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Short Communication
Exogenous antioxidants on quality of cabbage seeds

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Abstract
During seed germination there is production of reactive oxygen species, which, in a controlled way, are important to cell signaling and protection against pathogens, but, in excess, impair germination. Therefore, the objective of this study was to assess the action of different compounds on antioxidant mechanisms and enzymatic activation in cabbage seeds. Compounds like kojic acid, thymol and tyrosol were used to imbibe the cabbage seeds together with distilled water, and a control treatment without imbibition was used as well, with subsequent assessment by means of germination test, endosperm rupture, vigor, radicle protrusion, and assessment of seedling biochemical analyses by the activity of enzymes ascorbate peroxidase, catalase, superoxide dismutase and α-amylase. Data were subjected to analysis of variance and to the LSD means comparison test. Seeds treated with tyrosol presented higher results on the rupture of the endosperm, germination and vigor, and root development increased with use antioxidants. For the activity of antioxidant enzymes in seedlings, only kojic acid showed increase in the superoxide dismutase activity. There was also a reduction in the catalase activity with the use of thymol and tyrosol compounds compared to dry-seed assessments. After tyrosol treatment, ascorbate peroxidase enzyme was not detected, and water-imbibed seeds showed higher α-amylase activity. The use of antioxidant compounds has beneficial effects on cabbage seeds, and soaking with tyrosol led to better physiological quality, with activation of antioxidant defense mechanisms during germination.
Key words: enzymology; germination; oxidative stress.

Resumo
Durante a germinação das sementes, há produção de espécies reativas de oxigênio, as quais, de maneira controlada, são importantes para a sinalização celular e proteção contra patógenos, mas, em excesso, prejudicam a germinação. Portanto, o objetivo deste estudo foi avaliar a ação de diferentes substâncias sobre mecanismos antioxidantes e ativação enzimática em sementes de repolho. Substâncias como ácido kójico, timol e tirosol foram utilizadas para embebição das sementes de repolho juntamente com água destilada e um tratamento controle sem embebição, com posterior avaliação por meio do teste de germinação, ruptura do endosperma, vigor e protrusão da radícula, e análises bioquímicas em plântulas por atividade das enzimas ascorbato peroxidase, catalase, superóxido dismutase e α-amilase. Os dados foram submetidos à análise de variância e ao teste de comparação de médias de LSD. Sementes tratadas com tirosol apresentaram maiores resultados na ruptura do endosperma, germinação e vigor, e o desenvolvimento radicular aumentou com o uso dos antioxidantes. Para a atividade de enzimas antioxidantes em plântulas, apenas o ácido kójico apresentou aumento na enzima superóxido dismutase. Houve uma redução na atividade da enzima catalase com o uso de timol e tirosol, em comparação às avaliações de sementes secas. Para o tratamento com tirosol, a atividade da enzima ascorbato peroxidase não foi detectada e as sementes embebidas em água apresentaram maior atividade da α-amilase. Os usos de substâncias antioxidantes apresentaram efeitos benéficos nas sementes de repolho, e a embebição com o tirosol levou à melhor qualidade fisiológica, com ativação de mecanismos de defesa antioxidante durante a germinação.
Palavras-chave: enzimologia; germinação; estresse oxidative.

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Introduction

Seed germination naturally promotes the synthesis of reactive oxygen species (ROS) (Kumar *et al*. 2015), a response similar to that triggered by biotic and abiotic stresses (Gill & Tuteja 2010).

ROS at low concentrations play a vital role in cell signaling, which supports and makes germination viable, in addition to having a protective effect against pathogens. However, in excess, ROS accumulation impairs germination due to oxidative damage in proteins, lipids and deoxyribonucleic acid (Awasthi *et al*. 2017; Kumar *et al*. 2015), which, inevitably, promotes the activation of the vegetal defense system, synthetizing antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), all responsible for ROS elimination or reduction (Xiong *et al*. 2018; Beyaz *et al*. 2017).

Superoxide dismutase (SOD) is the first enzyme that acts on the defense mechanism against oxidative stress and breaks free oxygen into oxygen peroxide (H_2O_2), followed by the coordinated action of a set of enzymes including catalase (CAT) and ascorbate peroxidase (APX), which convert H_2O_2 into water and oxygen (Beyaz *et al*. 2017; Barreiros *et al*. 2006).

