.68****	**0.69****	**0.65****	**0.71****
CAT	**7.66****	37.5	**0.80****	**0.83****	**0.84****	**-0.63****	**0.70****	**0.65****	**0.65****	**0.65****

*, ** Significant at 5% or 1% of probability by F-test. F values and coefficients of variation (CV) from the ANOVA, with three Degrees of Freedom, of the enzymes activities and four high stressed lots for each cultivar

Table 6. Significant enzymes activities by F-test and correlated with physiological tests for both low and high stress treated seed lots.

Low stress conditions			
	'IAC Carioca Akytã'	IAC Carioca 80SH'	'Rosinha G2'
Year	SOD axis	SOD axis	SOD axis
0	0.001975b	0.001600ab	0.006025b
1	0.001500b	0.002125a	0.004325b
2	0.002675b	0.001175b	0.009425ab
3	0.004275a	0.001150b	0.012425a

High stress conditions				
	'IAC Carioca Akytã'		'IAC Carioca 80SH'	
Hours	CAT axis		PRX axis	SOD cot
0	0.0289 a[1]		0.0011 a	0.0019 a
48	0.0135 b		0.0008 ab	0.0018 ab
96	0.0106 b		0.0005 b	0.0012 bc
144	0.0109 b		0.0006 b	0.0011 c
	'Rosinha G2'			
	SOD axis	CAT axis	SOD cot	CAT cot
0	0.0078 a	0.0209 a	0.0027 a	0.0167 a
48	0.0052 ab	0.0150 ab	0.0016 b	0.0107 ab
96	0.0043 b	0.0097 bc	0.0018 b	0.0076 b
144	0.0034 b	0.0067 c	0.0013 b	0.0048 b

Mean values in columns followed by the same letters do not differ following Tukey's test at 5% significance.

1994), while sunflower seeds showed increased CAT and glutathione reductase activities and decreased SOD activity (Bailly et al., 2002). These results showed that SOD and CAT reactions were coordinated and have similar behaviours as they can act in the ROS degradation (Fig. 1). If they are negatively controlled there would be a ROS accumulation that will lead to cell death. PRX is involved in many cellular mechanisms and may serve as a trigger for what are termed, Systemic Acquired Resistance responses (; Siegel, 1993; Benhamou and Nicole, 1999; Jung et al., 2000; McCue et al., 2000). Because of these varied roles, there were fewer significant correlations of the activities of PRX with the vigour data, which is different from the other enzymes reported in this study (Table 4 and 5). This implies that ROS accumulation, either due to generation under stress or a lack of degradation by detoxifying enzymes, may play an important role in germination and initial growth by regulating plant defences and maintenance of redox status (El-Maarouf-Bouteau and Bailly, 2008). High SOD activity suggests that seed tissue (such as embryos and the cotyledon) maintains a tight control of the respiratory pathway. If SOD activity decreases, there is a rapid degradation of many molecules due to ROS accumulation, which leads to cell death and consequent germination failure. Liu et al. (2007) proposed that superoxide formation (O_2^-) provides an optimal method for following seed vigour because it is formed early in inhibitory events. While according to Bailly et al. (2008) there are some limits that restrict germination to an oxidative or stress window, above or below which germination cannot occur either due to damage or maintenance of dormancy (Fig. 1), where ROS accumulation could provide an alternative to dormancy avoidance (Oracz et al., 2007 and El-Maarouf-Bouteau and Bailly, 2008).The appearance of protein laddering occurred either due to degradation by ROS, peptidases or oxidation (Job et al., 2005; Rajjou et al., 2008), or peptide condensation, all of which would change the cultivar protein profile (Fig. 2) by increasing the chemical kinetics within the cell. These phenomena lead to degradation of storage proteins, cellular disorganization, with subsequent vigour loss and seed death. According to Machado Neto et al. (2001) and in the present study, the protein electrophoretic patterns of *Phaseolus vulgaris* seeds under low stress did not correlate with the vigour tests variations, because there was almost no loss of the banding pattern.

Materials and Methods

Plant materials

Phaseolus vulgaris L. seeds from two Brazilian groups, carioca (IAC Carioca Akytã and IAC Carioca 80SH) and colours (Rosinha G2) were used because IAC Carioca 80SH and Rosinha G2 are ancient cultivars and IAC Carioca Akytã was a modern breeding line.

