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Effect of *Bacillus subtilis* QM3 on β-amylase Isoenzyme in Early Germination of Wheat Seed

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Authors' contributions

This work was carried out in collaboration between both authors. Author YJL performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author QPH designed the study, managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Seed germination is the basis and initial stage in the process of plant growth and development. *Bacillus subtilis* QM3 is a plant growth promoting rhizobacterium (PGPR) with function of growth promoting, promoting, prevention of pathogens and pests attack and can improve plant resistance to different stress factors. Through the measurement of the early germination rate of wheat (*Triticum aestivam* L.). seeds under *B. subtilis* QM3 treatment, the results showed that *B. subtilis* QM3 can significantly promote the germination of wheat seeds, which has reached a significant level at 6h after seeds sowing or at 6 h of treatment, during imbibition. The β-amylase isoenzyme in early period of wheat seed germination was measured by denaturing polyacrylamide gel electrophoresis. The results showed that the band width and brightness of β-amylase isoenzyme of wheat seeds treated with *B. subtilis* QM3 increased during three to 6 h of imbibition and especially the effect was significant at 6 h. In the early period, the band of α-amylase isoenzyme could not be detected. It is suggested that the increase of β-amylase isoenzyme early band may be one of the main reasons for *B. subtilis* QM3 to promote wheat seed germination. Through the combination of *B. subtilis* QM3 with free and binding states of β-amylase, it was found that the former can increase the activity of β-amylase by either increasing free β-amylase or releasing binding β-amylaseisoenzyme. β-amylaseisoenzyme inhibitors can significantly inhibit β-amylase activity,

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nevertheless α-amylase activators and inhibitors have no significant effect on β-amylaseisoenzyme, which further proved that the β-amylase exerted the effects in the early period of wheat seed more precisely during the imbibition of the seeds.

Keywords: B. subtilis QM3; early seed germination; β-amylaseisoenzyme.

1. INTRODUCTION

Wheat (*Triticum aestivam* L.) is an important food crop and the second largest food crop after rice (*Oryza sativa* L.). It is widely distributed and has high yield and nutritional value. Wheat adds up to half of the calories in human food to meet people's biggest nutritional needs. The output of wheat accounts for 22% of China's crop output, and the planted area accounts for 25% of the crops. Wheat is the main food crop for human survival. China is a country with a large population, and wheat consumption accounts for most of the crop consumption [1]. The consumption of wheat in the world accounts for half of the consumption of food crops. In addition to being a major food crop, wheat is also an important industrial raw material and has important economic value. Each country in the world uses about 600 million tons of wheat for commercial purposes every year [2]. According to the estimates of the International Food Policy Research Institute (IFPRI), with the growth of the population and the progress of society, the living standards of human beings are getting higher and higher, and the requirements for food production and quality are growing, so, consumption, consumption of wheat will climb up, from 552 to 775 million tons by year 2020, ultimately results consumption up to 60% by the year 2050 [3].

Seed germination begins the life cycle of the crop. Timely germination and even germination are the key determinants of many crops in modern agricultural production systems. They are closely related to seed germination and subsequent plant growth and development, and ultimately affect the yield and quality of cereals. Germination is considered to be the most critical stage in the plant life [4]. Poor germination and decreased seedling growth result in poor establishment and occasionally crop failure [5]. As an important part of the life cycle, germination is the basis of crop growth. Starch is one of the main storage reserves of wheat seeds. Although the germination of cereal seeds is affected by many factors, the corresponding amylase is required to breakdown when the seeds germinate [6].Therefore, the research on amylase in the early stage of wheat germination (imbibition period) is extremely important.

