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Viability and performance of wheat seedlings after artificial seed aging

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ABSTRACT: Seed deterioration is a continuous irreversible process that affects cell structures and molecules and compromises the physiological quality of seeds. This study aimed to assess the effect of artificial wheat seed deterioration on germination and seedling performance. The TBIO Toruk wheat cultivar was used, with seeds submitted to different artificial aging times (2, 3, 4, 5, 6 and 7 days). The original and aged seed lots were submitted to laboratory germination and seedling performance tests (seedling length, seedling dry weight and endosperm dry weight). Alpha-amylase activity, electrical conductivity and the oxidative stress marker malondialdehyde were quantified. The exudate resulting from the electrical conductivity test was separated to quantify total soluble sugars, soluble proteins and phosphorus. The deterioration process increased lipid peroxidation and decreased initial alpha-amylase activity. During germination, the most deteriorated lots exhibited greater solute loss and lower alpha-amylase synthesis capacity. The lots with the greatest deterioration showed reduced viability and produced worse-performing seedlings.

Index terms: alpha-amylase, deterioration, malondialdehyde, physiological quality, *Triticum aestivum* L.

RESUMO: A deterioração de sementes é um processo contínuo e irreversível, atuando em estruturas e moléculas constituintes celulares e ocasionando a perda da qualidade fisiológica das sementes. Objetivou-se com este trabalho avaliar as alterações causadas pela deterioração artificial de sementes de trigo na germinação e desempenho de plântulas. Foram utilizadas sementes da cultivar TBIO Toruk, submetidas a diferentes tempos de envelhecimento artificial (2, 3, 4, 5, 6 e 7 dias). Após a obtenção dos lotes envelhecidos, juntamente com o lote original, foram conduzidos em laboratório os testes de germinação e desempenho de plântulas (comprimento total e massa seca total de plântulas e massa seca remanescente em endosperma). Foram quantificados o marcador de estresse oxidativo malondialdeído, a atividade da enzima alfa-amilase e a condutividade elétrica. O exsudato resultante da avaliação do teste de condutividade elétrica foi separado para a quantificação de açúcares solúveis totais, proteínas solúveis e fósforo. O processo de deterioração resultou em aumento da peroxidação de lipídeos e decréscimo da atividade inicial de alfa-amilase. Durante a germinação, os lotes mais deteriorados apresentaram maior perda de solutos e menor capacidade de síntese de alfa-amilase. Os lotes com maior nível de deterioração apresentaram decréscimo na viabilidade e formaram plântulas de menor desempenho.

Termos para indexação: alfa-amilase, deterioração, malondialdeído, qualidade fisiológica, *Triticum aestivum* L.

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INTRODUCTION

Wheat (*Triticum aestivum* L.) stands out as one of the main cereals grown worldwide and is an important source of carbohydrates in the human diet (Mieog et al., 2017; Tian et al., 2019). Most species farmed worldwide are propagated via seeds and the initial establishment of seedlings is critical to successful production (Finch-Savage and Bassel, 2016). Among the traits that determine this success, physiological quality is vital to initial establishment. Using high quality wheat seeds favors rapid and uniform emergence, which contributes to increasing crop yield (Abati et al., 2017).

The physiological quality of a seed lot is directly linked to its level of deterioration and seed aging compromises their physiological quality (Marcos-Filho, 2015). Seed deterioration is directly associated with the production and accumulation of reactive oxygen species (ROS) (Ebone et al., 2019). These molecules cause lipid peroxidation, protein oxidation and cytogenotoxicity (Soares et al., 2019), that is, they act on DNA, RNA, proteins and lipids (Mittler, 2017). Interaction between these molecules and ROS causes oxidative damage, compromising the physiological quality of seeds and affecting their performance during germination (Ebone et al., 2019). In seeds, lipid peroxidation is one of the most widely reported metabolic changes related to deterioration (Ebone et al., 2020). High lipid peroxidation caused by ROS results in malondialdehyde production due to interaction with cell membranes. As such, increased malondialdehyde production is an indicator of greater lipid peroxidation (Soares et al., 2019). In this respect, membrane damage is one of the main events during seed deterioration, resulting in inadequate solute exchange between the cell and the external environment and greater damage to the genetic material, increasing the consumption of reserves (Ebone et al., 2019).

