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Article

Oxidative Stress Parameters, Related Trace Elements Levels and Proteomics in Soybean Seeds in Order to Get a Better Assessment of Their Quality

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Antioxidant systems, Fe, Cu, Zn, Mn and Ni levels and proteomics in soybean seeds of different performance in field were assessed. These results were correlated with the *in vitro* tests required by regulatory organisms, and the actual behavior in field. Basal superoxide content, superoxide dismutase (SOD) activity (20-80 U mg^{-1} protein) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (4.6-7.0 mg antioxidant *per* g dry seed) permitted to study the oxidative stress of seeds. Essential metal levels were in accordance to previous reports, with the exception of Zn that was higher in some batches of good *in vitro* quality, but poor performance in field. Proteomic profile showed no differences between batches. Two of the studied parameters (basal superoxide content and Zn level) contribute to a better assessment of the health state of the seeds and predict a possible poor performance in field.

Keywords: soybean seed, oxidative stress, SOD, trace elements, proteomic

Introduction

Soybean seed is an important food source for humans and livestock worldwide, representing one of the most important protein and oil supply. In fact, 27.2 kg of soybean produces 21.8 kg of soybean protein meal and 4.99 kg of soybean oil. The inclusion of soybean in human daily diet increased in view of its speculated role in providing protection against illnesses such as atherosclerosis, diabetes, among others.[1,2]

For this reason along with new direct seeding techniques and high international prices, soybean crop went from being marginal to become the main agricultural crop in less than 10 years in producing countries such as Uruguay. Consequently, the reduction in the quality of seeds can unfavorably affect market value of the crop, producing great economic losses. There are several factors affecting seed's healthy development, and damage is caused both by biotic or abiotic factors. Abiotic factors include mechanical rupture of grains during harvest or transportation, climatic episodes like when it rains a lot previous to harvest so rapid hydration and dehydration of seeds produces partial degradation of coat, unbalanced content of salt and essential metals in soil, high temperature and humidity during storage that contribute to increase infections, and chemical contamination by pesticides. Biotic factors mainly include damage caused by insects and microorganisms.[3]

Because there are several factors affecting healthy development, interdisciplinary approaches are currently necessary to study the causes and consequences regarding this issue.

To ensure a good harvest, the Uruguayan government through the National Seed Institute (INASE) regulates the procedures for the evaluation of seed quality before marketing that consists of several *in vitro* tests such as germination, vigor and viability, heeding the International Seed Testing Association (ISTA) rules.[4]

Germination tests were designed to grow seeds under optimum conditions of oxygen, light, moisture and temperature, while vigor tests measure characteristics that are associated with the speed and uniformity of germination and seedling growth under unfavorable environmental

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conditions and the behavior to maintain the ability to germinate after storage. The objective of vigor analysis is to detect significant differences in the physiological quality of lots, complementing the information of germination tests. Certain lots of seeds that have high percentages of germination may have different behavior when they grow in field. This occurs because the seeds lose vigor before losing their ability to germinate. Viability tests measure viable embryos through different techniques determining living tissue in whole seeds.[5]

Despite the results of germination, vigor and viability *in vitro* tests required by INASE, producers in the region observed that certain batches of seeds of similar tests results did not produce good crops in the field, even when planted in similar soils and with the same climate as well as the same planting procedures.

In order to collaborate in obtaining knowledge that can shed light on this issue and to have good predictive power of seed development in the crops, we focus on the study of oxidative stress of soybean seeds with good and poor germination and in the determination of essential trace metals associated with antioxidant systems. To the best of our knowledge, few studies were performed in soybean seeds in the Mercosul (Mercado Comum do Sul) region and only a few references were found in the literature.[6-8]