The application of exogenous compounds with antioxidant action can protect seeds against ROS overproduction; thus, they stimulate their resilience, which will guarantee quality in germination due to the production of enzymes that sequester or degrade free radicals (Ratnam *et al*. 2006; Serkedjieva 2011). Among the few studies on the effects of exogenous application of antioxidant compounds on seeds, it has been observed that folic acid and ascorbic acid applied at concentrations of 0 to 500 µM improve the vigor of pea seeds, reflected in agronomical and biochemical responses, through the stimulation of the antioxidant response (Burguieres *et al*. 2007), and the use of tyrosol and kojic acid compounds protects against oxidative stress damage and promotes benefits to seeds metabolism (Macedo *et al*. 2018).

In light of the foregoing, further research on the use of antioxidant compounds and their effects on the germination process are important so that they become an alternative in agriculture. Therefore, the objective of this study was to assess the action of kojic acid, thymol and tyrosol, on antioxidant mechanisms and enzymatic activation in cabbage seeds.

Material and Methods

Cabbage seeds (Agristar/TopSeeds®) were sown and assessed under controlled conditions at the Laboratory of Vegetal Production Physiology and Metabolism, Federal University of Viçosa [Universidade Federal de Viçosa], Campus of Rio Paranaíba/MG. The seeds were subjected to treatments with compounds of recognized antioxidant potential.

The treatments analyzed consisted of: dry seeds (control), seeds imbibed in distilled water and in antioxidants solutions, kojic acid (MM: 142.11 g mol^{-1}, analytical purity ≥ 98.5%), thymol (MM: 150.22 g mol^{-1}, purity of 98.5%) and tyrosol (MM: 138.16 g mol^{-1} and purity of 98.0%), provided by Sigma®, and diluted at the concentration of 1 mg L^{-1}; the pH of the solutions were 9.94, 10.32 and 10.36, respectively. Screening tests with different doses allowed obtaining the best dose under study. Subsequently, the seeds were imbibed for 2 hours, then dried under ambient conditions in the laboratory, after which the tests were assembled.

The physiological quality of the cabbage seeds, conducted using four repeats of 50 seeds, was determined by the germination test in gearboxes with two sheets of germitest® paper moistened with distilled water, using a volume equivalent to 2.5 times the weight of the paper. The boxes were placed in a germination chamber, B.O.D type (Biochemical Oxygen Demand), regulated at a constant temperature of 20 ºC and photoperiod of 12 hours. On the fifth day, the first count test (vigor) was done, obtained by the number of normal seedlings, and, on the tenth day, the germination test was conducted (Brasil 2009). For both tests, results were expressed as percentage (%).

In the first 48 hours after test assembling, the endosperm rupture was measured, characterized by the cracking of the seed's tegument and emission of 0.2 mm of embryonic axis expansion, meaning visible radicle segment. The results were presented in percentage.

Daily and at the same hour, during the germination and vigor assessment period, the number of seeds with radicle protrusion was counted (Maguire 1962); only those with radicle emission measuring at least five millimeters in length were considered, and results were expressed as percentage.

At the end of the physiological quality tests, biochemical analyses were performed on cabbage seedlings. Total soluble proteins were sourced by macerating 100 mg of plant tissue in 1.5 mL of Tris-HCl buffer (Bradford 1976). To obtain crude enzymatic extract, 200 mg of seedlings were weighed and macerated in liquid nitrogen (Peixoto *et al*. 1999). Subsequently, the extract was used to measure the activities of antioxidant enzymes.

Ascorbate peroxidase (APX) activity was performed by taking a 37.5 µL aliquot of the enzyme extract, which was added to 1,500 µl potassium phosphate buffer 200 mM pH 7.0, 150 µl ascorbic acid 10 mM, 1,050 µl H_2O Milli-Q at 27 °C and still 150 µL H_2O_2 250 mM, for the moment of reading. Absorbance decrease at 290 nm was measured for one minute every 10 seconds and the enzymatic activity was calculated according to Nakano & Asada (1981).

For CAT determination, according to Anderson *et al*. (1995) and Havir & McHale (1987), a 37.5 µL aliquot of the enzyme extract was added to 1,500 µl potassium phosphate buffer 200 mM pH 7.0, 1,200 µl H_2O Milli-Q at 27 °C and 150 µl H_2O_2 250 mM. The enzyme activity was determined by measuring the absorbance reduction of the samples at 240 nm, due to H_2O_2 consumption.