Seed deterioration reduces enzyme activity, mitochondrial, protein and RNA synthesis, lowers stored reserves, compromises cell membrane integrity and causes protein denaturation (Marcos-Filho, 2015). Thus, artificial aging can be used to identify the mentioned results of deterioration in order to better understand the changes that occur during this process.

The enzyme alpha-amylase is associated with physiological quality in wheat seeds (Wen et al., 2018). As such, poor performance during germination may be associated with a decrease in alpha-amylase activity, lowering the consumption of reserves and, consequently, compromising seedling performance, an effect exacerbated by seed aging time.

In light of the above, this study aimed to assess the effect of wheat seed deterioration on germination and seedling performance.

MATERIAL AND METHODS

Seeds of the TBIO Toruk cultivar were used for the experiment. The seed lots were artificially aged in order to evaluate the effect of deterioration. Artificial aging was based on the accelerated aging methodology in saturated saline solution described by Marcos-Filho (2020), using plastic 11 x 11 x 3.5 cm germination boxes (Gerbox®). The seeds were evenly distributed in a single layer on an aluminium mesh in plastic boxes containing 40 mL of saturated saline solution at the bottom of each box (40% p/v of sodium chloride) and then covered with cling film. Next, the boxes were placed in an accelerated aging chamber at 41 ± 1 °C for periods of 2, 3, 4, 5, 6 and 7 days. After each time period, the seeds were dried in a forced-air oven at 30 °C until they reached 13% water content. Thus, seven lots with different levels of deterioration (days of artificial aging) were obtained from the original seed lot. A completely randomized design was used, with seven treatments and four replications.

After obtaining the lots, germination tests were performed and normal seedlings, abnormal seedlings and dead seeds were counted at four and seven days. The test was carried out with four replications of 50 seeds per lot, in a Mangelsdorf germination chamber at 20 ± 2 °C with a 12/12 h photoperiod. The seeds were placed on Germitest® paper moistened with distilled water at a ratio of 2.5 $mL.g^{-1}$ of dry paper. Normal and abnormal seedlings and dead seeds were counted at four (Brasil, 2009) and seven days after test onset.

Seedling performance was assessed in four replications of 20 seeds, with normal seedlings measured at three and five days after sowing to determine seedling length (SL) and seedling (SDW) and endosperm dry weight (EDW). The test

was conducted with seeds placed on the upper surface of rolls of Germitest® paper (Krzyzanowski et al., 2020). SL was measured with a digital pachymeter and expressed in centimeters per seedling ($cm.seedling^{-1}$). The seedlings assessed were separated from the endosperm to determine SDW ($mg.seedling^{-1}$) and EDW ($mg.seed^{-1}$) after drying at 80 °C for 24 h (Krzyzanowski et al., 2020).

Malondialdehyde (MDA) was determined after artificial aging at the previously described times and drying, using seeds with 13% water content for all the lots analyzed. The samples were prepared using approximately 3 g of seeds, ground with liquid nitrogen. For MDA extraction, 200 mg of the dried seed sample was added with 5 mL of 80% alcohol (v/v), ground in a mortar and pestle and centrifuged (Hodges et al., 1999). MDA was quantified using 1 mL of diluted extract added with 1 mL of 20% (w/v) trichloroacetic acid (TCA) solution containing 0.65% (w/v) of thiobarbituric acid (TBA). The samples were placed in a water bath at 95 °C for 25 minutes and read at 532 and 600 nm (Heath and Packer, 1968; Hodges et al., 1999).

Alpha-amylase activity (EC 3.2.1.2) was analyzed by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using the procedure described by Sun and Henson (1991), with some adjustments. The enzyme extract was prepared using 500 mg of fresh sample added with 5 mL of 100 $mmol.L^{-1}$ sodium acetate buffer (pH 5.5) containing 4 $mmol.L^{-1}$ of $CaCl_2$ and 0.005% (v/v) of Triton X-100. The samples were agitated for 1 h in ice and centrifuged, and the extract was incubated in a water bath for 15 min at 70 °C. Quantification was performed using 0.5 mL of enzyme extract and 0.5 mL of 100 $mmol.L^{-1}$ sodium acetate buffer (pH 5.5) containing 2.0% (p/v) soluble starch, with the resulting solution remaining in a water bath for 10 min at 40 °C. The reaction was stopped by adding 1 mL of DNS solution and the samples were then placed in a water bath at 95 °C for 6 min. Before reading, the samples were added with 8.0 mL of distilled water. Reading was performed in a spectrophotometer at 540 nm absorbance and the results expressed in units per milligram of protein ($U.mg^{-1}$ protein). One unit of enzyme was defined as the amount of enzyme needed to produce 1 µmol of maltose per minute.