As it is well-known, relatively high levels of reactive oxygen species (ROS) including the superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), can produce oxidative stress. These bioproducts are generated as a result of normal aerobic metabolism, but if the organisms are not able to control the intracellular ROS level, these species can produce damage in membrane lipids, proteins and nucleic acids leading to cell death.[9] In particular, the oxidative stress produced in seeds by drought, high temperatures, salinity and toxic metals among others, can cause molecular damage directly or indirectly through the formation of ROS.[10-13]

To scavenge ROS and avoid oxidative damage, efficient antioxidant systems, both enzymatic and non-enzymatic, are present in all cells. One of them is the group formed by superoxide dismutases (SOD) that catalyze the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. These enzymes are Cu and Zn, Mn, Fe or Ni dependent and it was reported that if the metal levels are low the activity of these enzymes decreases and different disorders can appear.[9,14]

Besides there are differences in SOD levels depending on seed genotype, location, climatic and storage conditions and presence of toxic metals, among others.[12,13,15,16]

Literature reports that genetic modifications change the proteome of the soybean seeds, as well as the metallome of the plant.[17]

Considering this, it was postulated that if the metallome changes in transgenic plants, also the ability of the plant to take in metals could change, thus affecting the production of ROS and the plant is more prone to oxidative stress.[18]

Barbosa *et al*.[8] reported that the SOD levels are lower in transgenic soybean seeds compared with non-transgenic ones and conclude that the genetic modification itself could be a stress factor; provoking changes in the activity of some enzymes as well as alterations in the soybean seed proteome. Nowadays, almost all soybean seeds commercialized in Uruguay are transgenic.[19]

In this work, soybean seeds of different performance both *in vitro* quality tests and in field were assessed for antioxidants, both enzymatic and non-enzymatic and superoxide content. Also as part of our research on metallomics involving metals in biomolecules, comparative proteomics[20] and Cu, Zn, Mn, Fe and Ni levels were determined. All these integrated results allowed us to study possible correlations between antioxidant capacity, *in vitro* quality parameters and actual development of the plants in the crop. Consequently, this work provides information about soybean seeds concluding that the incorporation of new *in vitro* tests could be useful for better prediction of the performance in field.

Experimental

Materials

All reagents and solvents were of analytical grade. Ultrapure water of 18.2 MΩ cm resistivity was obtained from a Millipore Simplicity 185 purifier. All glassware was soaked overnight in 10% (v/v) nitric acid and then rinsed exhaustively with distilled water.

Spectrophotometric measures were performed using a Thermo Scientific Evolution 60 spectrometer.

The determinations of the trace elements Fe, Cu, Zn, Mn and Ni were performed by means of flame atomic absorption spectrometry (FAAS) technique using a PerkinElmer Analyst 200 spectrometer. An acetylene-air flame was used for all the elements and the wavelengths (nm) were 248.3 (Fe), 324.8 (Cu), 213.9 (Zn), 279.5 (Mn) and 232.0 (Ni).

Seed samples

Transgenic yellow soybean seeds (Glycine Max (L) Merrill) were provided by Agropecuaria Valdense (Juan Jorge Ferreira, Colonia). Size for individual seeds ranged from 0.618 to 0.864 cm of diameter. Samples from 16 different batches were analyzed, categorized as follows:

(*i*) good germination (GG): 13 batches showed > 70% in germination test, ranging 74-97%. In spite of the fact that they have adequate germination results, they performed differently in field: good performance in field (GG-GPF), eight batches performed well in field, yielding a good harvest; and poor performance in field (GG-PPF), five batches performed poorly in field, yielding poor harvests that produced economic losses; (*ii*) poor germination (PG): three batches showed < 70% in germination test. These batches were not approved to be commercialized.

Enzyme extraction method

After the optimization of the extraction method, the following procedure was performed in the subsequent experiments: 1 g of milled and dried (80 °C, 24 hours) soybean seed was mixed with 0.2 g of polyvinylpyrrolidone (PVP) and 10 mL of extraction solution (buffer phosphate, pH 7.4), was added. The homogenate was stirred for 1 hour and then centrifuged at 28,000 g for 15 min. The supernatant was extracted using a PD-10 Sephadex G-25 column (Sigma). The eluate was collected in ten fractions of 2 mL each. These fractions were used to estimate protein concentration by measuring the absorbance at 280 nm and SOD activity. Elution profiles were plotted to determine the fraction of maximum protein concentration (FMPC).

