Impact of cold acclimation, de-acclimation and re-acclimation on carbohydrate content and antioxidant enzyme activities in spring and winter wheat

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ABSTRACT

The capability of overwintering in winter cereals results in their ability to withstand cold hardening during the autumn or de-hardening during temporary warm spells and then to withstand re-hardening when cold waves return. The present study was carried out to evaluate quantitative changes in the carbohydrate content in leaves, hydrogen peroxide (H_2O_2) production, and the activities of guaiacol peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The effects were studied on a less cold-hardy spring cultivar Pishtaz (LT_{50} = –6°C), and a cold-hardy winter cultivar CDC-Ospray (LT_{50} = –20°C) of wheat under cold acclimation (20 days at 4°C), interrupted by de-acclimation (10 days at 25°C) and then followed by re-acclimation conditions (10 days at 4°C). Hardening conditions induced the accumulation of carbohydrates in both cultivars and the de-acclimated plants exhibited a significant reduction. The activities of tested antioxidative enzymes were altered in both cultivars during the investigated periods. Cold acclimation induced elevation in the activities of SOD, APX, CAT and GPX in leaves of winter wheat, whereas in spring wheat a considerable increase was only recorded after the de-acclimation period. The results of the present experiment agreed with the conclusion that in hardy winter wheat cold acclimation may be associated with an improvement of the scavenging capacity for reactive oxygen species and carbohydrate accumulation in leaves.

Keywords: Cold hardening, Low temperature tolerance, Oxidative stress, Reactive oxygen species, Re-hardening, Triticum aestivum

YFIRLIT

Áhrif hörðnunar, afhörðnunar og endurhörðnunar á kolvetnisorkuforða og virkni andoxunarensíma í vor- og vetrarhveiti.

Geta vetrarkorns til að lifa af veturinn byggist á getu þess til að harðna að hausti eða mótstöðu gegn afhörðnun í hlákum og síðan á endurhörðnun þegar kólnar aftur. Þessi rannsókn var gerð til að kanna og meta breytingar á magni kolvetna í blöðum, framleiðslu á vetnisperoxíði (H_2O_2) og virkni guaiacol peroxidasa (GPX), superoxide dismutasa (SOD), catalasa (CAT) og ascorbate peroxidasa (APX). Rannsóknin var gerð á lítið vetrarþolnu kvæmi af vorhveiti Pishtaz (LT_{50} = –6°C) og vetrarþolnu kvæmi, CDC-Ospray (LT_{50} = –20°C). Hveitið var hert í kæli í 20 daga við 4°C, síðan afhert í 10 daga við 25°C og þá endurhert í 10 daga við 4°C. Á hörðnunartímanum jókst magn kolvetna í báðum kvæmunum en magnið minnkaði við afhörðnunina. Virkni andoxunarefnanna sem mæld voru breyttist í báðum kvæmunum á rannsóknartímabilinu. Á hörðnun-

artímanum jókst virkni SOD, APX, CAT and GPX í blöðum vetrarhveitisins, en í vorhveitinu varð aukning einungis við afhörðnun. Niðurstöður þessarar athugunar eru í samræmi við þá ályktun að frostþol í hinu þolna vetrarhveiti tengist aukningu á viðgerðargetu vegna skemmda af völdum sindurefna ásamt uppsöfnun kolvetna í blöðum.

INTRODUCTION

Low temperature (LT) is a major environmental limitation on plant geographical distribution and productivity (Boyer 1982, Larcher 1995). At high latitudes or altitudes the problem of coping with LT is exacerbated by the need to prolong the growing season beyond the short summer. Many changes in physiological and biochemical parameters have been observed during the exposure of plants to LT; modified levels and activities of enzymes from various metabolic pathways, accumulation of carbohydrates, amino acids and soluble proteins, as well as the appearance of new isoforms of proteins and altered lipid membrane composition (Thomashow 1999). A clear understanding of the molecular mechanisms through which plants respond to LT is of fundamental importance to plant biology. Knowledge about these mechanisms is also crucial for the development of rational breeding and transgenic strategies to improve stress tolerance in crops (Xiong et al. 2002).