Electrical conductivity (EC) was measured using the procedure described by Krzyzanowski et al. (2020). Four replications of 50 seeds per lot were placed in disposable cups previously cleaned with 5% HCl solution (v/v), added with 50 mL of deionized water and kept at 25 °C for 24 h. After the incubation period, electrical conductivity was determined, and the results expressed in $\mu S.cm^{-1}.g^{-1}$. The exudate was separated to quantify total soluble sugars, soluble proteins and phosphorus.

Total soluble sugars were quantified using the procedure described by McCready et al. (1950) with some changes in the quantification process. The reaction was performed using 1 mL of exudate and 3 mL of anthrone reagent followed by vortexing for 3 s. The samples were placed in a water bath at 95 °C for 450 s, then cooled in an ice bath and read in a spectrophotometer at 630 nm. The standard curve was obtained using anhydrous glucose and the results expressed in $\mu g.mL^{-1}$.

Soluble protein was analyzed using the method proposed by Bradford (1976) and quantified using 800 µL of diluted exudate and 200 µL of Bradford reagent. Reading was performed in a spectrophotometer at 595 nm. The standard curve was obtained based on a bovine serum albumin standard curve and the results expressed in $\mu g.mL^{-1}$.

Phosphorus was measured using the method described by Chen et al. (1956) with some changes to the quantification procedure, using 500 µL of exudate and 500 µL of C reagent (distilled water, H_2SO_4 3 M, 0.02 M, ammonium molybdate and 0.568 M ascorbic acid at a ratio of 2:1:1:1, respectively). The samples were incubated at 37 °C for 90 min and reading performed at 820 nm. The phosphorus standard curve was obtained from monobasic potassium phosphate and the results expressed in $\mu g.mL^{-1}$.

The data were submitted to analysis of variance (ANOVA) and the means compared with the Scott-Knott test ($p \leq 0.05$). The data were transformed for normality when needed. ANOVA and linear regression were performed in Sisvar software (Ferreira, 2011) and regression analysis with $p < 0.01$ and $p < 0.05$ using the t-test.

RESULTS AND DISCUSSION

The deterioration process compromised seed physiological quality, particularly considering the results obtained in the germination first count (FC) and percentage of normal seedlings (NS). The nonaged seed lots (0 days) exhibited an FC of 91% and the aged lots obtained lower values (78 to 11%), indicating the effect of deterioration on germination speed. The same response was observed for NS (Table 1).

There was an association between FC and NS, whereby lots with a lower FC percentage also obtained a low NS (Table 1). Artificially aged sunflower seeds also showed a decline in FC and NS values, demonstrating that deterioration has a negative effect on the ability to produce normal seedlings (Morais et al., 2021). A low FC percentage is initially associated with membrane disorganization and a low final germination percentage is related to a larger number of abnormal seedlings, resulting from tissue death in different parts of the seed, especially the embryonal axis (Marcos-Filho et al., 2015).

The percentage of normal seedlings is considered the germination percentage of a seed lot in seed analysis reports for commercialization purposes. In this respect, the nonaged seeds and those aged for 2 days were the only lots suitable for commercialization according to MAPA (*Ministério da Agricultura, Pecuária e Abastecimento*) Ordinance 45/2013, which establishes a minimum germination percentage of 80% (Brasil, 2013). Deterioration during storage can compromise seed lots and make them nonviable, since the first count percentage and ability to produce normal seedlings decline as seeds age (Table 1).

Seeds submitted to artificial deterioration exhibited a higher percentage of abnormal seedlings (AS) and dead seeds (DS), increasing with artificial aging time (Table 1). Among deterioration outcomes, lipid peroxidation, increased respiratory activity and damage to genetic material lead to reduced viability and vigor (Ebone et al., 2019).