The SOD method of extraction from seed was optimized by means of multivariate experiments (central composite design).[21] The variables stirring time (min) and volume of extraction solution (mL) were studied in three different levels, 15, 30 and 60 min for stirring time and 10, 20 and 40 mL for the volume of extraction solution. The effect of PVP concentration was evaluated in the range of 0.2 and 1 g.

Superoxide dismutases (SOD) activity

SOD activity (U mg^{-1} protein) was determined by the method based on the inhibitory effect of SOD over the reduction of nitrobluetetrazolium (NBT) by the O_2^{-} generated by xanthine/xanthine oxidase system, adapted from Beauchamp and Fridovich's method.[22]

To 0.1 mL of the fraction of maximum protein concentration (FMPC) obtained in 2.3 were added 0.2 mL of 4.6 mmol L^{-1} xantine, 0.1 mL of 0.75 mmol L^{-1} NBT, 0.1 mL of 3 mmol L^{-1} ethylenediaminetetraacetic acid disodium salt (EDTA), 0.1 mL of 1.5 mg mL^{-1} bovine albumin, 0.1 mL of 10 mg mL^{-1} xanthineoxidase and buffer phosphate (pH = 7.4) until a final volume of 2 mL. Simultaneously a control assay was carried out using buffer phosphate (pH = 7.4) instead of FMPC. After incubation at 28 °C for 30 min, the reaction was stopped with 0.1 mL of 6 mmol L^{-1} $CuCl_2$ and the absorbance was measured at 560 nm. Also absorbance at 560 nm of 0.1 mL of FMPC diluted in buffer phosphate (pH = 7.4) till 2 mL was measured as blank. All the determinations were performed in duplicate.

Estimation of basal superoxide anion level

To 0.1 mL of FMPC obtained as previously indicated, 0.1 mL of 0.75 mmol L^{-1} NBT and buffer phosphate (pH = 7.4) until 2.0 mL were added. To assess superoxide content the absorbance at 560 nm was measured. A blank was prepared with buffer phosphate. All the determinations were performed in duplicate.

Antioxidant capacity: 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

1 g of milled and dried (80 °C, 24 hours) soybean seed was mixed with 5 mL of ethanol, stirred for 4 hours and centrifuged at 2,054 g for 15 minutes. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of the extract was evaluated using an ethanolic solution of DPPH following Brand-Williams method.[23,24] To 0.2 mL of ethanolic extract, 2 mL of an ethanolic solution of DPPH (prepared in such a way that its absorbance at 517 nm was between 0.9 and 1) was added. After 30 min the absorbance of the mixture (A_s) was measured at 517 nm. The same procedure was followed with a control solution containing 0.2 mL of ethanol and 2 mL of DPPH solution.

Quenching (Q) was calculated as $Q = 100 - (A_s \times 100/A_c)$ where A_s is the absorbance of the sample and A_c is the absorbance of control. Ascorbic acid was used as control standard. All the determinations were performed in duplicate.

Comparative proteomics

The protein extraction, precipitation and separation procedures for 2 GG-GPF batches of soybean seeds and 2 GG-PPF batches were performed as previously reported by Brandão *et al.*[20] The program Image Master 2D Platinum was used to analyze the images.

Trace elements determination

Fe, Cu, Zn, Mn and Ni concentrations were determined in all the batches of seeds. The seeds were milled, and 5 g of the obtained flour was digested according to the AOAC 975.03 procedure.[25] All the determinations were performed in triplicate. To ensure the accuracy, a reference material

was also analyzed (National Institute of Standards and Technology-NIST 1567a Wheat Flour).