The extent of amylase activity probably determines germination ability of cereal seeds. Amylases have many isoezymes in plants. Studies have shown that varieties with fast germination rate have more isoenzymes bands than those with slower germination rate, indicating that isoenzymes have a certain relationship with seed germination rate [7]. Starch enzymatic hydrolysis power is closely related to its activity and is the most critical enzyme in starch degradation. In wheat grains, β-amylase generally exists in two forms, free and bound [8]. The active center of β-amylase consists of specific groups, which are involved in the conversion of enzyme and substrate complexes and the process of enzyme-substrate binding [9]. Amylopectin produces less maltose than amylose. When β-amylase acts on glycogen, the amount of maltose converted is only 40 % - 50 % of the syrup. β-amylase has strong acid resistance but is not heat-resistant, and is completely inactivated by holding it in a constant temperature water bath at 70°C for 15 min.

Bacillus subtilis is a kind of biological control bacterium, which can act on the rhizosphere, body surface or plant body of the plant. It secretes antibacterial substances to inhibit its growth, and induce the plant to start the defense system, which can not only promote the growth of the plant, but also improve the plant tress resistance. *B. subtilis* QM3 is a strain with biocontrol effect isolated from Qinghai cow dung. It has strong reproductive capacity and is a good biocontrol bacterium [10,11]. Previous studies have shown that *B. subtilis* QM3 can promote the germination and growth of wheat seeds, and it can alleviate the growth of wheat under salt stress. *B. subtilis* QM3 promotes early amylase isoenzyme changes during seed germination.

The purpose of this study was to investigate the effects of *B. subtilis* QM3 treatment on early amylase isozymes in wheat seed germination and to promote physiological and biochemical processes during seed germination, by *B. subtilis*

QM3. Molecular aspects provide some theoretical guidance, which lays a foundation for *B. subtilis* QM3 bacteriumto promote seed germination and further apply to agricultural production practices.

2. MATERIALS AND METHODS

2.1 Media

The commonly used media in this investigation were the beef extract peptone containing peptone 1.0%, beef extract 0.3%, sodium chloride 0.5%, agar 1.5% - 2.0%, pH 7.4 - 7.6 and sterilized 20 min at 121°C.

2.2 Bacterial Suspension Preparation

B. subtilis QM3 used in the present study come from the microbiological lab, the School of Life Science, Shanxi Normal University [11]. A culture of *B. subtilis* QM3 was obtained by transferring colony from the activated culture plate into a 250 mL flask containing 100 mL beef extract-peptone medium and shaking in an orbital shaker at 200 rpm at 37°C for 3 days. Then, it was diluted in sterile water solution in order to reach an optical density $(OD)_{600}$ nm of 0.8 [10^8 colony-forming units (CFU)]. Dilute its bacterial suspension liquid 10 times and 100 times to reserve [12].

2.3 Seed Materials

Wheat seeds (Linhan N. 9) were used came from Shanxi Academy of Agricultural Sciences. Healthy seeds of similar size and mass were selected. Prior to germination, the seeds were surface-sterilized in 5% sodium hypochlorite for 10 min and rinsed in distilled water. Seeds were divided into two large groups that soaked in sterile water (CK), 10^7 CFU mL^{-1} *B. subtilis* QM3 (X0) and 10^6 CFU mL^{-1} *B. subtilis* QM3 (X) for 3 h, respectively, and then transferred to Petri dishes for germination. Seeds were taken every three hours until the end of 12 h. The followings conditions have been assured temperature incubator (25°C day12 h; and 55% relative humidity). The experiment was repeated three times.

2.4 Germination Rate

Standard germination was conducted according to Ma [13]. Using 50 seeds for each repetition, repeated 3 times. The seeds were incubated in the growth box in the dark at 25˚C for 12 h, and under light at 25˚C for 12 h. Germination is based on the radicle penetrating the seed coat. From the beginning of seed germination, the germinated seeds have been counted every 3 h and the germination rate was recorded.

$$\text{Germination percentage (GP)} = (Gt/T) \times 100\%$$

Where Gt is the number of germinations in t days, and T is the total number of seeds used in the test.

