Botanica
ISSN 2538-8657
https://doi.org/10.35513/Botlit.2025.2.1
2025, 31(2): 54–63

Original research

Quantitative determination of amylase activity in germinating cereal grains using agar plates and ImageJ software

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Received: 7 February 2025. Accepted: 21 March 2025. Published online: 14 April 2025.

Abstract

Yastreb T.O., Shkliarevskyi M.A., Kolupaev Yu.E. 2025: Quantitative determination of amylase activity in germinating cereal grains using agar plates and ImageJ software. – Botanica, 31(2): 54–63. https://doi.org/10.35513/Botlit.2025.2.1

The main energy source during cereal seed germination is soluble carbohydrates formed by the hydrolysis of stored starch. This process is carried out by a complex of hydrolytic enzymes, the most important of which are α- and β-amylases. Classical methods of amylase analysis are based on the extraction of enzymes from homogenised grains with a buffer and subsequent determination of their activity by incubating the enzyme extract with starch solution and determining one of the reaction products (reducing sugars) or residual starch by spectrophotometric methods. At the same time, there are also methods for detecting amylase activity by incubating grain halves on starch-containing agar plates, with cleavage fixed by staining the surface with solutions containing I_2. However, these simple methods are only used for an approximate qualitative assessment of amylase activity. This work demonstrates the possibility of quantitatively determining the total amylase activity in germinating wheat and triticale grains by using ImageJ software to estimate the size of the non-iodine-stained halos formed on the plates because of the hydrolysis of starch by amylases. The analysis protocol and examples of the application of the method in the study of the effect on the amylase activity of wheat and triticale grains of priming with physiologically active substances that enhance the germination of grains (hydrogen sulphide donor NaHS and β-aminobutyric acid) are presented. The method is proposed to evaluate the effectiveness of physiologically active substances for priming seeds to increase their germination and for selecting seeds with potentially high germination energy.

Keywords: amylase, analysis of activity, cereal seed germination, ImageJ, seed priming.

INTRODUCTION

Seed germination in most plant species is critically dependent on amylase activity. Amylase activation is particularly important for the normal germination of cereal seeds. Grains of most cereals contain approximately 70–80% starch, and enzymes for its hydrolysis are synthesised in the scutellum or aleurone layer in response to germination induction (Joshi, 2018). Thus, in cereals, the digestion of starch stored in the

endosperm is the primary source of metabolites and energy required for seedling development (Damaris et al., 2019).

The complete hydrolysis of starch is achieved by the combined action of α-amylase, β-amylase, debranching enzyme and α-glucosidase. The enzyme α-amylase, classified as 1,4-D-glucanmaltohydrolase, is the predominant form of amylase and the most critical enzyme throughout the growth and life cycle of angiosperm plants. α-Amylase is a calcium-containing enzyme that functions at random sites in the starch chain to form α-maltose and α-glucose (Pujadas & Palau, 2001). In contrast, the function of β-amylase (1,4-D-glucan malt hydrolase) is to act from the non-reductive end of starch, hydrolysing the second α-1,4-glycosidic bond and cleaving one unit of maltose at a time (Yamaguchi et al., 1999).

It has been shown that α-amylase is synthesised *de novo* during seed germination in the presence of endogenous gibberellic acid from the embryo (Sugimoto et al., 1998; Damaris et al., 2019). In contrast, β-amylase is present in an inactive form before germination without gibberellic acid control, and its activation occurs by proteolysis from the carboxyl end of the molecule (Wang et al., 1996).

Amylase inhibition is one of the causes of reduced germination vigour and seed germination under adverse conditions (Joshi, 2018).