Results and discussion

Enzyme extraction method

Regarding the optimization of enzyme extraction, 10 mL of extraction volume and a stirring time of 60 min yielded the highest protein concentration. As expected, these experiments showed that the protein extraction was higher when the volume was reduced and the stirring time is increased, but when working below 10 mL of extraction buffer a semi-solid paste was formed and the stirring was not effective. As for PVP, the study showed that the use of more than 1 g produce an inefficient separation in the column.

Elution profiles for all samples showed that the fourth fraction was the most concentrated in protein (FMPC) and in SOD activity (Figure 1). Fractions 3 to 7 had to be diluted (1/10 or 1/100) in order to obtain a signal within the instrument scale. Mean absorbance at 280 nm of FMPC for seeds of different performance (GG-GPF, GG-PPF and PG) showed no significant difference (protein content ranging from 7.87 to 15.60 g mL^{-1}). Thus, total protein content in FMPC does not correlate to seed performance.

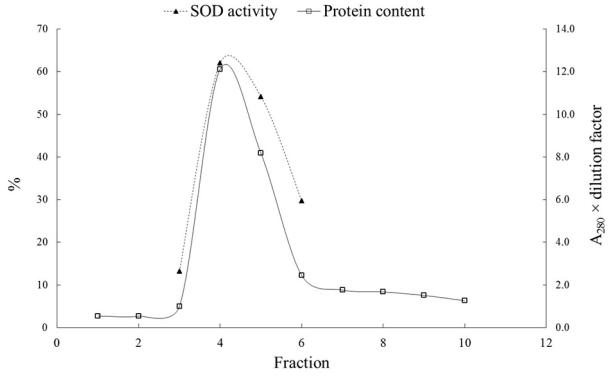

Figure 1. Elution profile for a particular batch.

SOD activity and superoxide level

SOD activity for seeds in Mercosul region ranged 20 to 80 U mg^{-1} protein. To our knowledge, SOD activity varies with latitude, genotype[8,12,14,15] and time post-harvest as well as storage conditions.[26,27] SOD activity for seeds in this study are in accordance with those reported by Barbosa *et al.*[8] for transgenic seeds in the region. No significant difference in SOD activity was found between GG-GPF and GG-PPF batches while SOD activity is significantly higher for GG batches (mean 49 ± 16, n = 13) as compared to PG batches (mean 17 ± 10, n = 3) (Table 1).

Table 1. Mean and standard deviation (SD) values for SOD activity and basal superoxide level of transgenic soybean seeds

Batch	SOD[a] activity / (U mg^{-1})	Basal superoxide level ($Absorbance_{560}$)
GG[b]	49 ± 16	0.016 ± 0.006
PG[c]	17 ± 10	0.023 ± 0.007
GG-GPF[d]	50 ± 14	0.012 ± 0.002
GG-PPF[e]	48 ± 20	0.022 ± 0.006

[a]Superoxide dismutase; [b]good germination (GG); [c]poor germination (PG); [d]good performance in field (GG-GPF); [e]poor performance in field (GG-PPF).

Reduced SOD activity for seeds that failed to germinate is in agreement with the fact that faulty SOD can affect physiological processes and consequently produce adverse effects preventing healthy germination.

Basal superoxide anion level showed no significant difference between batches of different germination test result (GG and PG in Table 1). However, when GG seeds were discriminated based on their performance in field, the ones with good performance (GG-GPF) presented significantly lower superoxide level than those of poor performance (GG-PPF). Therefore, for seeds of good performance in field superoxide level is significantly lower than for those seeds that failed to produce good crops or were not planted due to their poor germination test.

