The Influence of Barley Storage on Respiration and Glucose-6-Phosphate Dehydrogenase During Malting

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ABSTRACT

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Malt is produced by the controlled, but limited germination of barley. To produce good quality malt, the barley employed must be able to germinate rapidly and synchronously. Dormancy is a seed characteristic that can interfere with the rapid and uniform germination of barley, thereby reducing the resultant malt quality. Various studies have shown that post harvest storage can be used to remove dormancy and enhance barley germination characteristics and malt quality. Because of its complexity, the fundamental basis of dormancy induction, maintenance and termination remain unknown. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate limiting enzyme of the pentose phosphate pathway and has been associated with dormancy decay and increased seed vigor of a variety of different seeds. The aim of this study was to determine if changes in barley germination vigour were associated with respiration and/or G6PDH changes during malting. Commercially grown barley (cv. Gairdner) was obtained from various states of Australia and stored at room temperature for up to 7 months. At 1, 3 and 7 months, samples were taken and stored at –18°C. The germinative energy (GE) and germinative index (GI) of these samples were measured. Samples were micro-malted and the α-amylase activity, respiration rate, and G6PDH activity of the germinating grains were measured at various stages of malting. It was found that storage of barley for up to seven months significantly improved the germination characteristics and increased the α-amylase activity during malting. However, these improvements were not associated with concomitant changes in respiration rate or G6PDH activity during malting.

Key words: Barley, dormancy, germination, glucose-6-phosphate dehydrogenase, respiration.

INTRODUCTION

Malting is the limited germination of cereal grains under controlled conditions, to bring about the physical, chemical and biochemical changes that are collectively termed modification[12]. One of the key qualities of malting barley (*Hordeum vulgare*) is its ability to germinate rapidly and synchronously[4]. Dormancy can interfere with the rapid and uniform germination of barley, thereby reducing the resultant malt quality. Storage at room temperature is a well practiced means of overcoming dormancy of malting barley. However, although storage can remove dormancy, storage is expensive and can be troublesome when the grain starts to die and loose germination vigor. A better understanding of the physiological characteristics of dormancy decay may help predict the optimal maturity of barley for malting, which in turn would enhance malt quality.

Dormancy is defined as a state in which viable seed will not germinate when placed in conditions normally considered adequate for germination, that is, when provided with a suitable temperature, adequate moisture and sufficient oxygen. Even though a seed lot can be considered non-dormant, the rate at which the seed population germinates can vary. Dormancy may be broken and the germination vigour of the seeds improved by dry storage and/or exposure of dry seed to elevated temperatures, treatment with chemicals (e.g. gibberellins), chilling, leaching, scarification, light and exposure to fluctuating temperature[4,26]. Of these treatments, storage is one of the most practical and often unavoidable ways in which dormancy decays. Seed storage conditions, such as temperature and moisture content, are the major factors influencing the rate of dormancy decay.

Australian barley dormancy is not thought to be strong, but nevertheless it is a major factor influencing maltability and final malt quality. Woonton *et al.*[28] found that storage of Australian barley at room temperature for up to one year significantly increased the germination vigor (as measured by the Germinative Index) with a concomitant enhancement in the production of hydrolytic enzymes during malting and improvements in an array of malt quality parameters (diastatic power, malt extract, kolbach index (KI) and apparent attenuation limit). A study by Gothard[10] with European barley also showed that storage for up to one year increased malt quality parameters such as the Hot Water Extract and KI. Furthermore, a study with New Zealand barley found similar results[7].

Many hypotheses have been proposed to explain the mechanism of seed dormancy, including the hormone balance theory, the metabolic deficiency theory and changes in grain respiration[3]. However, because of its complexity, the fundamental basis of the induction, maintenance and termination of dormancy remains unknown[14]. It is thought