SOD activity was determined by adding 100 µl enzyme extract to 1,880 µl of a solution containing 1,000 µl phosphate buffer of potassium 100 mM pH 7.8, 400 µl methionine 70 mM, 20 µl EDTA 10 µM, 150 µl NBT 1 mM, 310 µl H_2O Milli-Q at 27 °C and 20 µl riboflavine. The reaction occurred in a chamber, under 15W fluorescent lighting, exposed for 10 minutes. The samples were taken to reading at a 560 nm wavelength. An enzyme unit was defined as the amount of enzyme required to inhibit the NBT photoreduction by 50% (Del Longo *et al*. 1993; Giannopolitis & Ries 1977).

For assessment of α-amylase enzyme, 1 gram of cabbage seedlings were collected and macerated in phosphate buffer solution (pH 6.9), following recommendations by Fuwa (1954), adapted by Duran *et al*. (2018).

Total soluble protein, enzymatic activity and α-amylase readings were performed on a PerkinElmer UV-VIS Spectrometer Lambda 25 spectrophotometer.

The design employed was completely randomized, with four repeats of 50 seeds for physiological quality tests, and three repeats for biochemical analyses. Data were subjected to analysis of variance and the means comparison test (LSD), at a 5% significance.

Results

The use of different antioxidant compounds had a significant effect on the germination and vigor of the cabbage seeds (Fig. 1a-b). It was evident that seeds treated with tyrosol presented higher mean values for germination and vigor (99%) and differed from the control, which expressed values of 95 and 92%, respectively.

The rupture of the seeds' endosperm (Fig. 2a) showed the same germination and vigor behavior; the treatment with tyrosol had the highest mean values observed (70%) and was significantly distinct from the dry-seed treatment (control with 52% of rupture). Root development (Fig. 2b) increased with the days and with the antioxidant treatments.The activity of antioxidant enzymes in seedlings from cabbage seeds treated with antioxidants compounds indicated that, for superoxide dismutase (Fig. 3a), only kojic acid induced significant increase in its activity (2.43 U mg^{-1} prot) compared to controls (1.60 and 1.75 U mg^{-1} prot for dry seeds and imbibed in water, respectively). Whereas there was a significant

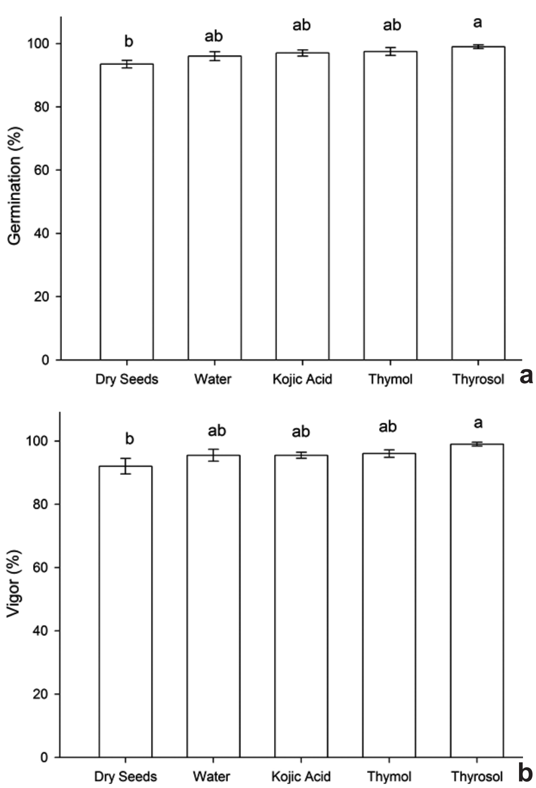

Figure 1 – a-b. Cabbage seeds treated with antioxidants – a. germination; b. vigor.

reduction in the catalase activity (Fig. 3b) with the use of thymol and tyrosol (27.90 and 28.76 µmol min^{-1} mg^{-1} prot, respectively) compared to dry-seed assessments (57.00 µmol min^{-1} mg^{-1} prot). Ascorbate peroxidase activity (Fig. 4a) showed no difference in the action mechanism for the antioxidants used, except for tyrosol, where APX production was not detected. For α-amylase (Fig. 4b), water-imbibed seeds showed higher activity (8.02 mg g^{-1} min^{-1}) and differed from the other treatments, while those imbibed in kojic acid, expressed lower activity (1.25 mg g^{-1} min^{-1}).

