Relationship Between Circadian Rhythm and Postharvest Quality of Soybean Sprouts During Storage

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（大豆モヤシの貯蔵中における概日リズムと収穫後品質の関係性）

2023

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I. INTRODUCTION

When plant organs detached from their modular structure such as postharvest fruits and vegetables, they are susceptible to fast deterioration. Because the intact structure of plants allows individual organs to remain biologically active and carry out metabolic processes, such as photosynthesis, respiration or several biological processes even after harvest (De Larrinaga et al., 2019; Liu et al., 2015). To consider this fact, fundamental research is needed on harvested vegetables and fruits that continue to sense and respond to diverse stimuli, similarly to intact plants, to promote postharvest quality.

Circadian clocks seem to be a common feature that has evolved genetic routes capable of maintaining approx. 24 h. From prokaryotes to eukaryotes, almost all living organisms are dominated by this amazing timekeeping system. The rhythm is made from the central self-regulatory vibrator with several components using environmental cues, such as light and temperature as an input element. The generated rhythm is applied to the output that regulates various physiological and biological phenomena, such as photosynthesis, growth, leaf and stomatal movements (Webb, 2003; Dodd et al., 2005). The circadian clock also regulates aspects of plant biology that may have human health impact, such as levels of carbohydrates, ascorbic acid, chlorophyll, and glucosinolates in edible plant species (Goodspeed et al., 2013; Hasperué et al., 2011; Kiyota et al., 2006).

The molecular basis of the circadian clock is well established and revealed to consist of various types of genes essential for phase-wise transcription and translation of several interlocking feedback loops (Srivastava et al., 2019). For example, the early morning expressed CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*), LATE ELONGATED HYPOCOTYL (*LHY*) directly represses the expression of evening genes such as TIMING OF CAB EXPRESSION 1 (*TOC1*),

GIGANTEA (*GI*), EARLY FLOWERING 4 (*ELF4*) and LUX ARRHYTHMO (*LUX*) (Alabadí et al., 2001; Kamioka et al., 2016). They also activated PRR family genes PSEUDO-RESPONSE REGULATOR (*PRR9, PRR7, PRR5*), which is expressed sequentially throughout the daytime. In turn, *PRR* family genes repress the rise of *CCA1/LHY,* and finally, the evening complex formed by *ELF3, ELF4, LUX* inhibits the expression of PRRs (Pokhilko et al., 2012). However, it has been reported that *CCA1, LHY*, and *TOC1* reduce the expression of *GI*, but *GI* promotes the expression of *CCA1* and *LHY* (Sanchez and Kay, 2016). Together, all components in the clock system regulate many activities by producing robust and accurate rhythms in plants.

Numerous studies have elucidated the role of plant circadian clock components, either biologically or genetically, giving us insight into their impact on many important physiological processes. For instance, in Arabidopsis, afternoon genes (*PRR9, PRR7, PRR5*) contribute to the regulation of flowering time and photosynthetic sugar signaling (Haydon et al., 2013; Nakamichi et al., 2007), and evening genes (*ELF3, ELF4, LUX*) act as direct regulators for controlling hypocotyl growth in the early evening (Nusinow et al., 2011). It has also become evident that circadian clock manipulation through environmental signals could potentially increase productivity in crop plants. The plant circadian clock is controlled by light/dark or temperature cycles that are prone to produce rhythmic behavior in several aspects of plant physiology. For instance, the rhythm of soybean and broccoli flowering are controlled by changes in day length (photoperiodism), which are ultimately governed by the clock genes *LCL1* and *GI*, respectively (Thiruvengadam et al., 2015; Wang et al., 2020). Clock-regulated genes have direct involvement in several biotic (insect, fungus, bacteria, virus) and abiotic (drought, saline, osmotic, heat or cold stress) stresses that help plants enhance stress tolerance as well as plant fitness levels (Srivastava et al., 2019). The clock controls expression of a large number of abiotic stress-responsive genes.

Conversely, abiotic stress results in altered the function of clock gene expression. It is evident not only in model plants Arabidopsis but also other plants such as soybean (Marcolino-Gomes et al., 2014), and barley (Habte et al., 2014) etc. Several circadian clock key regulators including *CCA1*, *LHY*, *CHE*, *TIC* and *TOC1* have been well documented as to execute the defense system of plants through the day and night cycle (Karapetyan and Dong, 2018). Nevertheless, while a well-known relationship exists between environmental stress and the circadian clock in the vegetative stage of plants, little information is available for harvested fresh fruits and vegetables. The knowledge about the existence of the circadian clock system and its response behavior during varying storage could give a better understanding for elucidating/manipulating clock mechanism in fresh produce to control their postharvest quality and shelf life during postharvest handling.

Here, soybean sprouts were selected, one of the most popular vegetables in Asian countries. Among edible vegetables, soybean sprouts contain highest amount of isoflavones associated with human health benefits. In addition, soybean sprouts are easy to grow and has short shelf life after harvest. Due to high respiration rate, soybean sprouts considered as high perishable vegetables that make it easy to deteriorate. To ensure high quality of sprouts, postharvest handling practices should be applied soon after harvest. The application of postharvest abiotic conditions involving cold, and controlled atmosphere, has potential impact not only on preserving quality but also extending shelf life of perishables produces (Pedreschi and Lurie, 2015). For soybean sprouts, low storage temperature also has potential of minimizing postharvest morphological and nutritional degradation during marketing (Lee and Kim, 2004).

In the case of soybean clock genes, some orthologs to the Arabidopsis circadian clock genes, have been identified in the soybean genome and have been shown to oscillate in a circadian manner in controlled preharvest situations. Previous studies in soybean have shown connections between the plant responses to abiotic stresses (e.g. drought) and circadian clock (Marcolino-Gomes et al., 2014). However, the impact of cold, and controlled atmosphere such as low O_2 and high CO_2 on specific clock components of soybean sprouts central oscillator remains largely unknown, once circadian clock responses to various environmental abiotic stresses. Therefore, in this dissertation, we investigated the effect of temperature, and controlled atmosphere (low O_2 and high CO_2) stresses on the clock gene expression and postharvest quality of soybean sprouts. It is hoped that this information will provide insight into the development of new postharvest preservation technology by controlling circadian rhythm.