A decrease in amylase activity is considered one of the signs and possible causes of seed senescence. It has been found that ageing rice seeds with reduced germination energy showed a decrease in amylase activity and total sugar content. Osmopriming (pre-exposure to polyethylene glycol) increased seed germination, amylase activity and sugar content (Lee & Kim, 2000). The authors have found a close correlation between amylase activity and seed germination (r = 0.91) and between enzyme activity and sugar content (r = 0.92). Germination of seeds, including those subjected to aging or germinated under unfavourable conditions, can be improved not only by osmopriming, but also by hydropriming and pre-treatment with various physiologically active substances, such as melatonin (Kolupaev et al., 2024), β-aminobutyric acid (Mostek et al., 2016), donors of gastransmitters, in particular hydrogen sulphide (Dai et al., 2024).

The critical dependence of one of the essential properties of seeds – their ability to germinate – on amylase activity makes it advisable to control it when selecting seeds with high germination for breeding purposes, as well as to assess the effectiveness of various physiologically active substances that can potentially contribute to grain germination. However, this requires the availability of inexpensive methods for the analysis of amylase activity. Several methods for determining amylase activity in plant material have been described in the literature. Still, most involve homogenising germinating seeds and extracting amylases from the homogenates using buffer solutions (see, for example, review: Muralikrishna & Nirmala, 2005). The enzyme-containing extracts are then mixed with the substrate (starch solution), and the amount of reaction products (reducing sugars) or residual unhydrolysed starch is determined, making the procedure cumbersome, at least for mass analysis.

Since the 1980s, alternative methods have been used in individual studies, particularly in determining amylase activity in cut germinating grains by incubating agar gels with added starch. However, these methods only visualise the effect representing the enzyme activity without quantifying it (Xie et al., 2007; Zhang et al., 2010; Liu et al., 2018) or very roughly estimating the diameter of the iodine-stained area around the grain after its incubation with a starch-containing gel (Ho & Shih, 1980). At the same time, modern digital image processing techniques allow such a method to be translated into a quantitative basis.

ImageJ is an open-source, broad-spectrum scientific imaging software developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin). As open-source software, researchers can create custom tools for any image manipulation task. Still, the basic version of the software contains enough advanced image analysis tools, including quantification, to make it ideal for our purposes without the need to develop special macros (Schneider et al., 2012).

The work aimed to evaluate the possibility of determining total amylase activity in germinating wheat and triticale grains using ImageJ processing of images obtained after incubation of grains on starch-containing agar. In addition, to assess the possibility of using the method to study the effect of germination-promoting compounds on cereal seeds, the

objectives of the work included the determination of amylase activity in seeds treated before germination with the hydrogen sulphide donor sodium hydrosulphide or beta-aminobutyric acid. Wheat and triticale seeds, which have had reduced germination due to ageing, were used as model objects to study the effects of these compounds. The effects of NaHS and β-aminobutyric acid on germination and amylase activity in aged cereal grains have not been specifically investigated.

MATERIALS AND METHODS

Plant material

The aim of the work was not only to study the possibility of using ImageJ software as a component of the method for determining total amylase activity, but also to evaluate the possibility of using the method to study the reaction of cereal seeds to the action of physiologically active substances. We used cereal seeds with a known reduced germination rate (Kolupaev et al., 2024; Shakhov et al., 2024). Seeds of winter bread wheat (*Triticum aestivum* L.) of the 'Scorpion' cultivar (the Czech Republic, Austria) and winter triticale (× *Triticosecale* Wittm.) of the 'Raritet' cultivar (Ukraine) of the 2020 generation were used as experimental objects. Their germination was reduced to about 30–50% by four years of storage in a room with non-factorostatic conditions (periodic temperature increase to 30–35°C in summer and temperature decrease to -4°C or -6°C in winter, as well as humidity fluctuations from 30% to 80%).

Before the experiment, the seeds were disinfected with 5% NaClO solution for 15 min and then washed eight times with sterile distilled water. The seeds of the experimental lines were then primed with physiologically active substances to enhance germination: 0.5 mM and 1 mM solutions of hydrogen sulphide donor sodium hydrosulphide (NaHS) (Zhou et al., 2018) or β-aminobutyric acid (Jisha & Puthur, 2016; Özkurt & Bektaş, 2022) for four hours. The control seeds were incubated in distilled water for the same period. The concentrations of these compounds, which cause a noticeable effect of increasing seed germination energy, were selected based on preliminary experiments.