High superoxide level and reduced SOD activity suggests that there is a level of oxidative stress in PG seeds in accordance with the *in vitro* quality tests performed by INASE. On the other hand, GG-PPF seeds show that superoxide levels are elevated but SOD activity still counteracts its effect, permitting its germination. In our study, seeds that performed poorly in field had normal SOD activity, did well in the *in vitro* germination test but had significantly higher superoxide level than those that performed well in field. So this level of oxidative stress was not detected by the *in vitro* quality test germination Gidrol *et al.*[28] observed in natural and artificially aged soybean seeds that at first both superoxide and SOD levels can increase, probably due to the fact that superoxide anion, besides being cytotoxic (depending on its concentration), also plays role as signal molecule in biochemical plant response. Thus, healthy seeds maintain a balance between SOD levels and ROS.

SOD activity and basal superoxide level for GG batches related to *in vitro* parameters as expected (Figures 2a and 2b).

Slope and R^2 for germination were in accordance with those reported in the literature.[26] There is a poor correlation found between the germination test and the biochemical parameters evaluated individually and no correlation was observed for the viability test (the less reliable test

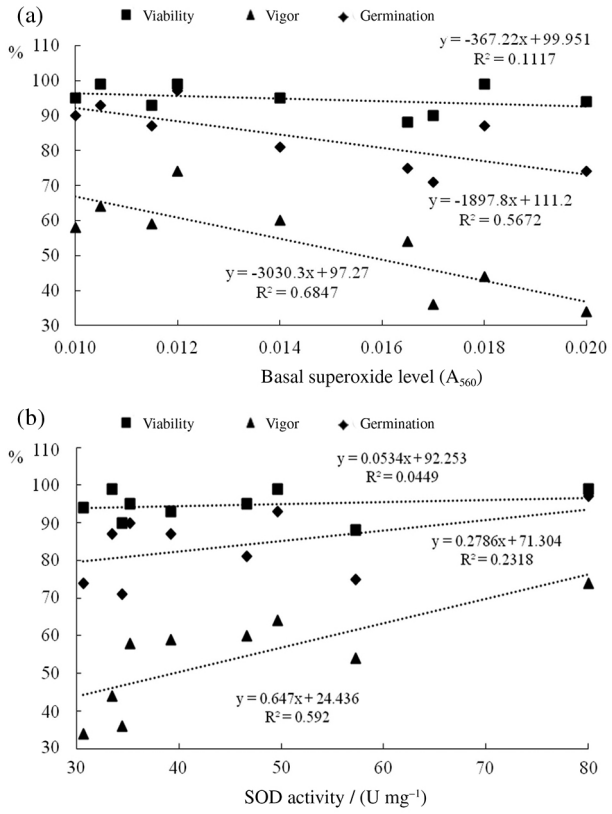

Figure 2. *In vitro* quality parameters related to basal superoxide level (n = 9) (a) and (b) SOD activity.

according to the Food and Agricultural Organization, FAO).[5]

Antioxidant capacity: DPPH scavenging activity

Antioxidant capacity of plant material is ascribed to the presence of phenolic compounds which are not only present in inner parts but also present at elevated amounts in the outer parts of fruits, leaves or seeds.[29] Yield and composition of extracts are dependent on method of extraction and solvents polarities. Methanol, ethanol, acetone, water, and their combinations are frequently used for the extraction of phenolic compounds.[30] So, using this method antioxidant level (non-enzymatic) was evaluated. The results expressed as Q (Figure 3) are in agreement with those reported for yellow type of soybean seeds.[29]

In the conditions of this experiment, no significant differences were observed for DPPH scavenging activity among the different batches of soybean seeds. Results ranged 4.6-7.0 mg antioxidant *per* g dry seed for GG seeds (n = 13) and 5.5-5.7 mg antioxidant *per* g dry seed for PG seeds (n = 3). No difference was observed when GG seeds were discriminated according to their behavior in field. Therefore, non-enzymatic antioxidant systems appear to be similar either for healthy or impaired seeds.