2.5 Determination of Isoenzyme

2.5.1 Preparation of amylase extract

There were used 0.5 g and these were grinded in ice bath with 5 mL phosphate buffer (pH = 7.5). The homogenate was centrifuged at 10000 g for 30 min. The supernatant was collected in an Eppendorf tube and it represented the total amylase solution extract. Utilizethe characteristics of α-amylase-resistant acid-resistant and heat-resistant β-amylase. Heated total amylase at 70°C for 10 min, and then centrifuged at 10000 g for 30 min. The supernatant obtained was designated as α-amylase. Added 4 $molmL^{-1}$ HCl to total amylase to make its pH less than 4, then centrifuged at 10000 g for 30 min. The supernatant obtained was designated as β-amylase [14].

Cancellation excluded half a grain of wheat germ, the method of reference Guerin, ice bath using Tris - HCl grinding into homogenate, with gauze filtration, the filtrate in 10000 g of centrifuge 30 min, remove the supernatant, precipitated by the Tris-HCl suspension centrifugal, repeated three times, merge the supernatant, known as beta amylase free state of crude enzyme liquid precipitation and Tris-HCl suspension, the suspension is a combination of β-amylase thick enzyme fluid state [15].

2.5.2 Isozyme extract by native PAGE

Amylase electrophoresis was assayed by the photochemical method described by Ming J. Wu [16]. Slight change, ten micrograms protein (10 μL) in each extract from each variety was mixed with an 5:1 volume of sample buffer (150 $mmolmL^{-1}$ Tris-HCl pH 6.8, 20% glycerol, 0.001% bromophenol blue) without boiling, and then loaded onto a native-PAGE gel which had a 10% separation gel with acrylamide: Bisacrylamide at a ratio of 29:1 in 150 $mmolmL^{-1}$ Tris-HCl pH 6.8 and a 5% stacking gel using 150 mmol/mL Tris-HCl, pH 8.8. Both gel components contained no

sodiumdodecyl sulfate (SDS). Electrophoresis was carried out at 80 V per gel for 1h for the stacking step and 110V per gel for 2.5 h for the resolving step in running buffer (25 mmolmL^{-1} Tris, 400 mmolmL^{-1} glycine) until the dye had run to the edge of the gel.

2.5.3 Dyeing

After electrophoresis, transferred the gel to phosphate-buffered saline (PBS) buffer containing 1% soluble starch, incubated at 37˚C for 3 h, rinse the gel surface with water, and immersed it in the configured I_2-KI solution for staining at 20 min, the results were photographed and recorded. The non-chromatographic band on the glue surface was the amylase isoenzyme band.

2.6 Data Analysis

All data are the average of three repetitions. The obtained results were analyzed statistically by SPSS (version 11.0) statistical software. Data were analyzed by the analysis of variance and treatment mean comparison by using least significance difference ($p < 0.05$) [17].

3 RESULTS

3.1 Effect of *B. subtilis* on the Germination of Wheat Seeds

The germination of seeds is closely related to the growth of plants. In this experiment, the germination rate was measured early in the germination of wheat, as shown in Fig.1.

As can be seen from Fig. 1, in general, in the early stage of germination (0-12 h), with the progress of germination time, the germination rate of CK group wheat seeds in the control group did not increase significantly, while the treatment group X0 and X treated wheat seeds the germination rate showed an upward trend. Compared with the control group, both X0 and X had a significant effect on promoting wheat germination ($P < 0.05$). There was already a significant difference at the 6th h of germination, and the growth was most significant at 12 h and the highest germination rate of wheat seeds compared with other treatment groups, the largest increase in the X treatment group. It shows that *B. subtilis* QM3 can obviously promote the early germination rate of wheat seeds, especially at a suitable concentration, that is, the concentration of *B. subtilis* QM3 is 10^6 CFU mL^{-1}.

3.2 Effect of B. Subtilis QM3 on A/B-Amylase Isoenzymes of Whole Wheat Seeds at Early Germination

Based on the above test results, we selected *B. subtilis* QM3 strain (10^6 CFU mL^{-1}) (X) treated with sterile water as the CK group for electrophoresis to analyze α/β-amylase isozymes.