One of the most important agronomic traits in winter cereals is winter-hardiness or LT tolerance, which can be enhanced by exposure to low non-freezing temperatures, a phenomenon known as cold acclimation (Guy 1990). During cold acclimation, several physiological changes occur, including alteration of lipid composition in plasma membranes, accumulation of protecting compounds and osmolytes, as well as the induction of new genes activity (Hughes & Dunn 1996). LT-induced overproduction of reactive oxygen species (ROS) brings about serious cellular damage by rapidly reacting with DNA, lipids, and proteins (Sattler et al. 2000). In order to avoid these oxidative injuries, plants have developed enzymatic systems for scavenging these highly active forms of ROS, superoxide is converted by superoxide dismutase (SOD) enzyme into H_2O_2, which is further scavenged by catalase (CAT) and various peroxidases. In several studies guaiacol peroxidase (GPX) and CAT were induced by LT (Janda et al. 2003, Scebba et al. 1998).

Although researchers have intensively studied various aspects of acclimation, the processes of de-acclimation and re-acclimation remain less understood. The term de-acclimation can be used to describe losses in hardiness due to such diverse factors as environmental stimuli (i.e. warm temperatures), phenological changes, and reactivation of growth. Furthermore, de-acclimation may be either reversible by subsequent re-exposure to low temperatures or result in a largely irreversible loss of hardiness (Kalberer et al. 2006). Some overwintering plants can return to previous levels of LT tolerance after de-acclimation, which has been referred as re-acclimation. Resistance to de-acclimation and the ability to re-acclimate may be of greater importance for winter survival, particularly during fluctuating winter conditions (Rapacz 2002).

This study was designed to examine the differential responses of spring and winter wheat cultivars during cold acclimation, de-acclimation and re-acclimation via determining antioxidant enzymes, hydrogen peroxide, and total soluble carbohydrates.

MATERIALS AND METHODS
Plant material and growth condition
The seeds of two wheat (*Triticum aestivum* L., 2n= 6x=42) cultivars were grown for 18 days in plastic pots filled with loamy soil under controlled conditions in a growth chamber. The cultivars included hardy winter wheat with a long vernalization requirement (Canadian winter wheat cv. CDC Ospray; LT_{50}= -20°C) and less hardy spring wheat with no vernalization requirement (Iranian spring wheat cv. Pishtaz, LT_{50}= -6°C under field conditions). Seedlings emerged and were grown under 340

μmol m^{-2}s PAR at soil level with a photoperiod 12 h day^{-1} provided by white fluorescent tubes and a constant air temperature of 25±1˚C. Once the wheat plants had reached the stage of a fully developed third leaf (18 days after planting) they were sampled as the control condition (non-acclimated plants); the remaining plants were transferred to a constant air temperature of 4±1 with 340 μmol m^{-2} s PAR and a photoperiod 12 h day^{-1} for 20 days as the acclimation condition. After taking samples on experimental day 38 the remaining plants were transferred in the growth chamber for de-acclimation with the same light intensity for a 12 h daily photoperiod at 20±1˚C for 10 days. On the 10th day after de-acclimation plants were sampled randomly. The remaining plants were re-acclimated under the conditions described above for 10 days. On experimental day 58 re-acclimated plants were harvested. After each sampling leaves were ground with mortar and pestle using liquid nitrogen and stored at -70˚C until analysis.

Carbohydrate content

Water soluble carbohydrates were determined using the phenol–sulfuric acid method (Dubois et al. 1956). Their concentration in the extract was determined at 490 nm in a spectrophotometer, using glucose as the standard.

Enzyme extraction

For SOD, CAT, APX and GPX extraction, leaf samples (0.5g) were homogenized in ice cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in a Beckman refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay (Esfandiari et al. 2007).