Discussion

The use of antioxidant compounds in seeds, though incipient, has presented favorable results for its application. In arabidopsis seeds, no phytotoxic action of tyrosol on seed quality was found (Reigosa & Malvido-Pazos 2007), while Macedo *et al*. (2018) reported beneficial action from this same compound on the quality of wheat seeds. In the present study, tyrosol increased the germination, vigor and endosperm rupture of the cabbage seeds.

The antioxidants did not have phytotoxic effect on the cabbage's root development, and it is possible to infer a constant and active increase in growth with the days and with antioxidant compounds applied. For a fast and reliable bioassay, plant species under study must germinate uniformly and present a relatively rapid growth (Dayan *et al*. 2000).

Beneficial effects derived from using these antioxidant compounds were also expressed in the activation of antioxidant defense mechanisms in cabbage seedlings, since, in the germinative process, different enzymes are formed and can act directly against ROS, produced from molecular oxygen resulting from normal cellular metabolism.

In this research, a greater expression of SOD activity was observed when there was exogenous application of antioxidant compounds, especially for kojic acid; this suggests that there was a significant increase in the presence of superoxide anion ($O_2^{\bullet-}$) in pre-germinated seeds subjected to imbibition in antioxidant compounds. Inversely, it

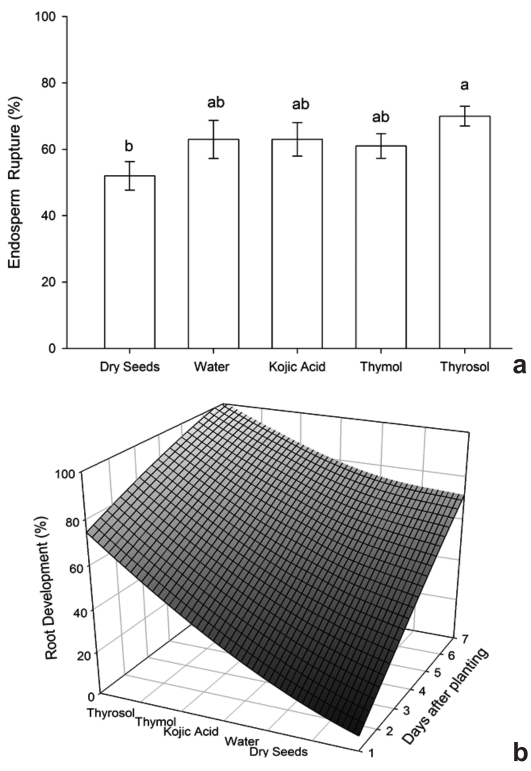

Figure 2 – a-b. Cabbage seeds treated with antioxidants – a. endosperm rupture; b. root development.

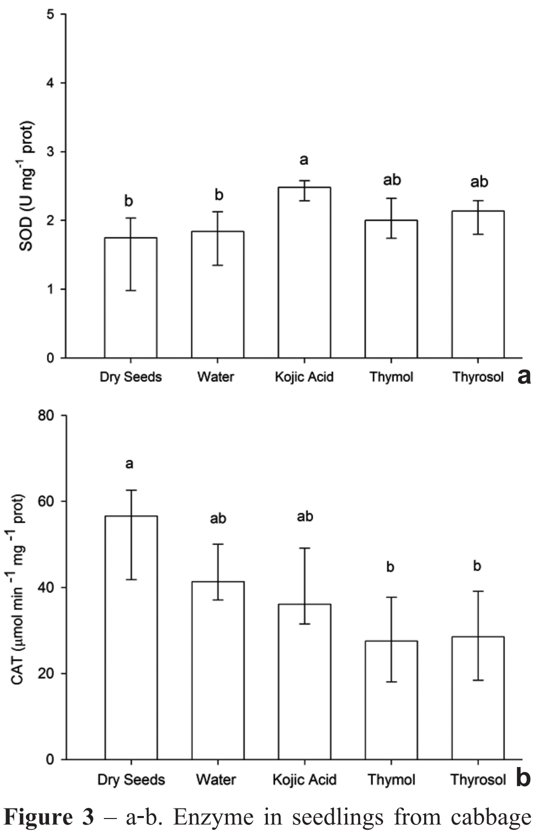

Figure 3 – a-b. Enzyme in seedlings from cabbage seeds treated with antioxidants – a. activity of superoxide dismutase; b. catalase.

can be seen that the use of the compounds reduced the activity of CAT enzyme, potentially due to the reduction of hydrogen peroxide in the seedlings tissues. It is ponderable to assume a biological balance mechanism in the production and control of free radicals by plant tissues, with evident benefits in the use of antioxidants compounds for an efficient system of excess ROS removal formed during germination (Kumar *et al*. 2015).