Ageing experiments

The cultivars were harvested from 4 seasonal harvests and these made up the 0, 1, 2 and 3 years of the natural ageing experiment. The seeds were harvested from the Universidade do Oeste Paulista (UNOESTE) experimental field and stored at 15±2ºC and 50%RH and constituted the material to be used in the natural ageing (low stress, Kranner et al., 2010) experiment. Along with fresh seeds (non-stored treatment) samples were sequentially removed from this environment after one, two and three years. Freshly harvested seeds from the non-stored lot, six months after harvested and stored, were artificially aged (high stress, Kranner et al., 2010) in a single layer over a sieve inside a plastic box containing 40 mL of water. These seeds, approximately 200 seeds per box, with four replicates per treatment, were exposed to 41ºC at 100% RH in a climatic chamber for 48, 96 or 144 h. Seeds were then re-dried at room conditions at 15±3ºC and 50%RH until their hygroscopic equilibrium had been re-established. A sample not submitted to these conditions was used as a control. To assess the effects of both stress environments, various physiological and biochemical parameters were measured.

Moisture content (MC)

The water content was measured throughout by drying in an oven at 105±3ºC for 24 h, using two replicates of fifty seeds per time sample for each seed lot (BRASIL, 2009).

Germination test (G and G5)

Four replicates of 50 seeds each were sown on a paper roll moistened with 2.2 times its original weight of water and kept in a germination chamber at 25ºC. Total Germination (G) was the sum of all germinated seed scored after eight days, while G5 recorded only the percentage of normal seedlings, with radicle and hypocotyl present five days after sowing.

V*igour classification (VC)*

This was defined by the proportion of normal seedlings which were morphologically complete and was measured after five days. Some seedlings appeared weak, or with minor defects, and these were omitted from. Only strongly growing seedlings were considered (Nakagawa, 1999).

Root and shoot length and dry weight (RL, SL, RDW and SDW)

These consisted of four replicates of ten seedlings taken from germination tests on day five. Dry weights of both the shoot and root, but excluding the cotyledons, were measured following oven drying with 0.001g of precision (Nakagawa, 1999).

Potassium leakage test (KL)

This test was conducted with four replicates of 25 seeds, which were weighed to a precision of 0.01 g, placed in a plastic cup filled with 100 mL of distilled water and stored at 25ºC for 60 minutes. The potassium leakage was determined using an Atomic absorption spectrophotometer (AAnalyst 200-Perkin Elmer), and the results were expressed as ppm $K^+.g^{-1}$ seeds (Custódio and Marcos Filho, 1997).

Enzyme extraction and analysis

One hundred seeds were taken from each treatment, soaked for 24h, the teguments removed and the embryo axis and cotyledon separated. These were separately ground at 4ºC in 0.1 M sodium phosphate buffer (pH 7.8) containing 0.4 g polyvinilpirrolidone, 2 mM dithiothreitol, 0.1 mM EDTA and 1.25 mM PEG4000. The extracts were centrifuged at 12,000 g for 20 minutes at 4ºC and the supernatant divided into four aliquots, one of which was used to quantify protein content (Bradford, 1976). The remaining aliquots were immediately

analysed or stored at -80ºC for subsequent use. They were used to measure the following enzymes:

Peroxidase (PRX, EC. 1.11.1.7)

One microgram of protein, adjusted to 10µL of extract, was added to 2.5 mL of 0.1 M phosphate buffer (pH 7.8) containing 13 mM of guayacol and 5 mM hydrogen peroxide, and the mixture was incubated for 20 minutes at 30ºC; the absorbance was measured at 470 nm using the procedure of Putter (1974).

Catalase (CAT, EC. 1.11.1.6)

Two hundred microlitres of the extract supernatant were added to 4.3 ml of 50 mM phosphate buffer (pH 7.0) containing 3.125 mM of H_2O_2. CAT activity was measured using H_2O_2 decay measuring absorbance at 240 nm and was expressed as nmol of H_2O_2 decay (mg protein)$^{-1}$ min^{-1} (Lei et al., 2005).