Changes in the number and brightness of amylase isozyme electrophoresis bands during wheat germination can largely reflect the activity of amylase. As can be seen from Fig. 2A and B, β-amylase has been present since the beginning of germination, but the presence of α-amylase

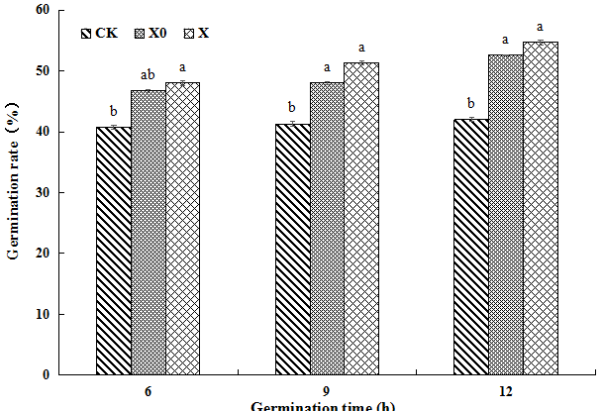

Fig. 1. The effect of *B. subtilis* QM3 on the germination rate during early germination of wheat seeds. CK, X0 and X represent sterile water treatment, *B. subtilis* QM3 (10^7 CFU mL^{-1}) and *B. subtilis* QM3 (10^6 CFU mL^{-1}) treatment, respectively. 6, 9 and 12 h are the germination time of wheat seeds. Different letters represent significant differences between different treatments at the same time ($p < 0.05$)

could not be detected. Three isozymes A_β, B_β, and C_β are observed in the gel of Fig. 2A, and most β-amylase activity can be attributed to C_β. The strip A_β is a narrow band, and the widths of the B_β and C_β bands are relatively large. At different germination times, the number and brightness of bands in group X were significantly different from those in group CK. The number and brightness of bands in group X at 6 h after germination were significantly different from other germination stages. This shows that during the germination of wheat seeds, group X can improve the activity of β-amylase isozyme, and it has the most obvious promotion effect at 6 h after germination, especially the specific B_β and C_β bands of B_β . Due to the increased brightness and width of the Bβ and Cβ bands, *B. subtilis* QM3 promotes the activity of β-amylase isoenzymes, thereby promoting wheat germination. In Fig. 2B, the activity of α-amylase isozyme did not change significantly in seeds treated with CK or *B. subtilis* QM3. The above conclusions indicate that *B. subtilis* QM3 can rapidly increase the content of β-amylase isoenzyme, but not of α-amylase.

In order to regenerate wheat seeds induced by *B. subtilis* QM3, β-amylase, rather than α-amylase, worked in the early stage of germination. Fig. 2C is the total amylase for the sample treated with *B. subtilis* QM3 for 6 h. Cut the slices into pieces and put them into Petri dishes contained α-amylase inhibitor ethylenediaminetetraacetic acid (EDTA), α-amylase activator (calcium chloride - $CaCl_2$) and β-amylase inhibitor (α-cyclodextrin, copper sulphate - CuSO4, mercuric chloride -HgCl). Incubated at 25°C for 30 min, rinsed three times with water, and stained with iodine-potassium iodide. The results showed that β-amylase inhibitors had a significant inhibitory effect on the total amylase of wheat seeds, while α-amylase inhibitors had no significant change in the activity of total amylase in wheat seeds. So, *B. subtilis* QM3 induced β-amylase, rather than α-amylase activity in wheat seeds during imbibition.