Enzyme activity assay

SOD activity was estimated by recording the decrease in absorbance of superoxidenitro blue tetrazolium complex by the enzyme (Gupta et al. 1993). About 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.01 ml

of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as controls. The reaction was started by adding 0.1 ml riboflavin (60 μM) and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped by switching off the light and covering the tubes with a black cloth. Tubes without enzyme developed maximal color. A non-irradiated complete reaction mixture which did not develop color served as a blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes. The SOD activity was expressed as units per milligram of protein per minute.

CAT activity was measured according to Aebi (1984), using about 3 ml of reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H_2O_2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. The reaction was started by adding H_2O_2 and the decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed and expressed as micromoles of destroyed H_2O_2 per milligram of protein per minute.

APX activity was measured according to Yoshimura et al. (2000) by monitoring the ascorbate oxidation in 3 ml of the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM sodium ascorbate, 6 mM hydrogen peroxide, and 0.2 ml supernatant. Oxidation of sodium ascorbate was started by adding 9.7 M hydrogen peroxide and it was followed at 290 nm for 1 min at 22°C. The APX activity was expressed as milimoles of ascorbate oxidized per milligram of protein per minute.

The activity of guaiacol peroxidase (GPX) was determined by measuring the oxidation of

guaiacol. The assay mixture contained 10 mmol L^{-1} potassium phosphate (pH 6.4), 8 mmol L^{-1} guaiacol, and 2.75 mmol L^{-1} H_2O_2. The increase in absorbance was recorded at 470 nm within 2 min (linear phase) after the addition of H_2O_2 (Huang et al. 2006). The GPX activity was stated as micromoles of destroyed H_2O_2 per milligram of protein per minute.

Statistical Analyses

All results reported were the means of three replicates. Data were analyzed by two-factor variance analysis using SPSS (15.0).

RESULTS

Leaf total water soluble carbohydrate content

The accumulation of carbohydrate during ac-climation, de-acclimation and re-acclimation is shown in Figure 1. Carbohydrate accumulation clearly increased in plants exposed to the acclimation condition in the leaves of either cultivar compared to the controls and declined after de-acclimation. Reexposing to the LT condition significantly improved accumulation of carbohydrate in winter wheat. The winter wheat cultivar appeared to surpass spring wheat in this accumulation. These results are consistent with the findings of other researchers (Perras & Sarhan 1984, Javadian et al. 2010) who reported the accumulation of soluble carbohydrate in wheat seedlings during cold acclimation.

Activity of antioxidant enzymes

The Activity of SOD in leaves of both winter
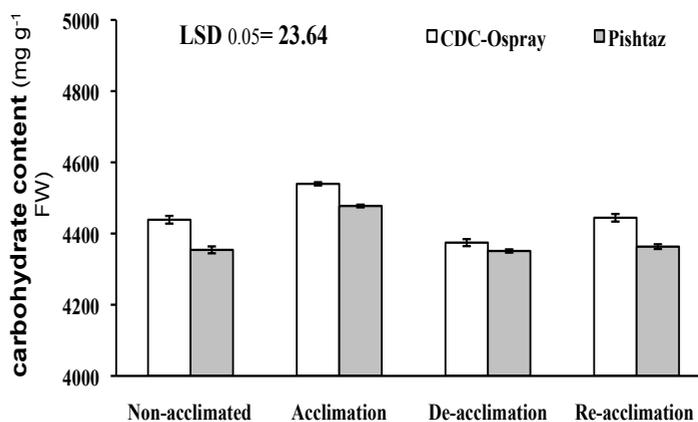
Figure 1. Changes in total water soluble carbohydrate content in leaf tissue of winter and spring wheat cultivars after cold acclimation (4 °C), de-acclimation (25 °C) and re-acclimation (4 °C) The values and standard errors (vertical bars) of three replications are shown.
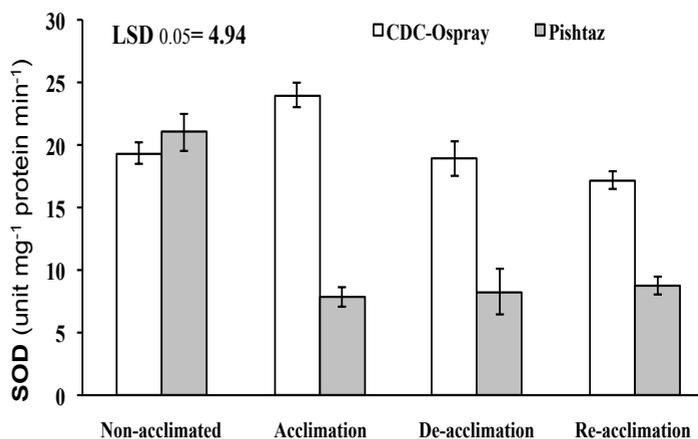
Figure 2. The effect of 20 days cold acclimation (4 °C) followed by 10 days de-acclimation (25°C) and 10 days re-acclimation on SOD activity in leaf tissue of winter and spring wheat cultivars.