II. LITERATURE REVIEW

2.1 Necessity for maintaining postharvest quality of sprouts

Globally, sprouts are of great interest to the consumers owing to their easy growth, beneficial traits, and health-promoting compounds. Sprouts being attractive for several amazing traits such as, unique colors, intense smells, delicate textures, and variable tastes (Galieni et al., 2020). Among the *Fabaceae* family, bean sprouts, such as mung bean and soybean sprouts, are the most consumed. They are also used for oriental and vegetarian diets. They are also used in Asian cuisine and vegetarian diets. Owing to their appealing nutty or bean-like flavor and crispy texture, bean sprouts have become a popular nourishment food in many Asian countries, including Japan, Korea, and China, over the years. Currently, alfalfa, buckwheat, red cabbage, and broccoli sprouts have also gained popularity and are consumed worldwide because of their beneficial health impacts (Aloo et al., 2021).

Sprouts are mainly used for homemade preparations and in fresh food markets. They can be eaten raw as appetizers and salads as well as added to stir fries, sautéed vegetables, pastas, and even desserts. Moreover, the nutritional value and sensory properties of certain food products can be enhanced by the addition of sprout powder. For example, soybean sprouts are used as additives in antiaging and cosmetic whitening products (Lai et al., 2012), and the use of wheat sprouts in tortillas enhance shelf life and sensory attributes (Liu et al., 2017). On the other hand, this type of processing might deteriorate the nutritional value of sprouts. However, a potential opportunity has been proposed for sprouts to be used as supplements in animal feed, which would then be transferred to humans (Dal Bosco et al., 2015).

Despite the increasing popularity of sprouts as a healthy food, their relatively high water content (up to 95%) and delicate texture make them susceptible to damage. Additionally, difficulties in storage management lead to rapid postharvest quality deterioration. For instance, sprouts easily dehydrate, have a high respiration rate, and rapidly lose certain nutrients after harvest (Chen et al., 2017; Lee et al., 2005). Diminished sprout quantity and quality also cause economic losses that subsequently limit market value. To consider this fact, fundamental research on suitable handling practices, and safety measures are required to extend shelf life and preserve quality of sprouts. Thus, in this literature review, a concise overview of postharvest quality aspects, emerging postharvest technologies emphasizing quality maintenance, and techniques for shelf life extension of sprouts are discussed.

2.2 Important quality attributes of sprouts

Postharvest quality attributes of fruits and vegetables are generally the result of chemical components or physical characteristics or a combination of these factors, including flavor (taste and aroma), appearance (color), texture, and nutritional compounds (Mahajan et al., 2017). In the case of sprouts, its unique sensory attributes vary between species. For example, sprouts from *Fabaceae* family seeds, such as lentils, peas, beans, and some legumes, taste bitter because of their high polyphenol content (Troszyñska et al., 2003). In contrast, high amounts of glucosinolates give a spicy taste to *Cruciferous* family sprouts such as radish and broccoli sprouts (Force et al., 2007; Gajewski et al., 2008). Color is another important external quality parameter that affects overall acceptability. A color change from green to yellow during postharvest storage is a major problem of green vegetables. The content of chlorophyll, a green-color-producing pigment, significantly decreases with storage time in broccoli and alfalfa sprouts and results in a yellow color (Fan and Thayer, 2001; Waje et al., 2009). Several studies have been conducted on the nutritional properties of sprouts as a quality parameter. Contents of titratable acid, ascorbic acid, carotenoids, soluble sugar, and protein were usually tested to evaluate the storage quality of mung beans and Brussels sprouts (Chen et al., 2018; Hasperué et al., 2016; Waje et al., 2009). These nutritional characteristics decrease over the storage period.

Edible sprouts are a good source of various phytonutrients and bioactive compounds. For instance, sulforaphane has been found in broccoli sprouts, isoflavone in soybean sprouts, resveratrol in pea sprouts, and glucoraphanin in rocket sprouts. High concentrations of these compounds contribute to antioxidant, antibacterial, and anticancer properties (Moreno et al., 2006). Biogenic amines such as histamine, tyramine, tryptamine, putrescine, cadaverine, and phenylethylamine are usually formed by decarboxylation of amino acids. They have psychoactive and vasoactive properties, which can cause toxicological effects on human health (Shalaby, 1997). The presence of biogenic amines in food is considered as an indicator of bacterial spoilage or the safety of food because high concentrations of amines such as histamine, putrescine, and cadaverine are associated with microbial activity (Martínez-Villaluenga et al., 2008; Shalaby, 1997; Simon-Sarkadi and Holzapfel, 1995). The relationship between intense microbial activity of *Enterobacteriaceae* and high accumulation level of biogenic amines has also been proposed in sprouts (Martínez-Villaluenga et al., 2008). Biogenic amines are also endogenously produced during the germination process of sprouts (Glória et al., 2005). The predominant increase in histamine concentration was found in lupin and fenugreek sprouts at 5 days of the germination process (Martínez-Villaluenga et al., 2006). In lentils and radish sprouts, a high putrescine level was detected during germination (Simon-Sarkadi and Holzapfel, 1995). Biogenic amines of sprouts were also monitored during storage conditions. For example, cadaverine and putrescine were found in broccoli sprouts during refrigerated storage (Vale et al., 2015). The content of total phenols, flavonoids, isoflavone, glucosinolate and biogenic amine and the accumulation of these compounds are also important as quality indices for sprouts. Therefore, it is necessary to evaluate changes in extrinsic and intrinsic properties during the postharvest storage of sprouts to optimize their quality and palatability.

2.3 Postharvest quality changes of sprouts during storage

Sprouts are characterized as highly perishable produce owing to their high respiration rate, high moisture content, high sensitivity to physical injury, and availability of nutrients for pathogenic organisms. Generally, the degree of degradation or perishability of harvested produce is proportional to its respiration rate. Sprouts that are stored at high temperatures exhibit higher respiration rates, which in turn result in decreased weight and nutritional quality and color changes (Hasperué et al., 2016; Jie et al., 2018; Lee et al., 2005). Additionally, sprouts have a fine texture and delicate structure that can easily deteriorate during harvesting and subsequent storage and transportation. Rotting caused by bacteria or fungal attacks may also occur due to improper handling practices, which could result in food loss and pose biohazard risks to human health (Yang et al., 2013).