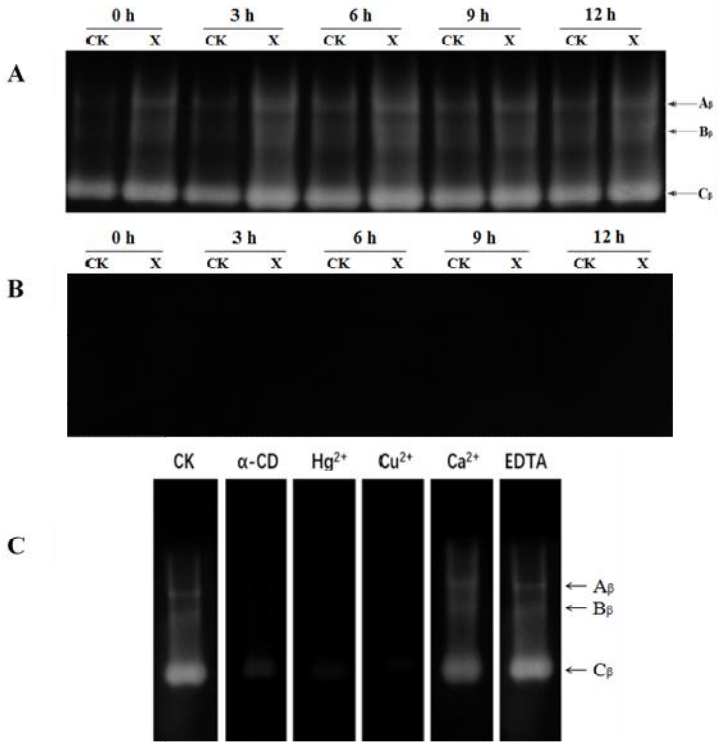

Fig. 2. A: Shows the effect of *B. subtilis* QM3 on β-amylase isozymes in the early germination of whole wheat seeds. CK and X represent sterile water treatment and *B. subtilis* QM3 (10^6 CFU mL^{-1}) treatment, respectively. A_β, B_β and C_β are three different β-amylase isoenzyme bands. 0, 3, 6, 9 and 12 h are the germination time of wheat seeds. B: Is the α-amylase activity during early germination. C: Shows the changes in the total amylase electrophoresis placed in inhibitors and accelerators

3.3 Effect of *B. subtilis* QM3 on A/B-Amylase Isoenzymes of Half - Grain Wheat Seeds at Early Germination

Using embryo-free half-grain wheat as the experimental material, and repeating the above experiment, we obtained similar results as shown in Fig. 3.

Fig. 3 A and B are the β-amylase isozymes in the early stage of wheat seed germination. The results of β-amylase isozyme and α-amylase isozyme of half-grain seeds are the same as those of whole wheat seeds. *B. subtilis* QM3 quickly induced an increase in the brightness and width of the β-amylase band in the early stage of seed germination, and basically detected less than the α-amylase band. It showed that *B. subtilis* QM3 had an effect on β-amylase, but not α-amylase, in the early stage of wheat seed germination. Fig. 3 C is *B. subtilis* QM3 treatment after 6 h of total amylase electrophoresis, placed in different activators or inhibitors, using α-amylase inhibitor (EDTA) and activator ($CaCl_2$) and β-amylase inhibitors (α-cyclodextrin, $CuSO_4$, HgCl). The results show that α-amylase inhibitors and activators have no significant effect on the total amylase isoenzyme band. β-amylase inhibitors can significantly inhibit the total amylase isozyme.