After treatment, all seeds were dried on a glass surface for one day in the dark in a thermostat TCO-80 (MICROmed, Ukraine) at 24°C to initial moisture content. The seeds of all variants were then placed in Petri dishes of 75 seeds, each on a double layer of filter paper moistened with 8 ml of distilled water for germination.

Evaluation of amylase activity

The analysis was carried out 24 and 48 hours after the start of seed soaking. Grains of the same size in which the germination process was visible were used. The grains were cut with a lancet, and the half without the embryo was placed, cut side down, on plates prepared in Petri dishes consisting of 1% agar with 0.2% starch. The samples were incubated in a thermostat at 24°C for 1–3 h. At the end of the incubation period, the gels were filled with 10 ml of diluted Lugol's solution (0.04% I_2 in 0.1% KI), the excess solution was removed with an autopipette after 5 min, and the lower part of the dish was photographed using a horizontal camera Samsung SM-N9750 at high-density resolution on a paper-covered glass with bottom illumination. The resulting photographs were then analysed using ImageJ software (version 1.54 g).

During the pre-processing phase, several key steps were taken to standardise and improve the images. First, contrast enhancement was performed using the histogram equalisation function in ImageJ to improve the clarity of regions of interest and accurate segmentation. Next, all colour images were converted to single-channel halftone images to remove variability caused by colour information and focus solely on intensity differences. However, the RGB Stack function was used for this procedure, rather than the default function of converting a colour image to an 8-bit single-channel image, as selecting only the red channel produces a more contrast-rich result (Fig. 1).

Masks of the brightened halos around the grains were then created on the pre-processed images using ImageJ's selection tools, excluding the cut areas of the grains themselves. The resulting masks were checked by two independent experts to minimise errors, and discrepancies were resolved by consensus.

The pixel area of the selected regions was measured using ImageJ's measurement tools. A correction factor based on the measured area of the lower half of