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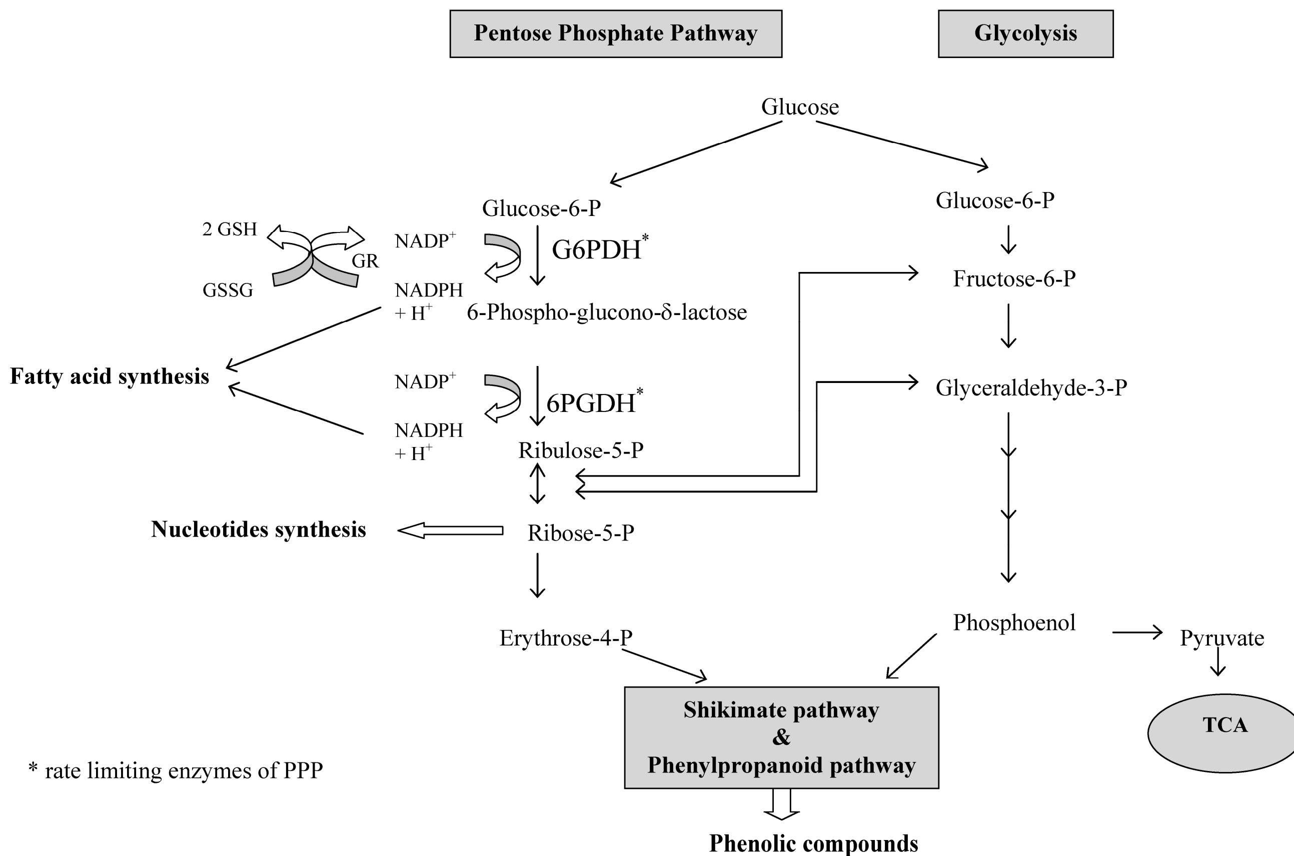

Fig 1. Schematic representation of the Pentose Phosphate Pathway (PPP) and its relationship to glycolysis, oxido reduction cycle involving glutathione reductase (GR), glutathione, synthesis of nucleotides, fatty acid and phenolic compounds. GR = glutathione reductase, GSH = reduced glutathione, GSSG = oxidised glutathione; TCA = tricarboxylic acid cycle, G6PDH = glucose-6-phosphate dehydrogenase, 6PGDH = 6-phosphogluconate dehydrogenase. (Modified from: Voet and Voet[25]; Zimmer[31]; Shetty *et al.*[21])

that there is a relationship between the respiration rate of the germinating grains and endosperm modification during malting. Respiration not only supplies the necessary energy and carbon sources for synthesizing new proteins, but also participates in the degradation of inhibitors of germination[11]. Yoshida *et al.*[30] found that a lowered respiration rate during steeping is correlated with slower malt modification. Thornton *et al.*[23] also ascertained that the respiration rate of embryos isolated from both dormant and non dormant grain was similar over the first 13 hours of imbibition, after which the oxygen uptake increased in the non-dormant sample and remained at the initial low level in the dormant sample. There is little information on dormancy decay, germination vigour and the respiration of barley during malting. The first aim of this study was to examine the relationship between germination vigour and barley respiration rate during malting.

Three respiratory pathways are assumed to be active in the imbibed seed, namely glycolysis, the pentose phosphate pathway (PPP) and the citric acid cycle[3]. The pentose phosphate pathway (Fig. 1) is an essential alternative metabolism for utilization of carbohydrates[25], and is thought to be the main oxidative pathway active during the initial stages of germination of various seeds such as lettuce, barley, oats, rice and kidney beans[18,19].

Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme of the PPP[8,25], and increased G6PDH activity has been associated with dormancy decay and increased seed vigor of a variety of seeds. Swamy and Sandhyarani[22] found that there was a gradual increase in G6PDH activity during dry storage of dormant peanut seeds (*Arachis hypogaea*). They also observed a distinct increase in the G6PDH activity when the dormant seeds were treated with kinetin, a peanut seed dormancy breaking hormone[17]. Likewise, Upadhyaya *et al.*[24] investigated the levels of G6PDH in genetically dormant and non-dormant pure lines of wild oat (*Avena fatua*) and found that after 24 h of imbibition there was a greater enzyme activity in embryos isolated from the non-dormant line compared to the dormant counter part. Furthermore, an increased germination vigour of cabbage, rye and tomato seeds has been associated with greater activities of G6PDH[2,11]. Although this literature indicates that the PPP is involved in dormancy decay, there has been little research examining the dormancy decay of barley, germination vigour and PPP activity. The second aim of this study was to examine the effect of barley storage on the rate limiting enzyme of the PPP; G6PDH.

MATERIALS AND METHODS

Barley samples

Commercially grown *Hordeum vulgare* cv. Gairdner (2002 harvest) was obtained from the Australian states of New South Wales (NSW), Victoria (VIC) and Western

Table I. Variety, harvest year, growing location, moisture content and protein content of the Australian barley samples employed in this study.

Variety	Harvest	Growing location	Moisture (%)	Protein (% d.b.)
Gairdner	2002	Victoria	9.7 ± 0.2	11.5 ± 0.1
Gairdner	2002	New South Wales	9.8 ± 0.2	10.5 ± 0.1
Gairdner	2002	Western Australia	10.3 ± 0.2	10.0 ± 0.1

(± the SE of the mean, n = 3)

Australia (WA). The moisture and protein contents of each sample were determined using EBC[9] methods 3.3 and 3.3.2, respectively (Table I). The barley samples were stored at room temperature (20–25°C) for 7 months and at 1, 3 and 7 months approximately 2-kg samples were sealed in polyethylene bags and, following a well established practice[6,27,28], stored at –18°C to halt further changes.

Germination testing

The 4 mL and 8 mL germinative energy (GE) and germinative index (GI) of the samples were determined according to EBC[9] method 3.7.

Malting

Before micro-malting in an Automated Joe White Malting Systems Micro-malting unit (Perth, Australia), samples (approximately 100 g) were removed from the freezer and left at room temperature for two hours in an air tight glass container. The samples were then micro-malted in two 50 g lots, one for respiration studies and the other for α-amylase and G6PDH enzyme activity assays. The malting program consisted of a 22 h interrupted steeping program (12 h submerged, 8 h air rest (17°C, 70% airflow) and 2 h submerged) at 17°C, and a 95 h germination program at 17°C and 70% airflow. To stagger the micro-malting of each sample, the first wet phase of the steeping process was carried out in a water bath at 17°C. Steeping air rest periods and the germination processes were undertaken in the Micro-malting unit. At 18 h of germination, 6 g of water was manually added to each sample. At 42 h and 66 h of germination, samples were made up to 46% moisture (w/w) by the manual addition of water.

Respiration measurement

At the end of steeping, and after 18 h, 42 h, 66 h and 95 h of germination the rate of grain respiration was measured using a Servomex 1400 Gas Analyser (Crowborough, Sussex, England) with a miniport pump (N72 KN.18, KNF Neuberger, Germany). Each sample was removed from the micro-malter, placed in the respiratory chamber and held at 17°C. The percentage carbon dioxide and oxygen in the circulating air was recorded at 2 min intervals over an 11 min period. The carbon dioxide production and oxygen consumption rates were determined by the increase in carbon dioxide and decrease in oxygen concentration over this period. The Gas Analyser was calibrated using atmospheric air (O_2 = 20.9%, CO_2 = 0.03%) and 9.2% CO_2 (0% O_2) at 101 kPa (BOC Australia).

From the observed changes in CO_2 and O_2, the carbon dioxide production rate and oxygen consumption rate were calculated and expressed in $mg \cdot h^{-1} \cdot kg^{-1}$ dry weight using the following equations:

$$\frac{dCO_2}{dt} = \frac{dk \times V \times s}{m}$$

$$\frac{dO_2}{dt} = \frac{dk \times V \times s}{m}$$

where

dCO_2/dt = carbon dioxide production rate ($mg \cdot h^{-1} \cdot kg^{-1}$)
dO_2/dt = oxygen consumption rate ($mg \cdot h^{-1} \cdot kg^{-1}$)
dk = change in CO_2 or O_2 concentration (% h^{-1})
V = volume of measurement chamber (dm^3)
s = Density of CO_2 (1.977 $g \cdot dm^{-3}$) or density of O_2 (1.345 $g \cdot dm^{-3}$)
m = Mass of sample (kg dry weight)