2.3.1 Browning

Browning of sprouts is a major problem during postharvest handling that adversely affects consumer acceptance. This undesirable event is especially relevant for sprouts as they are rich in polyphenols and are therefore highly susceptible to enzymatic browning. Polyphenol oxidase (PPO) is the key enzyme related to browning. Phenylalanine ammonia-lyase (PAL) is another enzyme that participates in the browning reaction by synthesizing phenolic compounds during storage. Generally, browning appears when subcellular de-compartmentation is occurred by stress or injury which leads to the contact between PPO enzymes and phenolic compounds. Fresh sprouts can be easily turned brown due to microbial growth, cold stress, hot air drying, and physical injury such as cutting (Gan et al., 2017; Kogo et al., 2018; Nishimura et al., 2012; Wang et al., 2013). Figure 1 shows a generalized scheme for enzymatic browning in sprouts since they have a similar browning mechanism to that of fresh-cut produce (Nishimura et al., 2012). According to the figure, postharvest stress causes damage to the cell membrane system and induces PAL activity by signaling molecules (Ma et al., 2022). This induction of PAL activity results in the biosynthesis of phenolic compounds, the substrates of browning reaction. It is reported that phenolic compounds (synthesized by PAL) are stored in vacuoles. When cell compartmentation is gradually disrupted, phenolic compounds consequently leak out from vacuole to cytosol. The eluted phenolics then interact with active PPO that present in cytosol, and ultimately leading to the form of quinone (browning compound) (Kogo et al., 2018). Quinone can undergo further non-enzymatic polymerization to form brown pigments that eventually appeared as tissue browning (Nishimura et al., 2012). In mung bean sprouts, flavonoids were identified as the main substrate for PPO activity, leading to browning (Zhang et al., 2018). Biochemical characteristics of PPOs from stored lentil sprouts were analyzed by Sikora et al. (2019) who found that the optimum pH for PPO was 4.5–5.5 and optimal temperature was 35 °C. They also reported that catechol was the suitable substrate for lentil sprout PPO. Therefore, characterization of PPO in different sprouts during postharvest storage is important to provide information for developing novel methods to effectively control browning.

2.3.2 Softening

oftening, or texture degradation, is a quality disorder that limits the shelf life of sprouts and occurs during the process of postharvest senescence. Several factors, such as weight (moisture) loss, cell wall modification, and heat treatment, result in softer sprouts. It has been reported that

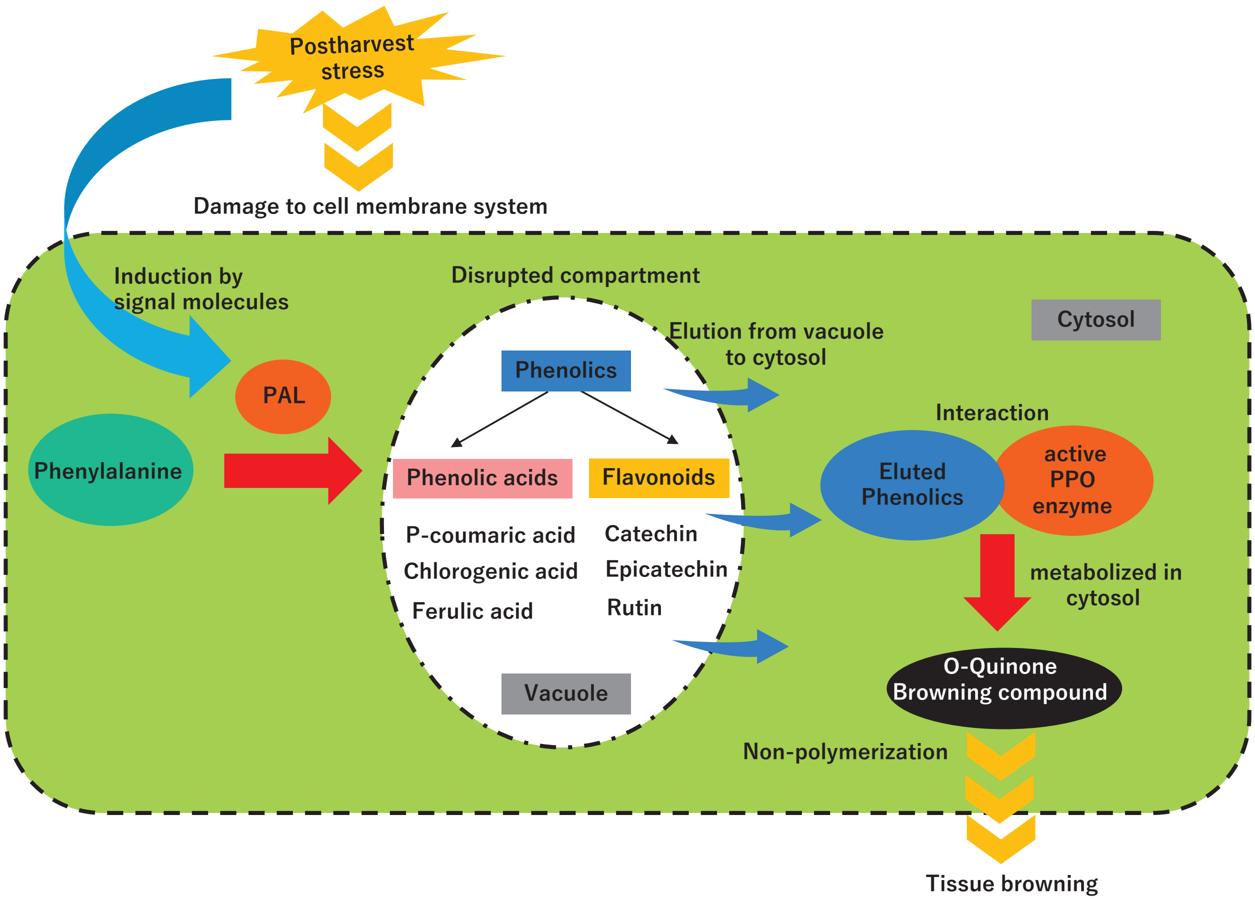


Figure 1: Schematic diagram of enzymatic browning of sprouts during postharvest cold storage modified from (Kogo et al., 2018; Nishimura et al., 2012)