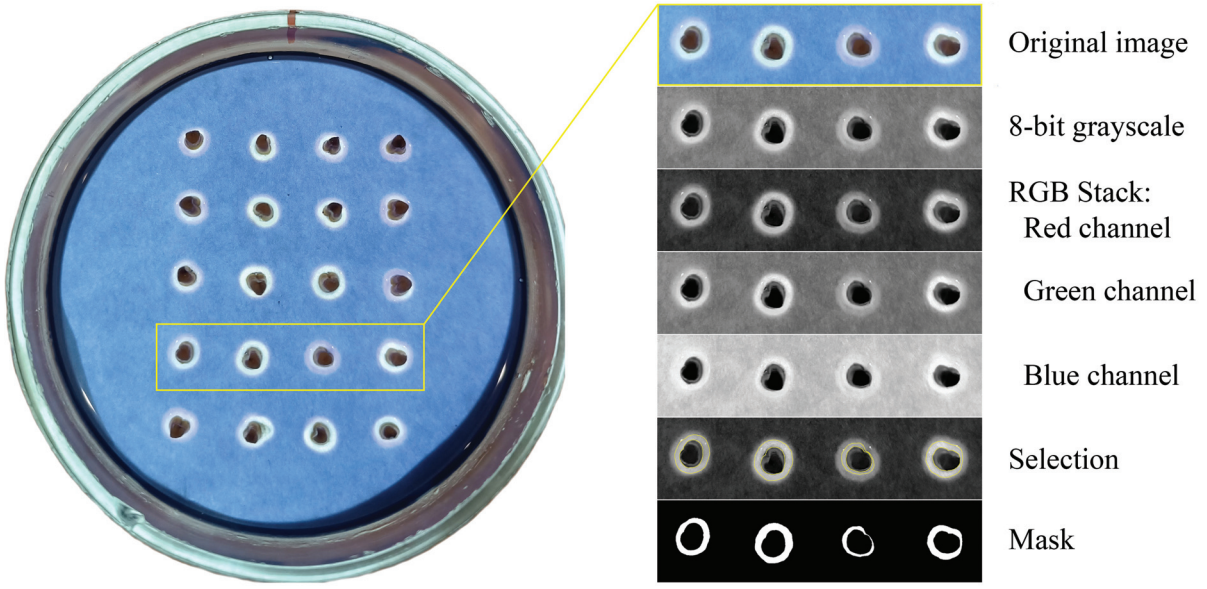

Fig. 1. Sample Petri dish after gel staining and image processing sequence using ImageJ tools.

the Petri dish was applied to equalise the pixel measurements in the different photographs. This correction ensured accurate scaling, allowing the samples to be matched prior to statistical processing. The results of the amylase activity estimation were expressed in conventional units representing one thousand pixels per grain.

In order to assess the accuracy and biological relevance of the image-based method, total amylase activity was determined from the amount of reducing sugars formed by starch hydrolysis in samples from the same series of experiments using known protocols (Goldstein & Jennings, 1975; Fawzi & El-Fouly, 1979) with minor modifications. A portion of the plant material (500 mg) was homogenised in 0.2 M acetate buffer (pH 5.6), and the homogenate was centrifuged in an MPW 350R centrifuge (MPW MedInstruments, Poland) at 8000 × g for 15 min at a temperature not exceeding 4°C to prepare the supernatant, which was then analysed. The supernatant was diluted the required number of times with the same buffer and used for analysis. The enzyme extract was mixed with 5 ml of 2% starch solution and incubated in a thermostat for 30 min at 25°C, after which the reaction was stopped by precipitating the proteins by adding 0.1 ml of 10% lead acetate and 0.1 ml of 16% sodium sulphate. In the control samples, these compounds were added before starch. The reducing sugar content of each sample (including the inactivated enzyme control) was determined by reaction with Fehling's reagent. After adding 5 ml of Fehling's reagent, the samples were boiled in a water bath VB-20 (MICROmed, Ukraine) for 10 min, cooled and centrifuged at 6000 g for 10 min. The optical density of the supernatant was determined on a UV-1280 spectrophotometer (Shimadzu, Japan) at a wavelength of 670 nm.

Determination of seed germination vigour

After 48 h from the start of seed germination, the relative number of germinated grains was determined. Seeds with a shoot size of at least half the length of the seed were considered germinated.

Replication and statistical processing of results

To determine total amylase activity using the ImageJ software, the halos of at least 20 grains (five Petri dishes, each containing four grains of the corresponding variant) were evaluated in each experiment variant. When total amylase activity was analysed using the method based on reducing sugar measurement, the analyses were performed in at least three replicates.

When germination vigour was considered, sprout size was estimated in four cups for each variant. Each cup contained 75 seeds.

The significance of the differences between the experimental variants was determined by Student's *t*-test. Figures and tables show means and their standard errors.

RESULTS

In the first series of experiments, we selected the optimum time for exposing wheat and triticale grains to agar plates to quantitatively determine total amylase activity. The analysis was carried out 48 hours after the start of seed soaking.

Amylase activity was higher in triticale grains than in wheat (Fig. 2). With increasing incubation time of the grains on agar plates, the size of the light halo, representing the enzyme activity, increased almost linearly with time. We also used a 2-hour exposure of grains on agar plates, which allowed us to obtain sufficiently large, but not merging, light halos, the size of which was conveniently determined using ImageJ software.