G6PDH activity

The G6PDH activity was measured at 0 h, at the end of steeping, and at 18 h, 42 h, 66 h and 95 h of germination using a method adopted from Shetty *et al.*[21]. Briefly, seeds (10) were homogenised in 10.0 mL of cold enzyme extraction buffer (0.5% w/v polyvinyl pyrrolidone, 3 mM EDTA and 0.1 M potassium phosphate buffer at pH 7.5) using a cold mortar and pestle. A 1.0 mL aliquot of the homogenate was pipetted into a 1.5 mL Eppendorf tube and centrifuged at 10,000*g* for 10 min at 3°C. After centrifugation, 50 μL of the enzyme extract supernatant was pipetted into a 1.5 mL plastic cuvette, to which a 1.0 mL aliquot of enzyme reaction mixture (0.38 mM β-NADP; 6.3 mM magnesium chloride; 3.3 mM glucose-6-phosphate; 5 mM maleimide and 50 mM Tris HCl at pH 8.0) was added. The mixture was held at 25°C and the change in absorbance at 339 nm was recorded every min over an 8 min period. The G6PDH catalytic activity was expressed in units (U) and calculated using the following equations:

$$b = \frac{\Delta A \times V \times 1000}{\varepsilon \times d \times v} \, \mu\text{mol} \times \text{min}^{-1} \times \text{L}^{-1} \text{ (U/L)}$$

$$\text{G6PDH (U/grain)} = \frac{b \times \text{enzyme extraction volume}}{\text{number of grains}}$$

where

ε = linear millimolar absorption coefficient of NADPH, $L \times mmol^{-1} \times mm^{-1}$
ΔA = rate of change of absorbance, min^{-1}
d = light path, mm
V = assay volume, L
v = volume of sample used in assay, L

α-Amylase activity

The α-amylase activity of all samples was determined at 95 h of germination using AACC method 22-05[1]. Briefly, seeds (10) were homogenised in cold enzyme extraction buffer (0.05M sodium malate, 0.05M sodium chloride, 2mM calcium chloride and 0.005% sodium azide) using an X620 homogeniser (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The samples were centrifuged at 1,000*g* for 10 min at 3°C and the supernatants were suitably diluted (1:80). After dilution, 0.10 mL of

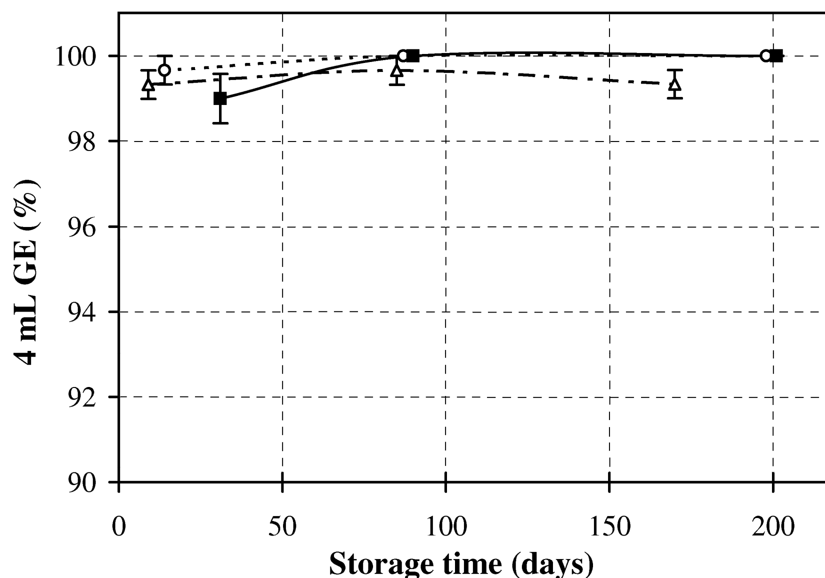

Fig. 2. Changes in the 4 mL GE of VIC (△), NSW (○) and WA (■) Gairdner samples stored for up to 7 months. Error bars represent the SE of the mean. Where error bars are not visible they are smaller than the symbol, n = 3.

the diluted enzyme extract was pipetted into a 10 mL V-bottom test tube (Technoplas Australia) and incubated at 40°C for 5 min. To each tube, 0.1 mL of non-reducing-end blocked p-nitrophenyl maltoheptaoside (BPNPG7) substrate solution (pre-incubated at 40°C for 15 min; Megazyme Ltd., Ireland) was added directly to the bottom of the tube and incubated for exactly 10 min. At the end of incubation, 1.5 mL of the stopping reagent (1% Trizma base (Sigma Aldrich) adjusted to pH 11.0 with 1M NaOH and/or 1M HCl) was added and the tube contents were vortexed for 10 s. The absorbance of the solution was measured at 410 nm and the α-amylase activity calculated according to AACC method 22-05[1].