Artificial deterioration had a negative effect on seedling performance. At three days of germination, physiological differences were observed in the plants formed, with the deteriorated seed lots obtaining lower seedling dry weight and length values. Plant performance declined when seed lots were exposed to longer deterioration, as demonstrated in regression analysis (Table 2). Among the methods for evaluating seed lot performance, growth parameters are quality indicators (Finch-Savage and Bassel, 2016). This was evident in the findings obtained, whereby low-quality seed lots resulted in poor seedling performance (Table 2).

For EDW, there was no significant difference between lots at three days of germination. However, regression analysis demonstrated high EDW in aged seed lots, indicating less hydrolysis and mobilization of stored reserves (Table 2). Starch is the main source of reserves for wheat seeds (Mieog et al., 2017), providing energy and metabolites that are hydrolyzed by alpha-amylase and mobilized to the embryonal axis, enabling seedling formation (Yu et al., 2015).

At three days of germination, alpha-amylase activity was lower in deteriorated lots, with a tendency to decline as aging increased (Table 2). The high alpha-amylase activity in lots with shorter deterioration times explains the low EDW. Thus, greater reserve hydrolysis capacity favored the formation of taller seedlings with high dry weight values (Table 2). The catabolic reactions triggered by starch degradation allow aerobic cellular respiration as the energy in chemical bonds is released. The ATP molecules produced during cellular respiration in the cytoplasm and mitochondria provide energy for seed germination and subsequent seedling formation and growth (Wang et al., 2016). Less deteriorated seeds exhibit intense cell metabolism, with greater oxygen and ATP consumption during germination, favoring the formation of superior seedlings.

Seedling performance and alpha-amylase activity assessed after five days of germination showed a similar response pattern to that observed at three days, whereby the greater the deterioration of seed lots the worse the seedling performance (Table 2). As seed deterioration progressed, germination rate, seedling growth and the mobilization of seed reserves declined (Mohammadi et al., 2011). Seed lots with higher deterioration levels displayed greater lipid peroxidation and damage to genetic material (Ebone et al., 2019), which took longer to repair or failed to fully repair (Marcos-Filho, 2015).

Table 1. Germination first count (FC), normal seedlings (NS), abnormal seedlings (AS) and dead seeds (DS) in seven wheat seed lots before and after 2, 3, 4, 5, 6 and 7 days of artificial aging.

Deterioration (Days)	FC	NS	AS	DS
		---------- % ----------		
0	91 a	94 a	3 c	3 e
2	78 b	86 b	7 c	7 d
3	59 c	77 c	12 b	11 c
4	49 d	72 c	14 b	14 c
5	38 e	61 d	20 a	19 b
6	25 f	55 d	22 a	23 b
7	11 g	24 e	24 a	52 a
y = ax + b	y = -11.73x + 95.25	y = 5.82x – 4.09	y = -9.01x + 102.12	y = 3.19x + 1.97
R^2	0.98	0.98	0.87	0.75
p-value	0.000	0.000	0.000	0.029
CV (%)	11.98	5.86	23.86	25.19

Means followed by the same letter in the column do not differ significantly according to the Scott- Knott test at 5% probability. n=28. R^2: Coefficient of determination. CV: Coefficient of variation.

The SDW results at five days indicated a difference in lots artificially aged for five days or longer; however, the difference in SL between lots was apparent at 2 days of aging (Table 2). Thus, it can be suggested that for wheat, SDW is less sensitive to deterioration than SL.

Considering seed lot response, the high alpha-amylase activity observed at five days resulted in low EDW. By contrast, the highest EDW was recorded in lots aged for six and seven days, which also showed low alpha-amylase activity (Table 2). Alpha-amylase activity is positively associated with vigor in wheat seeds and a determining factor in plant establishment (Wen et al., 2018). Thus, changes in alpha-amylase activity resulted in low starch hydrolysis and, consequently, high EDW, producing shorter seedlings with lower dry weight (Table 2).

In the nonaged seed lot, alpha-amylase activity was higher at three days than five days of germination, and as such, alpha-amylase activity at the former explains the superior performance observed at the latter (Table 2).