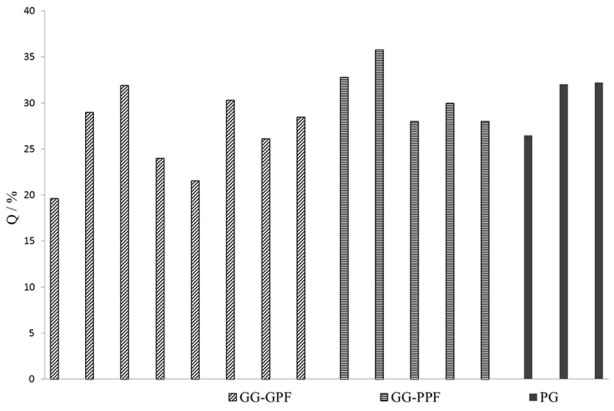

Figure 3. DPPH scavenging activity.

Comparative proteomics

The protein mass loaded was 500 μg for the 4-7 pH range gels (shown in Figure 4) and the number of the spots obtained for the four GG batches were 460, 442, 453 and 472 respectively. The obtained profiles are in accordance with the fact that they correspond to transgenic soybeans as compared with those reported by Brandão *et al.*[20]

The results show no significant difference in the number of spots for seeds of different performance in field (Figure 4). In all samples, the proteins were efficiently separated and evaluated, including those involved in nutrient storage like glycinin (subunits 30-45 kDa) and β-conglycinin (subunits 50-95 kDa) which upon germination of the soybean seed, will be digested, and the released amino acids will be transported to locations of seedling growth. In addition, this is good agreement with the fact that SOD activity is similar for these batches. According to this profile the four batches had the same probability of good performance in field in agreement with the fact that they had a percentage of germination higher than 70 in the *in vitro* tests. Thus, protein composition of the analyzed samples does not correlate to seed performance in field.

Trace elements levels

Soybean seeds presented ranges of: 47.0-90.6 mg kg^{-1}, 10.7-15.0 mg kg^{-1}, 19.5-24.1 mg kg^{-1}, 3.9-5.8 mg kg^{-1} for the SOD cofactors Fe, Cu, Mn and Ni, respectively (dry basis). Analytical precision was better than 6% in all cases (expressed as relative standard deviation, RSD, n = 3). These results are in accordance to the reported ranges in the literature.[31-34] No significant differences between the Cu, Fe, Mn and Ni concentrations were observed either for GG seeds compared to PG seeds or for GG with different field performance. Besides, similar levels of Cu, Fe, Mn and Ni in GG seeds are in accordance with the SOD activity