All experiments were conducted in triplicate and statistical analysis of variance (ANOVA) was undertaken using MINITAB software (Release 13.32).

RESULTS AND DISCUSSION

Germination parameters

The 4 mL GE is the percentage of grains that germinate over three days on filter paper soaked in 4 mL of water, and is generally considered a good test for the determination of barley dormancy. For malting barley, the requirement is that this value must be greater than 96%. The 4 mL GE of all samples was greater than 96% from harvest (time zero) until the end of the storage period, with no significant difference between values during this time ($p < 0.05$, Fig. 2). The observed high 4 mL GE values throughout storage indicate that the barley samples employed had minimal dormancy. These results are similar to Woonton *et al.*[28], who found that the 4 mL GE values of Australian Franklin, Arapiles and Schooner samples did not change significantly during twelve months of post harvest storage. Germination can be reduced if barley encounters excessive levels of moisture during germination, a phenomenon called water sensitivity[16]. The water sensitivity of barley is indicated by the 8 mL GE, which is obtained by adding an additional 4 mL of water (total of 8 mL) to the 4 mL GE test. In this study, storage for 7 months significantly improved the 8 mL GE values ($p < 0.05$) of the VIC and WA samples (Fig. 3), indicating that post harvest storage reduced the grain's water sensitivity. However, the NSW sample was not water sensitive from the very beginning and remained so throughout the 7 month storage period. Woonton *et al.*[28] and Briggs *et al.*[6] have indicated that both European and Australian grown barley overcome water sensitivity during post-harvest storage. The mechanisms involved in water sensitivity remain unknown, although microbes are thought to be involved[15]. Other factors may also be implicated, as treatment of barley with anti-microbial agents does not consistently overcome water sensitivity[13].

Seeds that do not show dormancy (high 4 mL GE values) can none the less have a reduced rate at which the seed population germinates. The GI is a measure of the rate at which the grains in a GE test germinate and high GI values are essential for high quality and homogenous malt. In this study, storage of the VIC, NSW and WA samples for 7 months significantly increased the GI values by 15%, 18% and 24%, respectively ($p < 0.05$, Fig. 4). The increase in GI values indicates that storage significantly improves the rate at which the grains germinate. Woonton *et al.*[28] found similar results and also revealed that the GI was correlated with the ability of the barley to produce enzymes during malting and various final malt quality parameters. The increases in the GI of these samples are likely to be associated with improvements in malt quality parameters (not measured).

α-Amylase activity

Post harvest storage of all samples lead to a significant increase in the α-amylase activity of the germinated grain ($p < 0.05$, Fig. 5). After 7 months of storage the α-amylase activity at the end of malting increased in the VIC

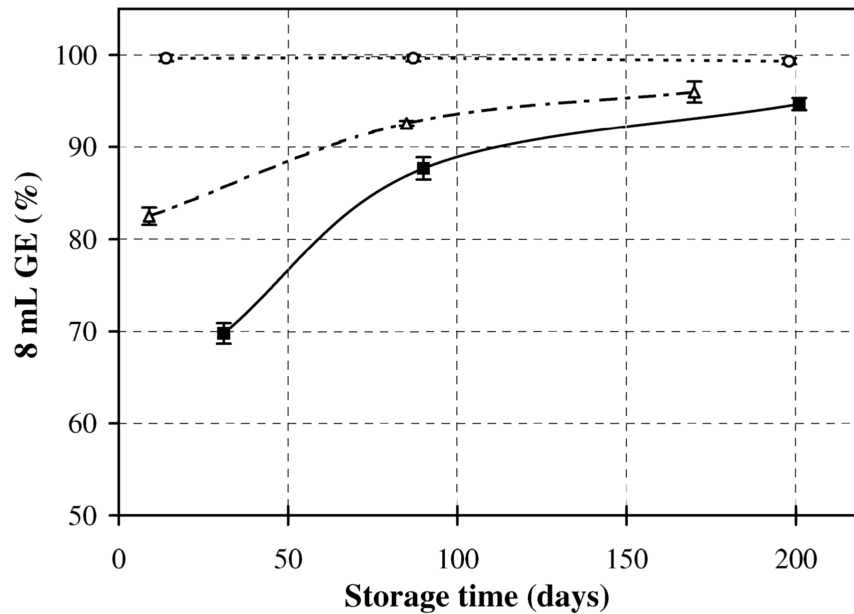

Fig. 3. Changes in the 8 mL GE of VIC (△), NSW (○) and WA (■) Gairdner samples stored for up to 7 months. Error bars represent the SE of the mean. Where error bars are not visible they are smaller than the symbol, n = 3.