In the next series of experiments, we evaluated the possibility of using the method to study the effect of the hydrogen sulphide donor NaHS on amylase activity in grains of two cereal species. For this purpose, the results obtained using ImageJ software were compared with data obtained using a known method based on spectrophotometric analysis of the amount of reducing sugars formed under the influence of amylase. In both control variants, the amylase activity in the grains increased with germination (Table 1). This effect was significant at $p \leq 0.05$ when activity was determined by both methods. The hydrogen sulphide donor caused an increase in enzyme activity in grains of both cereal species; this effect was evident at both 24 h and 48 h after the start of seed germination. No significant differences were observed in the changes in enzyme activity by using the different methods of analysis. The Pearson correlation coefficient between the 12 pairs of results presented in the table was 0.93, which is statistically significant at $p \leq 0.01$.

The main objectives of this work did not include the study of seed germination characteristics. However, since it is known that seed germination vigour is closely related to the activity of amylase in grains, we determined the value of germination vigour 48 h after germination (Fig. 3). Priming with hydrogen sulphide donor markedly increased the germination vigour of aged seeds of both cereal species. The Pearson correlation coefficient between amylase activity determined using ImageJ software after 48 h of seed germination and germination vigour values was 0.957 and was significant at $p \leq 0.01$. A high correlation coefficient ($r = 0.875$, significant at $p \leq 0.01$) was also observed between the values of amylase activity obtained by classical method with spectrophotometric assay and germination vigour. Thus,

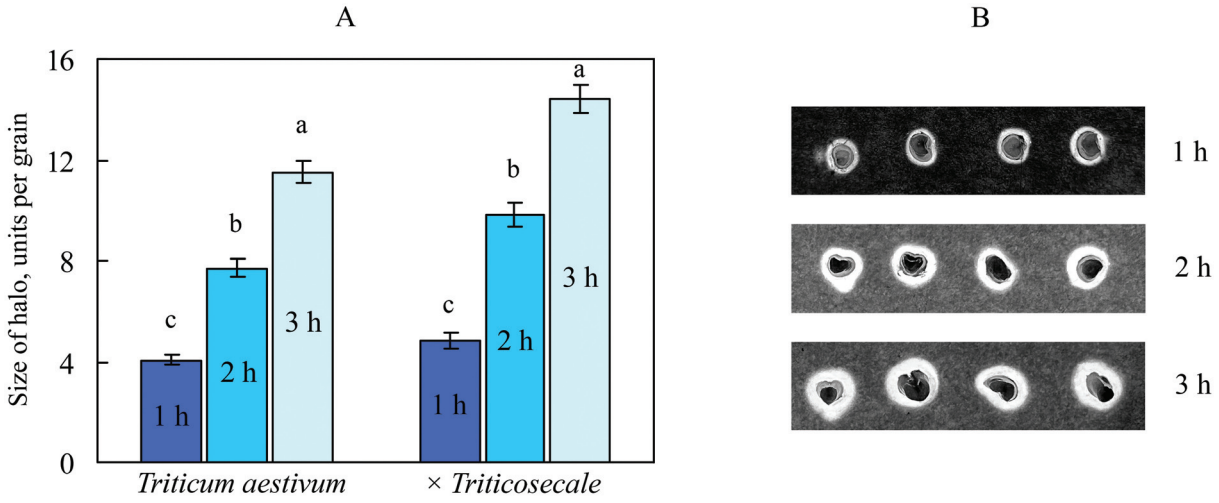

Fig. 2. Temporal dynamics of the increase in the size of the uncoloured halo expressing amylase activity during incubation of cut grains on starch-containing agar plates. A – Conventional value of the activity, expressed as units (thousand pixels) per grain; B – Halo samples after different incubation times (for *Triticum aestivum*). Different Latin letters for each type indicate values, the differences between which are significant when $p \leq 0.001$.

amylase activity estimated by different methods was closely correlated with seed germination vigour, and this index, as well as amylase activity, increased significantly under the influence of NaHS priming.

The data on changes in amylase activity obtained by us using the example of the action of hydrogen sulphide donor indicate the method's applicability based on the use of ImageJ software for such purposes. We also consider it appropriate to present the results of the approval of the above method in the study of the effect of treating aged triticale seeds with beta-aminobutyric acid on their germination and total amylase activity (Fig. 4).