the decrease in Brussels sprout firmness is inversely correlated with the increase in weight loss (Viña et al., 2007a). The reduced firmness of Brussels sprouts was also detected after immersion in 100 °C water for 4 min (Viña et al., 2007b). Loss of cell-cell connection in structural molecules is easily induced by heating, and heat treatment increases membrane destruction and subsequent leakage, leading to reduced firmness (IMAIZUMI et al., 2020). Polygalacturonase (PG) and pectin methylesterase (PME) are the major cell-wall-modifying enzymes in plants (Brummell and Harpster, 2001). However, the underlying mechanisms of cell wall changes that lead to softening of sprouts are complex and remain unknown. In a previous study, softening events of mung bean sprouts during storage were investigated transcriptionally, and the results revealed an increased expression of the pectin-degrading genes *PG* and *PME* (Chen et al., 2018). Additionally, the texture of mung bean sprouts can be maintained by stabilizing the structure and content of pectin (Chen et al., 2018). Another study on mung bean sprouts suggested that cell wall components play a role in changes in firmness. Increasing in the formation of cell wall components, including lignin, cellulose, and hemicellulose, which support elongation of mung bean sprouts during storage, is responsible for these textural changes (Zhang et al., 2018).

2.3.3 Weight loss

Weight loss is the preliminary indicator of quality deterioration of fresh produce after harvest, and higher weight loss of sprouts during storage has been described in previous studies (Chen et al., 2018; Fouzia et al., 2021; Tiziana et al., 2017; Zhang et al., 2018). These phenomena are primarily the result of water loss caused by transpiration and respiration processes. A previous study of mung bean sprouts found that during postharvest storage, weight loss consistently increased from 0% (after harvest) to 27.3% (at 3 days after storage) together with an increase in respiration rate (Chen et al., 2018). A progressive weight loss was also observed in other sprouts such as garlic sprouts, artichoke sprouts, and broccoli sprouts with extending the period of storage. The increased rate of dehydration and respiration was considered as a reason for their weight loss incidences (Amin et al., 2017; Fouzia et al., 2021; Hasperué et al., 2016)

2.2.4 Microbes attack

Sprouts contain a large amount of water and are rich in available nutrients. These factors provide favorable conditions that promote the rapid growth and survival of foodborne pathogens on sprouts (Yang et al., 2013). This quality was assessed by monitoring the microbial load of mesophilic bacteria, total coliforms, yeasts, and molds and by detecting the presence of *Salmonella* (Vale et al., 2015). A survey conducted on microbial contamination of sprouts found mesophilic (7.9 log CFU g^{-1}), psychrotrophic (7.3 log CFU g^{-1}), and *Enterobacteriaceae* (7.2 log CFU g^{-1}) microorganisms (Abadias et al., 2008). After harvest, sprouts can also be contaminated, or the growth of pathogens can increase depending on storage temperature, relative humidity (RH), and the gas composition of the storage environment. Sprouts infected by certain pathogens with an increased log concentration in different conditions throughout the storage period are summarized in Table 1. An increase in pathogens like *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes,* are responsible for the sprout-associated foodborne illness worldwide (Dechet et al., 2014). However, microbiological threats can be minimized through good agricultural practices (GAP) during sprouting as well as through good handling practices during harvest and postharvest processing (Galieni et al., 2020).

Table 1: Name and population of microorganisms during storage of sprouts

Sprouts	Storage conditions	Pathogens	log CFU g^{-1}	Ref.
Alfalfa	Refrigeration storage	Aerobic plate, coliforms	7.17–7.61, 5.52–6.65	(Kim et al., 2013)
	4 °C, 8 days in perforated packaging	Aerobic plate count, mold, and yeasts	5.71, 4.19	(Soylemez et al., 2001)
	4 °C, 8 days in MAP		7.32, 4.37	
	4 °C, 8 days in vacuum packaging		5.68, 5.67	
Broccoli	4-6 °C, 6 days	Aerobic bacteria, yeasts, and molds	7.3, 8.05	(Chen et al., 2019)
	4 °C, 12 days	Mesophilic bacteria, total coliforms, yeasts, and molds	9.95, 9.95, 8.94	(Vale et al., 2015)
	10 °C,14 days	*Enterobacteriaceae*, aerobic mesophilic bacteria, aerobic psychotropic bacteria, mold, and yeast	9.60, 10.04, 10.19, 8.59	(Baenas et al., 2017)
	5 °C,14 days	*Enterobacteriaceae*, aerobic mesophilic bacteria, aerobic psychotropic bacteria, mold, and yeast	9.15, 10.06, 9.58, 8.47	

Chickpea	8 °C, 12 days	Aerobic plate count, coliform count, *Staphylococci*, yeast, and mold	8.9, 8.1, 4.9, 5.4	(Nagar et al., 2012)
Dew gram	8 °C, 8 days		8.9, 8.6, 4.6, 4.3	
Lotus	4 °C, 8 days	*E. coli* O157:H7, *S. typhimurium, L. monocytogenes*	>10	(Wang et al., 2013)
Mung bean	4 °C, 8 days	Total plate count, lactic acid bacteria, yeast and mold, total coliform, *E. coli*	9.07, 7.93, 1.94,7.95, <1.00	(Gabriel, 2005)
Rapeseed	Refrigeration storage	Aerobic plate, coliforms	~8, ~7	(Kim et al., 2013)
Radish	10 °C,14 days	*Enterobacteriaceae*, aerobic mesophilic bacteria, aerobic psychotropic bacteria, mold, and yeast	9.47, 10.10, 10.23, 8.75	(Baenas et al., 2017)
	5 °C,14 days	*Enterobacteriaceae*, aerobic mesophilic bacteria, Aerobic psychotropic bacteria, mold, and yeast	9.63, 9.93, 9.28, 8.60	
Soybean	20 °C, 3 days	Aerobic bacteria	9.78	(Kim et al., 2017)
	4 °C, 5 days	Aerobic bacteria	8.89	