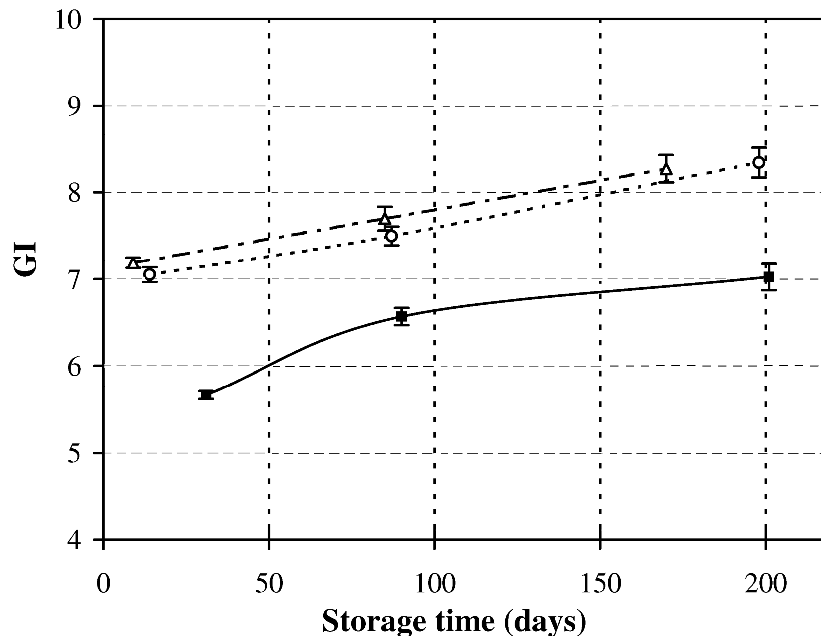

Fig. 4. Changes in the GI of VIC (△), NSW (○) and WA (■) Gairdner samples stored for up to 7 months. Error bars represent the SE of the mean. Where error bars are not visible they are smaller than the symbol, n = 3.

sample by 20%, in the NSW sample by 84% and in the WA sample by 45%. These findings are similar to those of Woonton *et al.*[28] and indicate that storage of these samples was influencing not only water sensitivity (8 mL GE) and germination rate (GI), but also enzyme synthesis during malting. It is interesting to note that the barley sample from WA had the lowest 8 mL GE values, the lowest GI values but the highest α-amylase activity at all storage times. This suggests that the growing location and/or environment have a large influence on the germination results and the ability of the grain to synthesise α-amylase during malting.

The changes observed in the 8 mL GE, GI and α-amylase activity in this study may be due to a large number of factors. During germination, the growing barley embryo synthesizes and secretes gibberellins into the grain which triggers the aleurone to synthesise and secrete a range of hydrolytic enzymes. It may be that the changes observed in this study are due to changes in gibberellin production[26,29]. However, Briggs[5] and Schuurink *et al.*[20] have

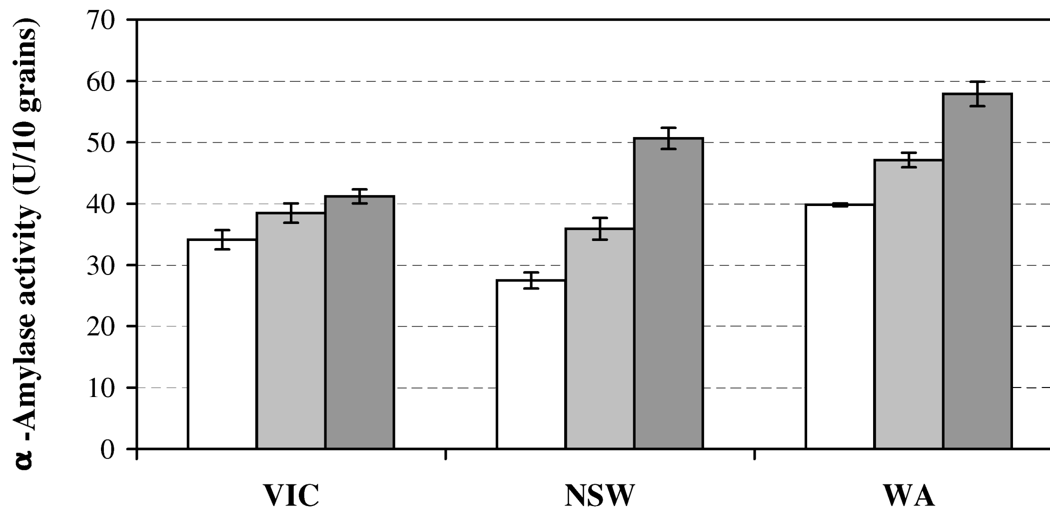

Fig. 5. The α-amylase activity at the end of germination of VIC, NSW and WA Gairdner samples stored for 1 month (white bar), 3 months (light grey bar) and 7 months (dark grey bar) after harvest. Error bars represent the SE of the mean, n = 3.