Superoxide Dismutase (SOD, EC.1.15.11)

Superoxide dismutase activity was measured using the method described by Lei et al. (2005), by adding50 µL of extract supernatant to 4.95 mL of 0.1 M phosphate buffer (pH 7.8) containing 13 mM methionine and 63 µM nitro blue tetrazolium (NBT), with 13 µM riboflavin as a modification to the procedure. The tubes were incubated at 25ºC for 15 minutes under fluorescent lamps, centrifuged at 10000rpmfor 5 min and absorbance was measured at 560 nm. Tubes containing the same medium without the extract, but not subjected to light, were used as a control. One unit of SOD (mg protein)$^{-1}$ was defined as the enzyme activity able to inhibit the NBT photoreduction to blue formazan by 50%. SOD data were normalized by protein content, determined by the Bradford method (1976).

Protein electrophoresis

To verify the quality of the storage proteins following low and high stress conditions they were examined through SDS-PAGE. Soluble proteins were extracted from five seeds, which were ground in liquid nitrogen and placed in a tube containing 2.0 mL extraction buffer (0.625 M Tris-HCl, pH 6.8; 2% sodium dodecyl sulphate; 5% 2-mercaptoethanol; 20% glycerol). The tubes were shaken and incubated at room temperature for 1 h and then boiled for 3 min. The solution was centrifuged at 9,500 rpm for 10 mins. The pellet was re-extracted once as described above, and the resulting supernatants were mixed and stored at -80°C until analysis. Protein was quantified according to Bradford (1976).
Electrophoresis was performed according to Laemmli (1970) in a system comprising gel containing 10% acrylamide-bisacrylamide (30:0.8), pH 8.8, and a stacking gel with 2.5% acrylamide-bisacrylamide, pH 6.8. Aliquots of 30 µg of protein were loaded into each well and run with an appropriate molecular ladder (Sigma Wide range; 205 up to 6,5 kDa). The running buffer was composed of Tris (25 mM), glycine (38 mM) and SDS (0.7 mM) at pH 8.8. The gel was run at 50 V and 2mA for 30 min and 100V and 20 mA for 4 h. The gels were fixed with a mixture of isopropanol, acetic acid and water (4:1:5) for 30 min and stained in the same solution (with the addition of 2% Coomassie Blue R250) until bands appeared. The gel was destained in 10% acetic acid and examined under white fluorescent light in a transilluminator; the image was analysed using an image analysis system (Quantum-ST4-1000).

Statistical analysis

The experiment was a completely randomized design with four replications per treatment. Data for germination, vigour classification and 5th day germination were arcsine transformed, while other data were not transformed prior to analysis.

An ANOVA F test was performed, and means were submitted to Tukey's multiple comparison test ($P<0.05$) using SISVAR software (Ferreira, 2008) for each type of ageing, physiological and biochemical measurement. Physiological data were tested for linear correlation with PRX, CAT and SOD activities using a regression *t* test at the 5% level of probability.

Conclusions

Enzymatic variation must be correlated with physiological test results if that information is to be used as a meaningful indicator of seeds' physiological status and quality. In this study, insufficient evidence was obtained to justify the use of enzymatic activity as an indicator of conservation or degradation status alone. It is likely that the lack of significant correlations is due to the way in which seed tissues respond to stress, and the effectiveness of repair mechanisms. small differences in quality among lowly stressed batches, which exhibited inconsistencies between the vigour tests as enzyme determinations increased. These results indicate that the tests are promising indicators of changes occurring within seeds under high stress. The potassium leakage test, vigour classification and 5th day germination were good predictors of seed quality when there were considerable differences due to high stress conditions. Nevertheless, when the differences among low stressed lots were not significant, tests based on the length and dry weight of the roots and shoots were more efficient. SOD and CAT determinations methods are easiest to perform but were only related to vigour tests in seeds under high stress conditions. SDS-PAGE exhibited differences in strongly stressed seeds. Thus, seedling development parameters are better than detoxifying enzymes to assess vigour evaluation on common bean. Vigour tests under development were not accurate when performed only on seeds that had been aged artificially. It is important that vigour tests will be confirmed in seeds that present naturally different physiological potentials. The rising divergences among the tests in lowly stressed seeds indicated the need of a continuous study and improvement of vigour tests to efficiently detect slight vigour differences, which could be automated to detect the deterioration process in its initial steps.

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