Priming of triticale seeds with β-aminobutyric acid significantly increased the germination vigour of aged triticale seeds, which was accompanied by an increase in amylase activity in the grains as determined by image processing using ImageJ software (Fig. 4). The effects of 0.5 mM and 1 mM solutions were not significantly different.

DISCUSSION

In the present work, the previously used method for the qualitative assessment of amylase activity in germinating cereal seeds (Zhang et al., 2010a; Liu et al., 2018) was transferred to a quantitative basis. This was made possible by using ImageJ software to process images of halos obtained on starchy agar plates after staining with Lugol's solution. Image processing

Table 1. Total amylase activity in germinating wheat and triticale grains determined by ImageJ software (units per cut grain (light halo size in thousand pixels after 2 h exposure on starch agar)) or spectrophotometric method (μmol maltose per 1 g dry weight of grain after 1 h exposure). Significant differences ($p \leq 0.05$) between experimental variants of the same species assessed using the ImageJ software method are indicated by lowercase letters, whereas differences between variants assessed using the spectrophotometry method are indicated by uppercase letters. The *p*-value indicates the significance of the differences between experimental variants and the corresponding control variant

Variant	Amylase activity							
	After 24 h from the start of seed germination				After 48 h from the start of seed germination			
	ImageJ software	*p*	Spectrophotometry	*p*	ImageJ software	*p*	Spectrophotometry	*p*
Triticum aestivum								
Control	4.17 ± 0.14 e		9.70 ± 0.30 E		7.76 ± 0.21 c		15.1 ± 0.60 B	
NaHS (0.5 mM)	4.75 ± 0.19 d	0.0395	12.9 ± 0.22 C	0.0010	9.23 ± 0.35 b	0.0070	17.9 ± 0.31 A	0.0132
NaHS (1 mM)	5.13 ± 0.15 d	0.0016	11.6 ± 0.22 D	0.0069	10.8 ± 0.44 a	0.0002	19.4 ± 0.82 A	0.0133
× *Triticosecale*								
Control	6.62 ± 0.27 c		18.3 ± 0.52 C		9.72 ± 0.25 b		22.0 ± 0.30 B	
NaHS (0.5 mM)	9.24 ± 0.32 b	0.0002	22.2 ± 0.40 B	0.0040	12.1 ± 0.31 a	0.0003	24.5 ± 0.44 A	0.0093
NaHS (1 mM)	9.94 ± 0.41 b	0.0001	22.1 ± 0.26 B	0.0028	12.2 ± 0.52 a	0.0023	25.6 ± 0.82 A	0.0153