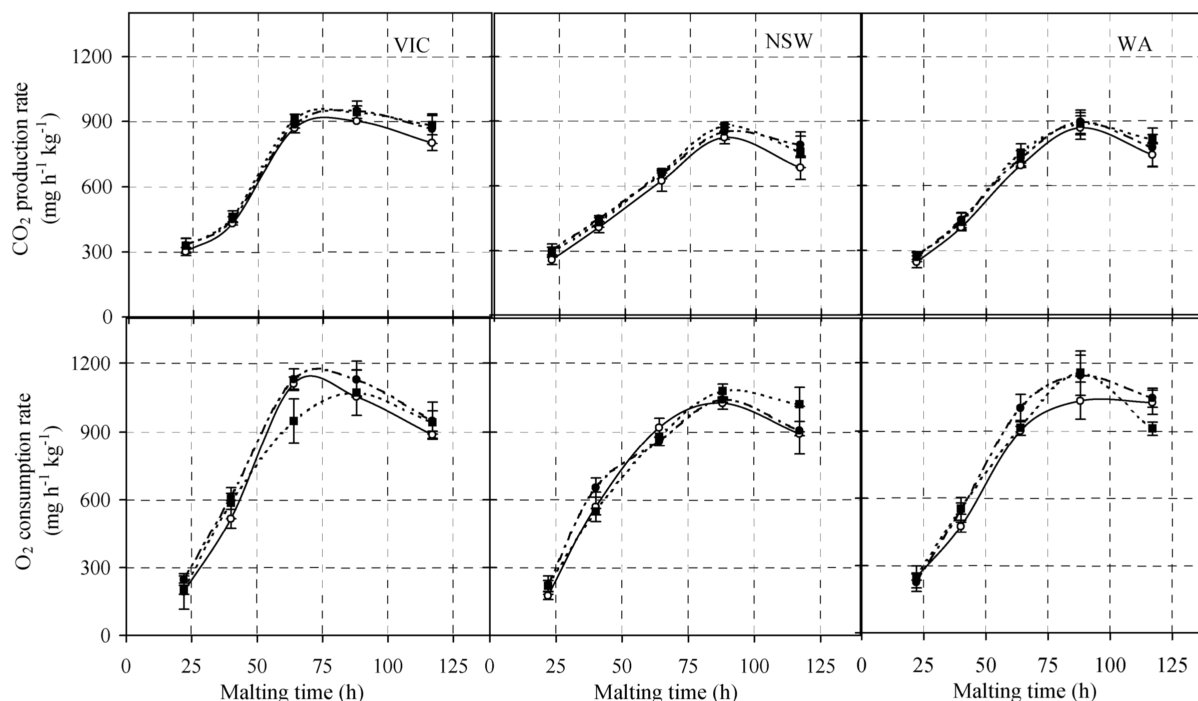

Fig. 6. CO_2 production rate and O_2 consumption rate during malting of VIC, NSW and WA Gairdner samples after 1 month (○), 3 months (●) and 7 months (■) of post-harvest storage. Error bars represent the SE of the mean. Where error bars are not visible they are smaller than the symbol, n = 3.

found that the dormancy state of the barley grain is correlated with the gibberellin responsiveness of isolated aleurone layer. Thus, the observed increase in the α-amylase activity during malting of stored samples may be due to an increased aleurone sensitivity to gibberellin.

Respiration rate

Respiration is the main source of energy for germination and has been linked with the dormancy decay of European barley. For all samples the carbon dioxide production rate at the end of steeping (22 h) was approximately 300 $mg \cdot h^{-1} \cdot kg^{-1}$ (dw) and increased to approximately 900 $mg \cdot h^{-1} \cdot kg^{-1}$ (dw) by 88 h of malting, after which it declined slightly (Fig. 6). Similarly, for all samples, the oxygen consumption rate at the end of steeping was 300 $mg \cdot h^{-1} \cdot kg^{-1}$ (dw) and increased to approximately 1200 $mg \cdot h^{-1} \cdot kg^{-1}$ (dw) by 88 h of malting. After this time the oxygen consumption decreased by a small extent. The observed respiratory pattern was similar to that found by others with barley[5].