The lots submitted to deterioration exhibited higher MDA concentrations than nonaged seeds ($p \leq 0.05$), indicating a rise in MDA concentration and consequent increase in seed deterioration. However, there was no significant difference between aged seed lots (Table 3).

Tian et al. (2019) observed a decline in the physiological quality of wheat seeds during aging, whereby an increase in MDA concentration during storage was negatively correlated with seed lot viability. These findings corroborate those observed in MDA quantification (Table 3) and physiological quality assessment (Table 1), where higher MDA levels may be related to poor physiological quality. However, the absence of a significant difference in MDA content between the aged lots makes it difficult to determine seed physiological quality based solely on MDA concentration, which is not an efficient test to establish the physiological quality of a lot.

There was no strong association between electrical conductivity (EC) and seed physiological quality for nonaged lots and seeds aged for 2 and 3 days (Table 3). Lots 2 and 3 obtained lower FC and NS values than those of nonaged seeds and these three lots had the same EC, despite exhibiting different physiological quality (Table 1). These findings suggest that electrical conductivity testing is efficient in assessing wheat seed physiological quality under accentuated

Table 2. Seedling length (SL), seedling dry weight (SDW), endosperm dry weight (EDW), and alpha-amylase activity at three and five days of germination in seven wheat seed lots before and after 2, 3, 4, 5, 6 and 7 days of artificial aging.

3 Days of Germination				
Deterioration (Days)	SL cm.seedling^{-1}	SDW mg.seedling^{-1}	EDW mg.seed^{-1}	Alpha-amylase U.mg^{-1} protein
0	6.78 a	3.59 a	24.40 a	3.34 a
2	5.20 b	2.96 b	25.14 a	2.98 a
3	4.62 c	2.98 b	26.86 a	3.15 a
4	4.21 c	2.34 c	26.50 a	2.56 b
5	3.48 d	2.30 c	26.39 a	2.41 b
6	3.04 d	1.99 c	26.35 a	2.31 b
7	1.10 e	1.18 d	27.52 a	1.66 c
y = ax + b	y = -0.72x + 6.86	y = -0.31x + 3.69	y = 0.37x + 24.72	y = -0.23x + 3.50
R^2	0.95	0.93	0.73	0.88
p-value	0.000	0.000	0.004	0.000
CV (%)	12.42	16.49	5.23	12.19
5 Days of Germination				
Deterioration (Days)	SL cm.seedling^{-1}	SDW mg.seedling^{-1}	EDW mg.seed^{-1}	Alpha-amylase U.mg^{-1}
0	17.04 a	7.92 a	15.80 b	9.32 a
2	14.92 b	7.67 a	16.03 b	7.84 b
3	14.42 b	8.01 a	16.78 b	6.55 c
4	12.22 c	7.44 a	17.32 b	6.08 c
5	9.94 d	5.60 b	17.42 b	6.67 c
6	7.44 e	4.31 c	21.07 a	6.01 c
7	5.02 f	3.22 d	22.97 a	5.41 c
y = ax + b	y = -1.75x + 18.32	y = -0.71x + 9.06	y = 0.99x + 14.37	y = -0.51x + 8.81
R^2	0.94	0.98	0.76	0.85
p-value	0.000	0.001	0.004	0.000
CV (%)	11.34	10.94	8.99	8.60

Means followed by the same letter in the column do not differ significantly according to the Scott- Knott test at 5% probability. n=28. R^2: Coefficient of determination. CV: Coefficient of variation.

deterioration conditions (e.g., lots aged for 5, 6 or 7 days), since differences in this parameter were only observed at advanced deterioration stages (Table 3).

ROS production is the main cause of deterioration, with the accumulation of these molecules triggering lipid peroxidation and resulting in higher MDA concentration (Ebone et al., 2020). Thus, lipid peroxidation indicated by MDA quantification is widely used to determine the occurrence of oxidative damage (Soares et al., 2019). The results indicated a higher MDA content in artificially aged seeds, suggesting an increase in ROS after aging (Table 3).

The lack of a significant difference in MDA concentration may indicate the activation of repair mechanisms that

Table 3. Malondialdehyde (MDA), alpha-amylase activity and electrical conductivity (EC) in seven wheat seed lots before and after 2, 3, 4, 5, 6 and 7 days of artificial aging.