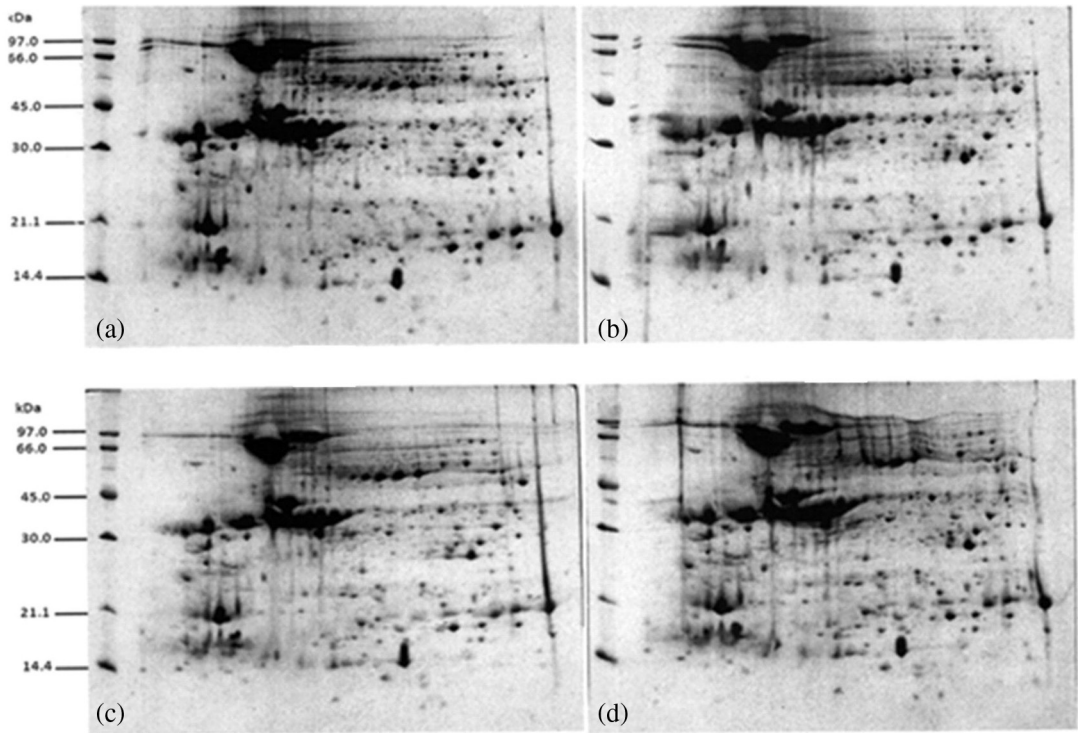

Figure 4. 2-D PAGE gels analyzed with Image Master 2D Platinum program. (a) and (b) GPF seeds; (c) and (d) PPF seeds.

presented in section SOD activity and superoxide level and with the proteomic profiles in the analyzed samples of section Comparative proteomics.

For Zn, the results were in the range 33.9-51.5 mg kg^{-1} (RSD < 5%) for all the batches, except two of them corresponding to GG-PPF seeds presenting values of 64.5 and 65.2 mg kg^{-1}. SOD activity for these batches were 71 and 68 U mg^{-1} protein, respectively. The results of this study show that high levels of Zn do not affect SOD activity enzyme. In addition, the explorative proteomic comparative study confirms no significant difference in the proteomic profile for seeds of different Zn level.

However, as reported by Wang *et al*.,[35] high levels of Zn can have negative effects on plants. Excess of Zn inhibits seed germination, plant growth and root development. It can also induce oxidative stress, by suppression of the uptake of other nutrient elements (secondary deficiency).[36] These facts are in accordance with the respective high results for superoxide (A_{560} for these batches 0.024 and 0.020) and suggest a nutritional imbalance that may cause and oxidative stress by Zn, contributing to damage the seeds and thus further inhibit plant growth.

Besides, it was reported that heavy metals such as cadmium and lead, could affect the uptake and the translocation of some nutrients in plants. Cadmium is a toxic element associated with the environment and seeds can accumulate it. In a previous work, Cd level in the same batches of soybean seeds was determined. The results were in the range of 0.02 and 0.11 mg kg^{-1} which meet the requirements of the regulations of the region for food (< 0.2 mg kg^{-1}). So Cd level is not responsible for the behavior of these batches of seeds.

Conclusions

This study provides objective information on the possible causes for different performances of some soybean seeds.

For PG seeds, rejected by INASE, loss of SOD activity and high superoxide level, evidencing oxidative stress, correlates to *in vitro* tests, being either the cause or consequence of other processes of seed deterioration. The loss of detoxifying enzyme activity is not due to metal deficiency since SOD cofactors Cu, Zn, Fe, Mn and Ni levels are adequate.

Some commercial seeds approved by INASE, with poor performance in field showed high levels of Zn and basal superoxide radical. Since the diminished quality was not detected by the *in vitro* tests, the determination of complementary parameters such as superoxide radical and Zn levels could be useful to predict a possible poor performance in field.

Commercial seeds approved by the regulatory governmental organism, with good performance in field showed no abnormal results for all parameters and correlate to *in vitro* tests.

To sum up, relevant information based in metallomics and biochemistry helps to know more about the conditions that affect the quality of a food that has a high impact on the economies of the countries.

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