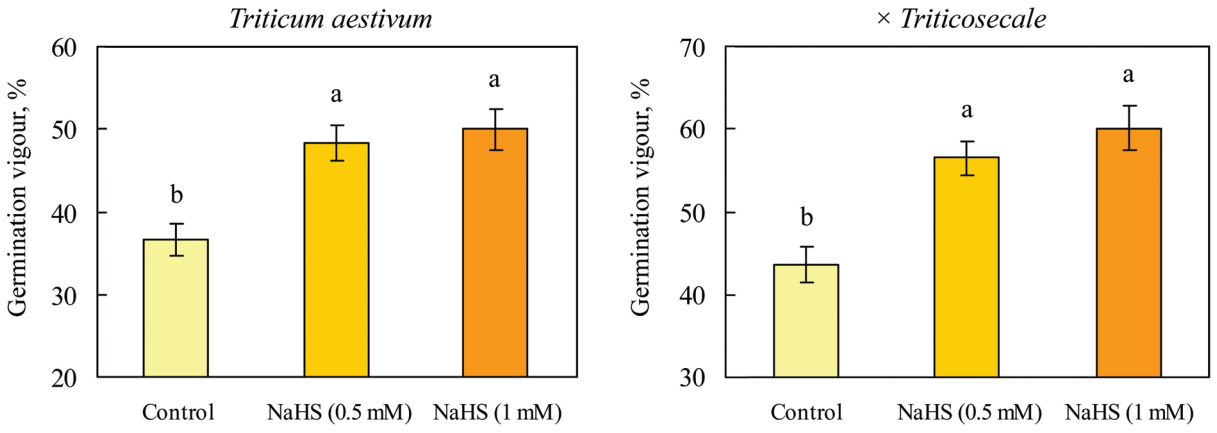

Fig. 3. Germination vigour of aged wheat and triticale grains under the action of hydrogen sulphide donor. Mean values and their standard errors for three replicates are presented; different letters above columns indicate significant differences ($p \leq 0.01$).

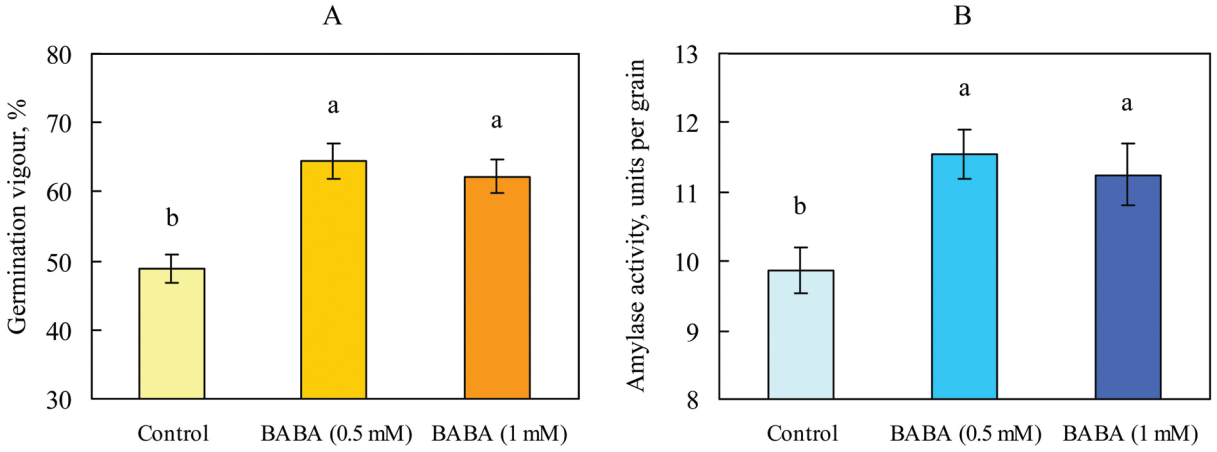

Fig. 4. Effect of β-aminobutyric acid (BABA) on seed germination vigour (A) and amylase activity (B) in aged triticale grains 48 h after germination. Mean values and their standard errors for three replicates are presented; different letters above columns indicate significant differences ($p \leq 0.01$).

included contrast enhancement using the histogram equalisation function in ImageJ, conversion of colour images to single-channel grayscale images using the RGB Stack function, and the creation of masks of brightened halos around the grains using ImageJ's selection tools, excluding the cut areas of the grains. The approach showed high reproducibility, with an average error not exceeding 4–5%, apparently due to variations in enzyme activity in individual grains (Table 1). At the same time, the values of the standard errors of the image-based method only slightly exceeded the corresponding errors of the classical biochemical method, where they amounted to about 4% of the mean.

The method using ImageJ software showed its efficiency in studying the effect on amylase activity of physiologically active compounds capable of improving seed germination as hydrogen sulphide donor NaHS and β-aminobutyric acid (Table 1, Fig. 3 and Fig. 4).