2.2.5 Changes in bioactive compounds

Sprouts are a rich source of various bioactive compounds. However, storage condition has a considerable effect on endogenous bioactive compounds of sprouts. For instance, refrigerated storage for more than 7 days revealed a significant decline in glucosinolate concentrations in Brassica and rocket sprouts (Force et al., 2007; Vale et al., 2015). Level of toxic biogenic amines such as histamine, tyramine, tryptamine, putrescine, and cadaverine can be induced during sprouting time. Factors influencing the biogenic amine production in sprouts include the presence of microorganisms that can decarboxylate amino acids, and the favorable conditions of such microorganisms for the growth and production of their enzymes (Shalaby, 1997; Simon-Sarkadi and Holzapfel, 1995). The presence of individual amines during the germination of some leguminous seeds is well established. However, the profiling and quantitative evaluation of biogenic amines and their changes in stored sprouts are very limited. Vale et al. (2015) only investigated the amount of biogenic amine content in four varieties of *Brassica* sprouts and indicated that increase in amine content was the result of microbial activity during storage. A comparative analysis of biogenic amines in sprouts stored under refrigeration and at room temperature has not been performed yet in any study. Since high content of biogenic amines can cause hazardous effects on human health, it is necessary to ensure the safety of sprouts. Therefore, it would be interesting to determine the influence of different storage conditions on the individual and total biogenic amine content of sprouts and their level of acceptance.

2.4 Application of postharvest technologies on various aspects of sprouts

2.4.1 Postharvest intervention technologies for sprouts decontamination

Sprouts are characterized by limited shelf life and are highly sensitive to harvest and postharvest handling practices. To prevent the occurrence of microbiological contamination of sprouts, the first step is to follow safety measures, including GAPs, and to carefully harvest using hygienic procedures and tools. All the strategies should be implemented, beginning with seed treatment, as the transmission pathway of contamination begins with seeds and spreads to the sprouts (Miyahira and Antunes, 2021). Extensive literature indicating the sources of contamination during preharvest and postharvest has been reviewed by Yang et al. (2013) and Sikin et al. (2013), and the effect of current measures on seed and sprout disinfection, including physical, biological, and chemical applications, were also examined.

Physical intervention methods include temperature control, light exposure, gamma irradiation, and treatment with ultrasound, plasma, or high hydrostatic pressure. Biological interventions are based on antagonistic microorganisms and antimicrobial metabolites. Chemical interventions include a wide range of chemical disinfectants and sanitizers, such as ozone and chlorine, as well as electrolyzed water. However, although various studies have focused only on the elimination of pathogen growth on sprouts, their consequences after harvest on sprout nutritional and sensory properties are limited. This review therefore presents information on postharvest interventions for ensuring microbial quality control in sprouts (Table 2).

The application of chlorine at 50–200 ppm concentration is widely used for postharvest disinfection. Chlorous acid, chlorine dioxide, and sodium chlorite are commonly applied to fresh postharvest produce. These substances provide outstanding disinfection effect and reduction of *Salmonella* spp., *L. monocytogenes*, *S. typhimurium*, and *E. coli*, without changes in visual quality parameters (Inatsu et al., 2017; Jin and Lee, 2007; Lee et al., 2002). However, the primary obstacle to disinfecting sprouts using these substances is the inability of the antimicrobial solution to reach the location of the pathogen. For instance, in a previous study, active chlorine, even at 20,000 ppm concentration, failed to inactivate *Salmonella* in cowpea sprouts, primarily because of a lack of chlorine penetration into sprout tissues where many *Salmonella* were located (Singh et al., 2005). Because chlorine-based sanitizers naturally produce polluting and carcinogenic compounds (Yang et al., 2013), highly concentrated chlorine may have undesirable effects on sprout quality and human health. In this regard, organic acids such as malic acid, lactic acid, and acetic acid have been introduced as alternatives, which have much greater potential for ensuring the quality and safety of sprouts (Singla et al., 2011; Wang et al., 2013).

Typically, the so-called Hurdle technology (combination of preservation methods) can control microbial growth in sprouts, resulting in an extended shelf life. For example, a combined treatment of malic acid and ozone was more effective in inactivating *Shigella flexneri* with no adverse effects on the quality of radish and mung bean sprouts than a single disinfectant treatment (Singla et al., 2011). However, treatment timing is a critical factor related to the elimination or elevation of microorganisms while using ozone. For instance, a 2 min treatment using water-containing ozone showed promising results when applied to alfalfa sprouts for controlling *L. monocytogenes*. However, a 5–20 min treatment resulted in the deterioration of the sensory quality of sprouts during subsequent storage at 4 °C for 7–11 days (Wade et al., 2003). In the future, more factors, including effects on sprout quality, treatment time, and concentration, should therefore be considered to optimize sanitizer delivery to sprouts.

Emerging physical techniques, including electron beam or gamma ray irradiation, as well as combinations with ultrasound, blanching, and ascorbate dip, have proven to be potentially useful for ensuring the hygiene and safety of mung beans, chickpeas, and lucerne sprouts, along with extending their shelf lives (Kumar and Gautam, 2019). Ultraviolet (UV) treatment is another effective physical treatment that is now exploited in the food industry as it does not leave any residues. However, owing to its low penetration power, it is commonly used only for surface decontamination of food material (Mir et al., 2021). In a previous study, when UV treatment was used with a sanitizing mixture of aqueous chlorine dioxide and fumaric acid, its decontamination efficiency for inactivating pathogens on sprouts was significantly improved (Chun and Song, 2013).