The action mechanism of ascorbate peroxidase (APX) seems not to have been triggered, which can be confirmed by the non-significance of the treatments and by the efficiency of tyrosol on the non-production of this enzyme. Because catalases are responsible for H_2O_2 removal, and its conversion into water and oxygen (Hasanuzzaman *et al*. 2012; Beyaz *et al*. 2017), this fact may have reduced ROS production, which evidences the non-action of APX when tyrosol solution is applied.

For Borba *et al*. (2014), seeds that presented lower APX activity also expressed higher physiological activity and, consequently, lower ROS production, which characterizes a lower disruption of the membrane system and a lower level of seed deterioration. These results agree with those presented in the present study, since cabbage seeds treated with antioxidant tyrosol did not express APX production and showed better physiological quality.

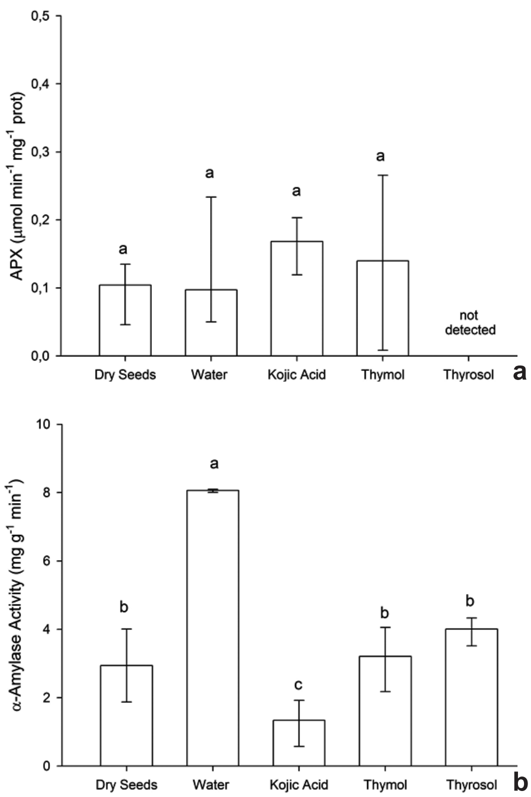

Figure 4 – a-b. Activity of enzymes on seedlings from cabbage seeds treated with antioxidants – a. ascorbate peroxidase; b. α-amylase.

As for the activity of antioxidant enzymes (CAT, SOD and APX), their important role in rapid defense responses of plant cells against oxidative stress is evident (Kusvuran & Dasgan 2017). However, it is worth noting that a succession of mechanisms occurs concomitantly during germination, such as the degradation of seed reserves for the growth of the embryo.

This study also evidences a higher α-amylase activity in cabbage seeds imbibed in water. This process seems to be natural because, among hydrolytic enzymes, there is α-amylase, which degrades amide reserves (Buckeridge *et al*. 2004). This effect was not observed for treatments with antioxidant solutions and dry seeds, which may be justified by the slower water imbibition; for Barroso *et al*. (2010), water absorption is impaired by excess of soluble salts that reduce water potential.

Although a lower water absorption is evident for cabbage seeds treated in antioxidants, and for dry ones, the use of antioxidant compounds in solution did not show reductions in the rates of reserve amide intake, since the germination of the seeds was not impaired.

In light of the above, the use of exogenous antioxidant compounds may be an excellent source against oxidative stress and to preserve seed quality during the germination process, as evidenced by studies suggesting antioxidant enzymes as reliable biological markers to monitor seeds' quality (Donà *et al*. 2013; Kumar *et al*. 2016; Singh *et al*. 2018).

Conclusion

The use of antioxidant compounds has beneficial effects on cabbage seeds, and soaking with tyrosol led to better physiological quality, with activation of antioxidant defense mechanisms during germination.

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