and spring wheat was similar to the control condition (non-acclimated plants). However, cold acclimated winter wheat plants showed a significant increase in SOD activity in their leaves compared with the control (Figure 2). In contrast, acclimation caused a drastic reduction in the activity of SOD in spring wheat and a stable activity state was observed during future sampling. A significant loss of SOD activity was detected in winter wheat after the

de-acclimation period. Re-acclimation could not affect SOD activity in either cultivar when compared with de-acclimated plants.

As shown in Figure 3, APX activity in winter wheat during the whole sampling time was higher than for the spring cultivar. Winter wheat plants subjected to acclimation showed a significant increase in APX activity compared to the 25°C-grown control plants. However, cold acclimation induced a small reduction in the APX activity in spring wheat leaves. After returning to optimal conditions APX activity increased in the spring cultivar, whereas behavior of the APX was stable in winter cultivar. Re-exposing the winter wheat plants to the acclimation condition significantly increased the APX activity.

There were alternations in CAT activity during specific periods (Figure 4). Similar to APX, CAT activity in winter wheat was considerably higher than in the spring cultivar. Cold acclimation caused obvious slow increases in the activity of CAT in the leaves of winter wheat cultivars. Although after the period of de-acclimation CAT activity was relatively constant in the winter cultivar, it showed a notable increase in the spring cultivar. The leaves of the re-acclimated winter cultivar had nearly twice the CAT activity than the de-acclimated plants.

The GPX activity increased remarkably in the LT-treated leaves of the winter wheat cultivar compared with the previous optimum condition, while its activity decreased in the spring
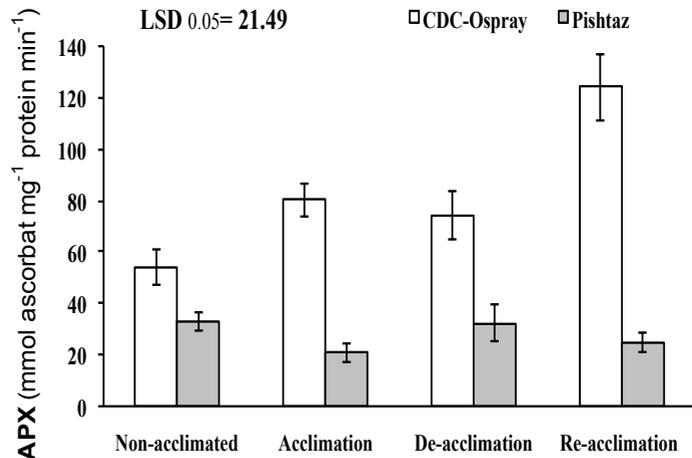
Figure 3. The effect of 20 days cold acclimation (4 °C) followed by 10 days de-acclimation (25°C) and 10 days re-acclimation on APX activity in leaf tissue of winter and spring wheat cultivars.
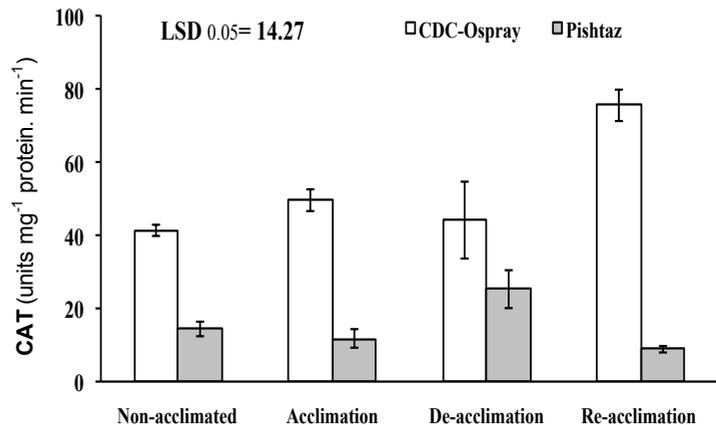
Figure 4. The effect of 20 days cold acclimation (4 °C) followed by 10 days de-acclimation (25°C) and 10 days re-acclimation on CAT activity in leaf tissue of winter and spring wheat cultivars.