Table 2: Postharvest interventions for microbial safety and corresponding quality in sprouts

Postharvest interventions	Sprouts	Experimental condition	Storage condition	Name of pathogen and reduction level (log CFU g^{-1})	Effect on postharvest quality	Ref.
Chemical	Mung Bean	Chlorous acid (268 ppm) for 10 min at 22 °C	4 °C for 9 days	*S. typhimurium* (not detected), *L. monocytogenes* (5)	No changes in visual quality (observation)	(Lee et al., 2002)
	Mung bean	Organic acids (1% concentration, pH 1.52)	-	Total aerobic microorganism (3.4), and *Enterobacteriaceae* (6.3)	Caused degradation, color change, and unpleasant odor (observation)	(Baker et al., 2019)
	Lotus	Lactic acid (2%)	4 °C	*E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* (2.3)	Improved the color and safety	(Wang et al., 2013)
Physical	Broccoli	Far-red light (730 nm)	5 °C for 15 days	Psychrophilic bacteria (8.1), enterobacteria (5.8), mesophilic bacteria (8.0), and molds and yeasts (5.3)	Better quality	(Castillejo et al., 2021a)
	Chickpea, and dew gram	Radiation (1 and 2 kGy)	8 °C for 16 days	Coliform count (2 and 4), yeasts and molds (1 and 1.5), and *Staphylococci* (1 and 1.5)	No negative effects on sensory and nutritional qualities	(Nagar et al., 2012)

	Mung bean, dew gram, chickpea, and garden pea	Radiation (2-kGy)	4 and 8 °C up to 12 days	Complete elimination of (10^4) *S. typhimurium*, and (10^3) *L. monocytogenes*.	No negative effects on texture, nutritional, and organoleptic qualities (unpublished data)	(Saroj et al., 2006)
	Mung bean	Plasma-activated water for 30 min	4 °C, 6 days	Total aerobic bacteria (2.32), total yeasts and molds (2.84)	No significant changes in the total phenolic and flavonoid contents, and the sensory characteristics	(Xiang et al., 2019)
Hurdle	Radish, mung bean	Malic acid (2%) + Ozone (2 ppm)	28 °C for 10 days	*Shigella* spp (4.4 and 4.8)	No significant changes in physicochemical properties	(Singla et al., 2011)
	Broccoli	Lactic acid (2%, v/v) + sodium hypochlorite (4 mg/L)	4–6 °C for 6 days	*Listeria innocua* (1.19)	No negative effects on the storage quality	(Chen et al., 2019)
	Mung Bean	Chlorine Dioxide (100 ppm) for 5 min at room temperature + MAP (CO_2 gas packaging)	5±2 °C for 7 days	*S. typhimurium* (>2), and *L. monocytogenes* (2.9)	Maintained quality and extend the shelf life	(Jin and Lee, 2007)
	Alfalfa	Chlorine (200 ppm) + Perforated atmosphere packaging	4 °C for 8 days	Total coliforms (5.7)	No changes in visual quality	(Soylemez et al., 2001)
	Bean	Sodium hypochlorite (100 mg/L) + phytic acid as secondary wash	10 °C for 4 days	*E. coli* O157:H7 (4) and *L. monocytogenes* (4)	Retained the color of bean sprouts	(Inatsu et al., 2017)

	Soybean	Slightly acidic electrolyzed water + Fumaric acid + Ultrasonication at 40 °C	4 °C for 7 days	*L.monocytogenes* (4), and *E.coli* O157:H7 (4)	Maintained good quality until the end of storage with slight deterioration due to ultrasound usage	(Ngnitcho et al., 2018)
	Mung bean and chickpea	Ultrasonication (4–10 min; 40–50 °C) + blanching (50–70 °C for 4–10 min using potable water) + ascorbate dip (0.25%, 5% and 1% up to 10 min at 4 ± 1 °C) + gamma irradiation (1–2.5 kGy)	4±1 °C for 35 days	Total aerobic plate count, yeast and mold count, and *Staphylococcus* (not detected (< 10))	Retained nutritional, physico-chemical and sensory attributes	(Kumar and Gautam, 2019)
	Lucerne		4±1 °C for 21 days			

2.4.2 Postharvest intervention technologies for maintaining quality and extending shelf life of sprouts

Short shelf life and susceptibility to spoilage are the key factors affecting sprout quality. Spoilage occurs sooner and more frequently in sprouts when proper storage is not provided by distributors or retailers. Table 3 provides a summary of implications of existing technologies on the quality and shelf life of sprouts.

2.4.2.1 Cold storage

Generally, low temperature storage can minimize postharvest quality degradation and extend shelf life by slowing the rate of respiration, senescence, and spoilage growth (Xiao et al., 2014b). However, the selection of optimum storage temperatures broadly depends on respiration rates and organoleptic qualities. For example, in radish sprouts (without radicle), visual quality was better maintained when stored at 4 °C because of the lower constant respiration rate (Berba and Uchanski, 2012). In contrast, the membrane structure of mung bean sprouts was heavily disrupted during 4 °C storage, which allowed eluted phenolics and oxidative enzymes to move from vacuoles to cytosol, eventually resulting in browning (Kogo et al., 2018). Thus, it has been a long-term challenge to select appropriate temperatures to extend the shelf life of sprouts while simultaneously overcoming these limitations.

The nutritional quality of bioactive phytochemical compounds was also better maintained when sprouts were stored at cold temperatures. For instance, the isoflavone content of 4-day-old soybean sprouts after cold storage (4 °C) lasting a week was generally equal or higher than that of fresh sprouts (Świeca et al., 2020). Additionally, storage temperature of 4–5 °C has been recommended to avoid extreme losses of bioactive compounds, and to maintain shelf life up to 14 days in broccoli and radish sprouts (Baenas et al., 2017). Genotypic variation and time of harvesting may also influence the accumulation of bioactive composition in sprouts. Total glucosinolate level (GLs) in 9-day-old *Brassica oleracea* sprouts (cultivars include red cabbage, broccoli, Galega kale, Penca cabbage, etc.) showed a significant reduction over 12 days when stored at 4 °C (Vale et al., 2015). However, Force et al. (2007) did not find any significant loss of GLs in 7-day-old sprouts of broccoli, kohl rabi, white radish, and rocket under the same conditions (4 °C for 3 weeks).