Deterioration (days)	MDA ($nmol.mL^{-1}$)	Alpha-amylase ($U.mg^{-1}$ protein)	EC ($\mu S.cm^{-1}.g^{-1}$)
0	1.08 b	0.18 a	37.40 c
2	1.22 a	0.15 a	36.97 c
3	1.21 a	0.13 a	36.58 c
4	1.25 a	0.08 b	41.23 b
5	1.27 a	0.09 b	48.66 a
6	1.33 a	0.10 b	41.74 b
7	1.28 a	0.07 b	42.21 b
y = ax + b	y = 0.0292x + 1.124	y = -0.0155x + 0.175	y = 1.1291x + 36.33
R^2	0.72	0.82	0.41
p-value	0.000	0.012	0.000
CV (%)	5.44	37.88	6.05

Means followed by the same letter in the column do not differ significantly according to the Scott- Knott test at 5% probability. n=28. R^2: Coefficient of determination. CV: Coefficient of variation.

prevented the accumulation of this oxidative stress indicator (Table 3). Oxidative stress enzymes such as superoxide dismutase contribute to ROS control and help maintain germination (Silva et al., 2018). The production and accumulation of the superoxide radical cause oxidative damage during seed aging (Tian et al., 2019). These results suggest that the action of enzymes related to the antioxidant system during artificial aging prevents a significant increase in MDA over the artificial aging periods analyzed.

Alpha-amylase activity showed a tendency to decline as the seeds were exposed to longer artificial aging times. Seeds aged for 4, 5, 6 and 7 days displayed lower initial alpha-amylase activity (Table 3). The deterioration process is associated with the production of ROS, which act on proteins (Mittler, 2017; Soares et al., 2019). ROS interaction with alpha-amylase during deterioration may have degraded the enzyme, which showed lower activity in aged seeds, particularly in lots submitted to longer aging times (i.e., 4, 5, 6 and 7 days).

The EC test indirectly assesses cell membrane integrity and showed no significant difference between nonaged lots and seeds aged for 2 and 3 days, as previously mentioned (Table 2). Corroborating the loss of cell membrane integrity, analysis of the EC exudate indicated the release of soluble sugars, soluble proteins and phosphorus. The longer the exposure of seed lots to deterioration, the greater the loss of these components (Table 4).

Electrolyte leakage (total soluble sugars, soluble proteins and phosphorus), demonstrated by EC, indicates that lots with a greater level of deterioration submitted to soaking exhibit higher solute and ion concentrations due to low cell membrane integrity (Silva et al., 2020).

As such, the increase in solutes leached from soaking seeds and the rise in MDA concentration demonstrate the peroxidation of lipids from the membrane (Table 3).

Based on the results obtained, low membrane stability (Table 3), loss of solutes (Table 4), reduced initial alpha-amylase activity and low initial alpha-amylase synthesis during germination (Table 2) compromised the physiological performance of the lots assessed.

Table 4. Total soluble sugars (TSS), soluble protein (SP) and phosphorus (P) in the electrical conductivity exudate of seven wheat seed lots before and after 2, 3, 4, 5, 6 and 7 days of artificial aging.

Deterioration (days)	TSS ($\mu g.mL^{-1}$)	SP ($\mu g.mL^{-1}$)	P ($\mu g.mL^{-1}$)
0	13.04 b	120.10 b	0.77 b
2	14.72 b	139.63 b	0.76 b
3	17.67 b	135.91 b	0.76 b
4	24.02 a	168.43 a	0.87 a
5	30.58 a	166.97 a	0.90 a
6	29.55 a	148.60 b	0.89 a
7	31.83 a	169.66 a	0.96 a
y = ax + b	y = 3.126x + 11.00	y = 6.527x + 124.73	y = 0.029x + 0.73
R^2	0.90	0.67	0.79
p-value	0.000	0.001	0.002
CV (%)	19.87	10.40	11.19

Means followed by the same letter in the column do not differ significantly according to the Scott- Knott test at 5% probability. n=28. R^2: Coefficient of determination. R^2: Coefficient of determination. CV: Coefficient of variation.

CONCLUSIONS

The seed deterioration process compromises the viability and performance of wheat seedlings as a function of increased peroxidation of lipids from the cell membrane and a decline in the initial activity and biosynthesis of alpha-amylase.

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