wheat with cold acclimation (Figure 5). The response pattern of GPX activity to the de-acclimation condition was opposite in the cultivars, so that exposure to optimum conditions could increase its activity in the less cold-hardy spring cultivar but was decreased in winter wheat. Re-exposing of plants to LT could considerably induce GPX activity in the hardy winter wheat cultivar, but the spring cultivar showed the opposite reaction. .

Analysis of the hydrogen peroxide changes

during sampling dates revealed that the less cold-hardy spring cultivar had a higher concentration than did the winter wheat (Figure 6). The H_2O_2 concentration after the acclimation period was obviously higher than that of non-acclimated seedlings in both cultivars. On the other hand, returning the winter wheat plants to optimal conditions increased the H_2O_2 concentration, which conversely decreased after the re-acclimation period.

DISCUSSION

Descriptive ecological and agronomic studies have uncovered a strong correlation between sugar concentrations and LT tolerance (Sasaki et al. 2001, Guy 1992, Perras & Sarhan 1984, Gudleifsson 2010). Carbohydrate accumulation can create a lower osmotic potential (O'Neill 1983). Carbohydrates have been shown to function as protectants of plasma membranes and proteins from the effects of freezing and dehydration (Santarius 1973, Steponkus 1984) and they may act to suppress ice nucleation (Mackenzie 1977). The sugar concentrations in transformants with an antisense inhibition of cytosolic fructose-1,6-bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS) expression, or with over-expression of maize SPS, has been shown to correlate with the extent of LT tolerance after cold acclimation (Stitt & Hurry 2002). Cold acclimation is also associated with the accumulation of other soluble solutes like proteins (Javadian et al. 2010). In wheat LT led to
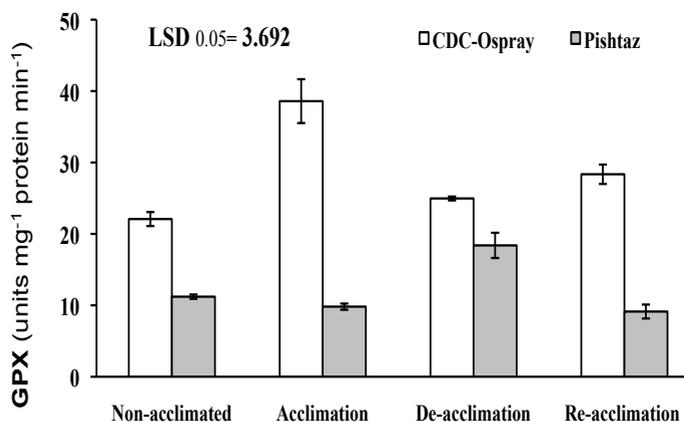
Figure 5. The effect of 20 days cold acclimation (4 °C) followed by 10 days de-acclimation (25°C) and 10 days re-acclimation on GPX activity in leaf tissue of winter and spring wheat cultivars.
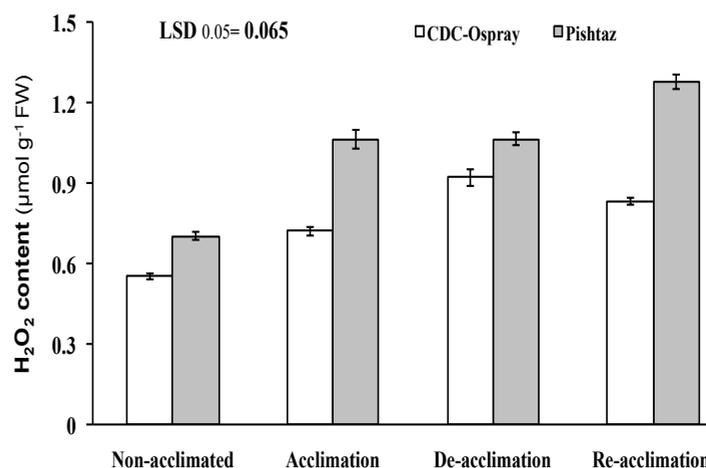
Figure 6. The effect of 20 days cold acclimation (4 °C) followed by 10 days de-acclimation (25°C) and 10 days re-acclimation on H2O2 concentration in leaf tissue of winter and spring wheat cultivars.