It should be noted that the effect of hydrogen sulphide on the germination of cereal seeds subjected to ageing has not been investigated so far. However, it has been reported that NaHS germination of wheat seeds increased under the influence of priming against the background of stress factors – toxic doses of copper (Zhang et al., 2008) and chromium salts (Zhang et al., 2010b). At the same time, the activity of amylase in grains increased under the influence of hydrogen sulphide. The authors consider this effect as a probable reason for the increase in seed germination (Zhang et al., 2008, 2010a, 2010b). In our experiments, the positive effect of hydrogen sulphide donor on amylase activity in grains was also accompanied by an increase in seed germination of aged wheat and triticale seeds (Fig. 3).

The effect of β-aminobutyric acid on the germination of cereal seeds, especially triticale, remained almost unexplored until the time of our research. However, the results were consistent with the data on the positive effect of its treatment on wheat seeds, which increased germination and activated root growth of seedlings (Özkurt & Bektaş, 2022). It has also been shown that β-aminobutyric acid seed priming enhances biomass accumulation and linear growth of *Vigna radiata* (L.) R. Wilczek seedlings under normal conditions and osmotic stresses induced by polyethylene glycol and NaCl (Jisha & Puthur, 2016). The effect of β-aminobutyric acid on amylase activity also has not been investigated in these works. However, an increase in total sugar content has been observed in *Vigna radiata* seedlings under the influence of priming with this compound under normal and stress conditions, indirectly indicating that their grain uptake is enhanced due to amylase activation (Jisha & Puthur, 2016).

In general, the studies show that the results of the amylase activity assay performed using the ImageJ software can be one of the criteria for evaluating the action of physiologically active substances capable of enhancing seed germination.

The method described is intended to determine

the total amylase activity. However, if necessary, it can be adapted for the separate determination of β-amylase activity. For this purpose, a calcium chelator, 2 mM ethylenediaminetetraacetic acid, can be added to the agar and the 0.2% starch solution (Zhang et al., 2010). Adding ethylenediaminetetraacetic acid to the medium inactivates the calcium-dependent α-amylase, allowing β-amylase to be detected separately. In this case, α-amylase activity can be approximated by the difference between total amylase activity and β-amylase activity, provided they are assayed simultaneously under identical conditions. As mentioned above, these two major forms of cereal grain amylases are involved in seed germination but differ significantly in both their catalytic and regulatory mechanisms *in vivo* (Wang et al., 1996; Damaris et al., 2019). However, in this case, a significant increase in the duration of grain incubation on agar is likely to be required, as well as additional verification of this modified methodology by alternative biochemical methods.

CONCLUSION

This paper describes a quantitative method for analysing total amylase activity in germinating cereal grains during incubation on starch-containing agar plates. The method is based on determining the size of a halo of hydrolysed starch around the grain, which remains virtually colourless after staining with iodine. The size of the selected areas is measured in pixels after a specific image processing using ImageJ software tools. Comparison of the data obtained by this method with the results of amylase activity determination by spectrophotometric method (by the amount of reducing sugars formed) showed their high correlation ($r = 0.93$, $p \leq 0.01$). The method was successfully tested by determining the effect on amylase activity of priming seeds of cereals with reduced germination with compounds that activate amylase and enhance germination – hydrogen sulphide donor NaHS and β-aminobutyric acid. The results allow us to consider it reasonable to use the method when evaluating the effectiveness of physiologically active substances intended for seed priming to increase germination. The method may also help select seeds with potentially high germination vigour.

ACKNOWLEDGEMENTS

This study was carried out with the partial support of the project 2-24-26 BO "Development of measures to ensure sustainable productivity of agrophytocenoses under the influence of abiotic and biotic stress factors" (State budget of Ukraine, state registration number 0124U000457) and the project 14.00.02.06.P "Development of methods of seed priming of cereal grains by the action of donors of gasotransmitters and compounds with hormonal activity" (State budget of Ukraine, state registration number 0124U000126).

Author contributions. TOY Investigations, methodology, writing and editing. MAS Methodology and data curation. YEK Writing, project administration. All authors have read and approved the final version of the article.

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