3.4 Effect of *B. subtilis* QM3 on Free and Bound B-Amylase

B. subtilis QM3 reacts with free β-amylase for 3, 6, 9, and 12 h.

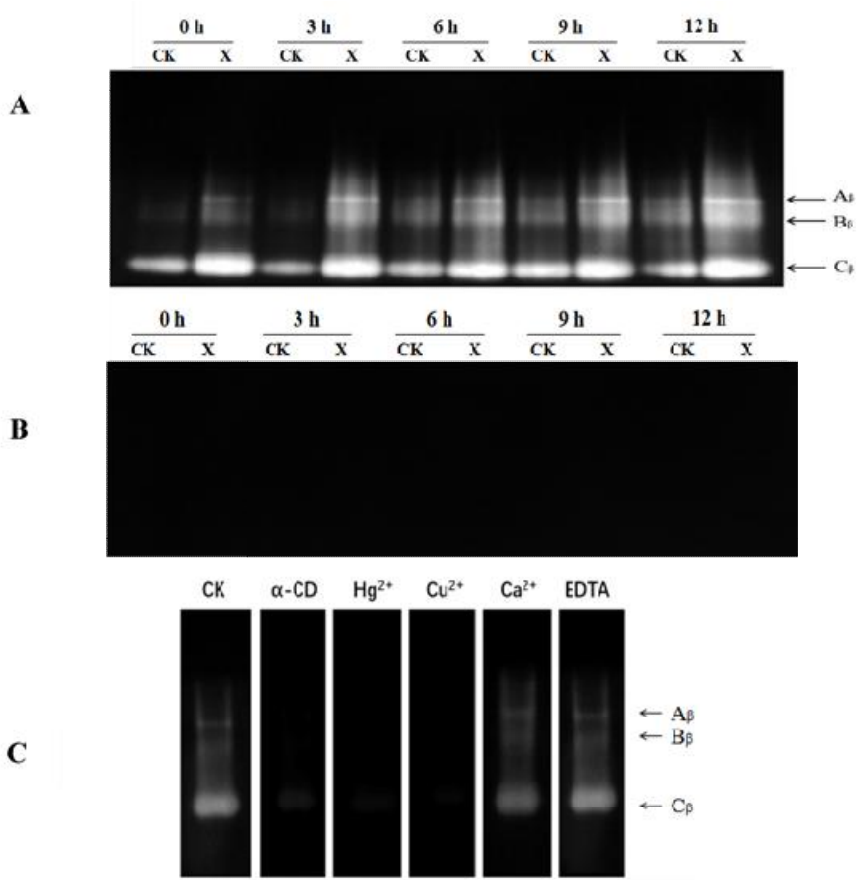

Fig. 3. A: Shows the effect of *Bacillus subtilis* QM3 on β-amylase isozymes in the early germination of half-grain seeds. CK and X represent sterile water treatment and *B. subtilis* QM3 (10^6 CFU mL^{-1}) treatment, respectively. A_β, B_β and C_β are three different β-amylase isoenzyme bands. 0, 3, 6, 9 and 12 h are the germination time of wheat seeds. B: Is the alpha-amylase activity during early germination. C: Shows the changes in electrophoresis of total amylase placed in the inhibitor and accelerator

The results are shown in Fig. 4 A. Compared with the CK group, the amylase bands in group X are significantly brighter than those in the control group (CK). In particular at 6 h, in the seeds treated with *B. subtilis* QM3, low-activity large-molecular-weight β-amylase monomers were depolymerized into high-active small-molecular-weight β-amylase monomers. It is suggested that the depolymerization of free β-amylase into monomer is one of the ways to improve the activity of amylase.

β-mercaptoethanol can dissociate the free β-amylase into monomers [18]. Treated the free β-amylase with low-concentration of β-mercaptoethanol for 3 h, then add *B. subtilis* QM3 to react for 3 h, the results are shown in the figure as shown in Fig. 4 B, the band brightness of the free β-amylase was not significantly different from that of the control, indicating that *B. subtilis* QM3 did not increase the free β-amylase monomer. In addition, we used *B. subtilis* QM3 to treat the crude enzyme solution of β-amylase binding state, as shown in Fig.4 C, and found that the band brightness of X was significantly higher than that of CK, indicating that β-amylase is elevated.

4. DISCUSSION

Starch is the main storage material in wheat and other cereal seeds. Amylase plays an important role in the process of seed germination, being a key enzyme of this physiological process [18,19]. The amylase activity affects the wheat seeds germination rate and the seedlings survival [20]. In the process of germination, the activity of amylase may be affected by various environments. Microorganisms can increase plant seed germination by increasing amylase activity during seeds germination [21]. Studies by Zhang and others found that β-amylase can increase the activity of β-amylase [8]. Sun and other studies found that β-amylase is one of the important factors to promote wheat seed germination [18]. According to different products, amylase can be divided into α-amylase and β-amylase, and β-amylase plays a major role in the early stage of wheat germination.