fructan synthesis (Tognetti et al. 1989). The present study with wheat plants showed that total carbohydrate content also increased on exposure to LT, especially in the cold- hardy cultivar. However, it is not clear to what extent the LT tolerance of plants depends on the carbohydrates accumulated at low temperature.

As previously shown by other authors (O'Kane et al. 1996, Guo et al. 2006) much of

the injury caused to plants during cold stress can be associated with reactive oxygen species (ROS), especially in chilling sensitive plants. Plants have developed effective oxygen-scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase (GPX) and catalase, and non-enzymatic antioxidants, such as ascorbic acid and reduced glutathione. These antioxidants protect membranes from the deleterious effect of ROS. It has been shown that LT tolerant cultivars have higher activities of antioxidant enzymes than less cold-hardy cultivars in several crops, such as rice and maize, results comparable to those found in the present study (Anderson et al. 1994, Guo et al. 2005). During both cold- and chilling-acclimation, a plant activates scavenging enzymes which helps to detoxify the cell (Apel & Hirt 2004, Gadjev et al. 2006) and then results in an increased tolerance to LT stress.

However, some part of recorded changes in antioxidant activity can result from plant development. In wheat plants LT tolerance decreases after the vegetative/reproductive transition, but some mechanisms such as the requirement for vernalization and responses to photoperiods can slow down the rate of phenological development and lengthen the vegetative phase (Mahfoozi et al. 2001). Accordingly the length of the vegetative stage affects the duration of LT-tolerance gene expression (Limin & Fowler 2006). Winter wheat cultivars, given their high vernalization requirement and a higher sensitivity to short days, can stay in the vegetative phase during winter and therefore conserve their re-acclimation ability. In contrast spring wheat cultivars lack the mechanisms and exhibit faster phenological development than winter wheat. In the present study it seems that for the winter cultivar, due to its longer vegetative phase, antioxidant genes were expressed for a longer time and at a higher level than in spring wheat. These features may bring an efficient scavenging system for winter wheat under LT stress (Ruelland et al. 2009).

It is interesting to note that in spite of the relatively small alternation of H_2O_2 in the less cold-hardy spring cultivar its increase after low temperatures was obvious. It was found that the activities of the scavenging enzymes in the less cold-hardy spring cultivar could be lowered by low temperatures, and the scavenging systems would then not be able to counterbalance the ROS formation that is always associated with mitochondrial and chloroplastic electron transfer reactions (Cheeseman 2007, Ruelland et al. 2009). The results presented here indicate that enzymatic antioxidative systems of the cold-hardy winter wheat worked faster against the acclimation, de-acclimation and re-acclimation periods than did the spring wheat.

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