Although post harvest storage of all samples for up to seven months influenced the rate of germination and α-amylase activity, it did not significantly change the carbon dioxide production rate or the oxygen consumption rate

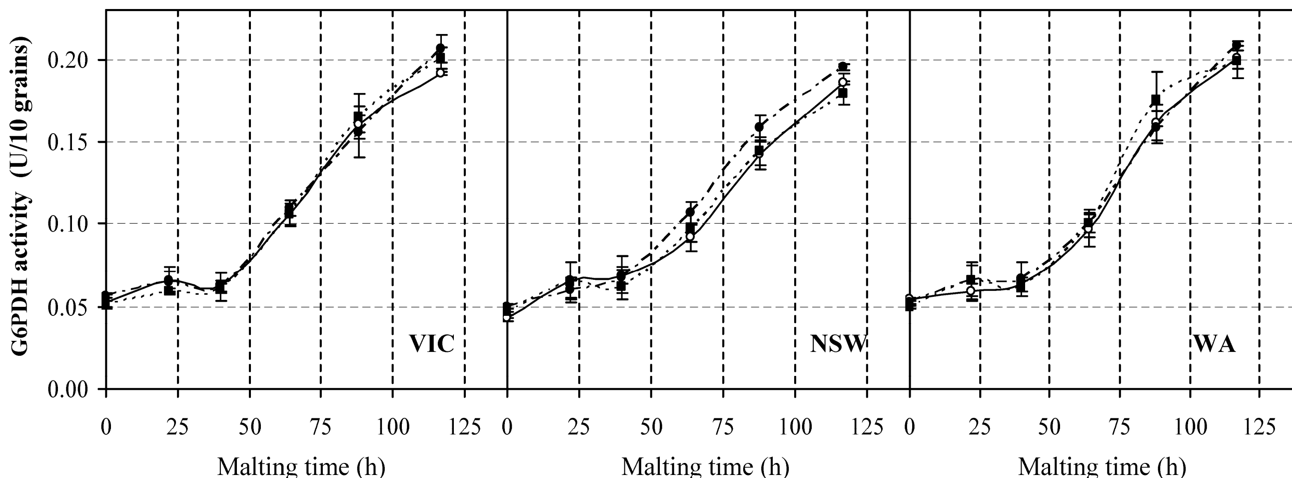

Fig. 7. G6PDH activity during malting of VIC, NSW and WA Gairdner after 1 month (○), 3 months (●) and 7 months (■) of post-harvest storage. Error bars represent the SE of the mean. Where error bars are not visible they are smaller than the symbol, n = 3).

during malting ($p > 0.05$). These results are in contrast to those of Thornton *et al.*[23] who found a difference in the respiration rate between dormant and non-dormant European barley samples. Typically, European barley has a more pronounced dormancy when compared to Australian barley, possibly due to the short days and colder climates in Europe[20]. The samples employed in this study had minimal dormancy, with no changes in the GE values over the seven month storage period. The differences between our results and those of Thornton *et al.*[23] could be due to this fact.

The growing environment (VIC, NSW and WA) had no significant effect on carbon dioxide production rate and the oxygen consumption rate at various stages of malting ($p > 0.05$, Fig. 6). This finding suggests that the environmental variations between these Australian states do not affect the respiration rate of the barley grains during malting.

G6PDH activity

All of the samples had a small amount of G6PDH activity before malting (Fig. 7) and a small increase in G6PDH activity during the first 40 h of malting (i.e. during steeping and up until 20 h of germination). After this time, the G6PDH activity of all samples increased until the end of malting (Fig 7). This sudden increase in G6PDH activity coincided with the rapid growth of the acrospire (shoot) during the malting process. The increase in the amount of living tissue within the grain after 40 h of malting could be a possible explanation for the rapid increase in G6PDH activity after this time.

Storage of all samples had no significant effect on the G6PDH activity during malting ($p > 0.05$). This contradicts the findings of Betty and Finch-Savage[2], Gui *et al.*[11] and Swamy and Sandhyarani[22], who found that increased activity of G6PDH was correlated with increased germination vigour of cabbage, rye, tomato and peanut seeds. This discrepancy may be due to barley having different dormancy decay and germination vigour pathways. Alternatively, the favorable growing conditions in Australia may lead to barley with minimal dormancy, minimal vigour differences and, no or only very minor differences in G6PDH activity during malting.

The G6PDH activities of barley from different growing locations were significantly different at time zero and at 117 h of malting ($p < 0.05$). At both time points, the NSW samples had lower G6PDH activities compared to the VIC and WA samples. However, there were no significant differences in the G6PDH activity among the samples from different locations at 22 h, 40 h, 64 h and 88 h of malting. It would seem that growing environment has some small influence on G6PDH activity before malting and at the end of the malting process.

CONCLUSIONS

Storage of barley with minimal dormancy at room temperature causes significant improvements in water sensitivity, germination vigour and α-amylase production during malting. However, storage of such barley does not significantly influence the respiration rate or the G6PDH activity during malting.

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