The experimental results showed that in the early stage of seed germination, with the increase of wheat germination time, the band of β-amylase isozyme in the seed increased, the band brightness increased and widened. Enzymatic enzyme bands have enhanced expression activity. After soaking with 10^6 CFUmL^{-1} *B. subtilis* QM3, the β-amylase isoenzyme band in wheat seeds increased and widened, thereby enhancing the expression activity of the enzyme band. Among them, the sixth h of wheat seed imbibition, the brightness and number of bands increased the most. However, as the imbibitions progressed, the α-amylase isoenzyme band of wheat did not change significantly, even the

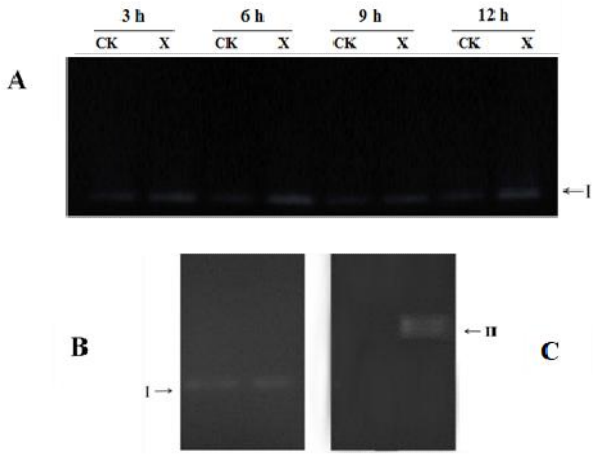

Fig. 4. A: Is treated with *B. subtilis* QM3 for free crude β-amylase enzyme solution for 3, 6, 9, and 12 h, where CK is the control group, that is, seeds treated with sterile water, and X is treated with *B. subtilis* QM3. Of wheat seeds, 3, 6, 9, 12 h represent the reaction time with crude enzyme solution for 3, 6, 9, 12 h, respectively. B: Shows the treatment of free β-amylase with β-mercaptoethanol for 3 h, and then adding *B. subtilis* QM3 for 3 h, 2-ME for β-mercaptoethanol for 3 h, and 2-ME + X for β-mercaptoethanol. After 3 h, add *B. subtilis* QM3 for 3 h. C: Shows that the bound β-amylase was treated with *B. subtilis* QM3 for 6 h, indicated by X

B. subtilis QM3 treated seeds indicated that *B. subtilis* QM3 could be enhanced by increasing the activity of β-amylase isozyme germination of wheat seeds, α-amylase has not been synthesized in the early stage of wheat germination.

In the early stage of seed germination, with the increase of time, the band of free β-amylase isozyme in the seed increases, and the brightness of the band increases and widens. The enzyme band has enhanced expression activity. After treatment with 10^6 $CFUmL^{-1}$*B. subtilis*QM3, the β-amylase isoenzyme band in wheat seeds increased and widened, thereby enhancing the expression activity of the enzyme band. Among them, the width and brightness of the band increased most obviously in the 6th h the germination of wheat seeds. However, with the extension of time, there was no significant change in the band of β-amylase isozyme in wheat, even the seeds treated with *B. subtilis*QM3. The results showed that the free β-amylase isoenzyme activity during seeds germination can be enhanced by *B. subtilis* QM3.

5. CONCLUSION

Soaking seeds with *B. subtilis* QM3 can increase the β-amylase activity during wheat seed germination, and promote the germination process by increasing in special of the free fraction of β-amylase enzyme. Choosing the appropriate concentration of bacterial solution is more conducive to promoting the germination of wheat seeds, thereby increasing the yield of crops. This phenomenon may be related to the combination of bacteria and wheat seeds, but the deeper reason needs further study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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