2.4.2.2 Modified atmosphere packaging

Modified atmosphere packaging (MAP) is one of the most effective technologies which can provide good quality and longer shelf life of vegetable crops by decreasing oxygen (O_2) and increasing carbon dioxide (CO_2) partial pressures in package headspace (Kim et al., 2004). However, improper packaging may be ineffective and reduces the produce quality in terms of shelf life and visual quality. The design of a successful MAP system depends on the product weight and respiration rate, atmospheric composition, appropriate packaging film and its permeability, and storage temperature (Fonseca et al., 2002). Packaging materials such as polypropylene, low-density polyethylene, and polyethylene have proven successful for extending the shelf life of sprouts. However, sprouted vegetables benefit more when they are stored in packaging film combined with cold temperature near their genotypic chilling tolerance. Kou et al. (2013) observed that buckwheat sprouts (without radicle) stored in MAP at 5 and 10 °C had smaller microbial populations and less tissue electrolyte leakage than those stored at 15 and 20 °C. The balance between O_2 and CO_2 in packaging film is also critical for MAP storage, and an optimal ratio is required for each specific sprout. For instance, low O_2 levels in package headspace may result in anaerobic conditions which may encourage the formation of ethanol and acetaldehyde which is responsible for off-odor development, and high CO_2 levels cause irreversible membrane damage

(Turner et al., 2020). In these cases, high oxygen transmission rate (OTR) film has shown an amazing capacity to retard off-odor development by achieving desired CO_2 and O_2 equilibrium conditions inside packages of radish sprouts (without radicle) (Xiao et al., 2014b). MAP combined with both active and passive packaging is useful to extend shelf life and preserve quality of postharvest fresh produces. A recent study found active packaging at 0 °C, with gas concentrations of 15% CO_2 and 7.5% O_2, was an optimum solution for extending shelf life based on the lowest count of aerobic mesophilic bacteria, prevention of off-flavor development, and discoloration of sprouts (Amin et al., 2017). However, there still remain insufficient published studies on the use of optimum active packaging technologies and their impacts on biochemical composition, another important quality trait of sprouts.

2.4.2.3 Light exposure and Irradiation

Previous research of strategies using postharvest exposure to visible spectrum lighting in young plants during their storage period is quite limited. Hasperué et al. (2016) evaluated the effect of white-blue light-emitting diode (LED) on Brussels sprouts at room temperature, and they found that light-treated sprouts had decreased respiration rates after 0 to 5 days of storage, and maintained the lower rate, as well as the green color in tissues until 10 days. Light treatment preserved higher levels of glucosinolates and phenolic content in broccoli sprouts, whereas yellow LED light showed better performance than green and white LED light. This phenomenon was also demonstrated in cases of UV exposure, where moderate UV exposure showed potential benefits to the biosynthesis of health-promoting bioactive compounds. For instance, when harvested broccoli and radish sprouts were exposed to 15 Wm^{-2} UV-B and 9 Wm^{-2} UV-C individually, the UV-B exposed produce showed the highest total phenolic content and total antioxidant capacity after 10 days of storage. UV-B also increased glucosinolate content >30% (Martínez-Zamora et al., 2021).

Conversely, continuous light exposure during postharvest storage had a negative effect on the sensory quality and amount of bioactive compounds found in radish sprouts (without radicle) by increasing the O_2 level in the package headspace (Xiao et al., 2014a). Thus, postharvest light conditions (spectral composition and intensities) need to be carefully considered in order to maintain desired concentrations of bioactive compounds and consumer acceptability.

Light plays an important role in elucidating the molecular mechanism of secondary metabolites biosynthesis in sprouts. Lim et al. (2020) specifically found the upregulation of chalcone synthase-encoding genes (*CHS6, CHS7*, and *CHS8*) in response to UV-B irradiation during the sprouting time of soybean sprouts. Involvement of light in defense mechanisms of soybean sprouts was previously studied by Dhakal et al. (2015). They suggested that light treatment increases the resistance against rotting disease caused by *Pseudomonas putida* 229. This developed resistance was observed by the accumulation level of *isochorismate synthase* and *pathogenesis-related 1* gene expression that regulated the pathway of salicylic acid and jasmonic acid biosynthesis. However, further research is needed to understand the biosynthetic pathway and stress tolerance using genetic approaches during postharvest storage of sprouts.

Irradiation is another very effective method for reducing foodborne pathogen in sprouts. However, very few studies have found irradiation effective in maintaining the sensory and nutritional qualities of sprouts. In one case, gamma irradiation, even at low doses, appeared to be a useful technique for preserving the visual quality of garlic sprouts and for delaying the oxidation of total phenols, total ascorbic acid, and chlorophyll degradation during storage (Fouzia et al., 2021). In contrast, UV-irradiation, another radiation source, did not have any impact on sprout quality and shelf life (Goyal and Siddiqui, 2014).

2.4.2.4 High Pressure Processing

High pressure processing (HPP), as a novel and non-thermal physical processing technology, has shown significant potential to preserve bioactive substances in sprouts. In general, the conversion of predominant glucosinolates to isothiocyanates and sulforaphane is essential to enhance their bioactivity. HPP treatment at 600 MPa exhibited the highest conversion (85%) of this beneficial compound in broccoli sprouts stored at 6 °C for 12 days after undergoing pressurization (Westphal et al., 2017). HPP technology is frequently utilized to reduce microbial growth on sprouts. In practice, pressures up to 700 MPa, and treatment times varying from a few seconds to several minutes are used to inactivate microbial cells (Woldemariam and Emire, 2019). However, there are no prior reports on the effects of HPP on sensory quality and shelf life of sprouts in combination with inactivation of pathogenic and spoilage microorganisms. Additionally, HPP at 300 to 700 MPa has some serious limitations, such as the difficulty of controlling and managing such high operating pressures, high equipment cost, and safety (Sikin et al., 2013).

2.4.2.5 Chemical Treatment

Some preharvest applications, such as chitosan and calcium treatments, have shown promising results and increased defense functions against oxidative stress during the storage of sprouts. In broccoli sprouts, a 10 mM CaSO4 spray application dramatically enhanced antioxidant enzyme activities including superoxide dismutase, peroxidase, catalase, and glutathione peroxidase, and increased antioxidant capacity including phenolic compounds and ascorbic acid. Moreover, this treatment also led to an increase in glucosinolate content, especially glucoraphanin, during growth, and prevented its loss during storage (Guo et al., 2018). Chitosan is characterized as a biodegradable and biocompatible polysaccharide extracted from natural resources, and is an alternative coating material for a wide range of food products. Supapvanich et al. (2018) observed improved antioxidant and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, and increased total phenols, flavonoids, and ascorbic acid contents, in chitosan-treated sunflower sprouts. The effect of chitosan treatment on transcription level of genes such as PAL, CHS, and isoflavone synthase which involved in phenylpropanoid pathway has been uncovered in soybean sprouts. Chen et al. (2009) concluded that chitosan treatment was ineffective for increasing isoflavone content in soybean sprouts because isoflavone biosynthetic gene expression level negatively responded to chitosan treatment in the high-isoflavone cultivar. However, a postharvest dip treatment using non-toxic adenosine triphosphate (ATP) and ascorbic acid was established as a potential inhibitor of PPO enzyme activity. Moreover, this did not adversely affect the taste and odor of stored mung bean sprouts(Chen et al., 2018; Sikora and Świeca, 2018). Nevertheless, postharvest dip/wash and drying processes profoundly reduced shelf life of these delicate vegetables due to mechanical damage (Kou et al., 2015).

2.5 Circadian rhythm and postharvest quality of sprouts

Sprouts like vegetables, once harvested and stored on refrigerator or supermarket shelves, continue to perform biological activities due to their modular nature and their ability to retain physiological autonomy. They can live after being harvested. Therefore, it is necessary to examine whether circadian rhythm continue to operate in postharvest fresh produces during postharvest storage such as temperature, packaging, and controlled atmosphere and if so, whether postharvest quality could be controlled by the circadian system.

Table 3: Overview of developed postharvest technologies on quality and shelf life of sprouts

Postharvest technologies	Sprouts	Treatment conditions	Shelf life	Outcome	Ref.
Cold storage	Radish (without radicles)	4 °C	3 weeks	Maintained constant respiration rate and visual quality	(Berba and Uchanski, 2012)
		10 °C	14 days		
	Soybean	4 °C	4 days	Minimized postharvest morphological degradation	(YH, 2004)
	Mung bean	1 °C	4 days	Hypocotyls remained whiter	(DeEll et al., 2000)
		6 °C		Cotyledon color was better	
	Soybean	4 °C	7 days	Increased isoflavone content and reducing power	(Świeca et al., 2020)
	Green pea, lentil and mung bean	4 °C	7 days	Elevated starch digestibility and antioxidant activities	(Świeca and Gawlik-Dziki, 2015)
	Broccoli and radish	5 °C	14 days	Maintained nutritional quality and acceptability for consumers	(Baenas et al., 2017)
	Broccoli, kohl rabi, white radish and rocket	4 °C	3 weeks	Maintained the glucosinolate level	(Force et al., 2007)
MAP	Buckwheat (without radicle)	16.6 pmol m^{-2} s^{-1} Pa^{-1} OTR film at 5 °C	21 days	Freshest appearance with lowest tissue electrolyte leakage	(Kou et al., 2013)

	Artichoke	15% CO_2 + 7.5% O_2 at 0 °C±2 °C	18 days	Avoided the development of off flavor, prevented discoloration in sprouts and gave good appearance	(Amin et al., 2017)
	Quinoa	5% O_2 + 20% CO_2 at 5 °C	7 days	Helped to retain texture profile and odor	(Tiziana et al., 2017)
	Brussels	Polyvinylchloride film at 0 °C	42 days	Minimized browning and losses in weight and firmness, maintained ascorbic acid and total flavonoid content while increasing the radical-scavenging activity	(Viña et al., 2007a)
	Chickpea	Perforated polypropylene and low-density polyethylene at 10 °C	7 days	Maintained color quality and removed off-odor	(Singh et al., 2014)
	Mung bean	Moderate vacuum packing (↓0.04 mPa) at 4 °C	7 days	Restricted sprout elongation and maintained the firmness, slowing down the browning	(Zhang et al., 2018)
	Radish (without radicle)	29.5 pmol m^{-2} s^{-1} Pa^{-1} oxygen transmission rate (OTR) packaging at 1 °C	28 days	Lowest tissue electrolyte leakage, aerobic mesophilic bacteria, yeast and mold count, and off-odor score	(Xiao et al., 2014b)
Light exposure and irradiation	Brussels	White-blue light-emitting diodes at 22 °C	10 days	Lower respiration rate, remained greener, better visual quality	(Hasperué et al., 2016)
	Broccoli	Far-red LED (730 nm) at 5 °C	15 days	Increased the biosynthesis of phenolic compounds	(Castillejo et al., 2021a)
	Broccoli	Continuous 35 μmol m^{-2} s^{-1} yellow illumination at 5 °C	15 days	Enhanced and maintained the biosynthesis of glucosinolates and phenolic compounds	(Castillejo et al., 2021b)

	Broccoli	50% of 15 kJ m^{-2} UV-B applied after harvest and on the first day of storage at 4 °C	10 days	Highest total phenolic content and total antioxidant capacity, enhanced the glucosinolate content	(Martínez-Zamora et al., 2021)
	Garlic	Gamma irradiation (1.25 kGy) at 3±1 °C, RH 85%	15 days	Maintained the external appearance, texture, and appeal, enhanced the content of total phenols, and also delayed the oxidation of total phenols, total ascorbic acid, and chlorophyll degradation	(Fouzia et al., 2021)
	Alfalfa	Gamma irradiation (1.71 and 2.57 kGy) at 5 °C, and then stored at 6 °C	7 days	Increased carotenoid content compared to non-irradiated sprouts	(Fan and Thayer, 2001)
High pressure processing	Broccoli	400−600 MPa for 3 min at 30 °C and stored at 6 °C	12 days	Increased isothiocyanate formation	(Westphal et al., 2017)
	Brussels	200 MPa for 3 min at 5 °C and stored at 4±2 °C	4 days	Increased the essential amino acids (isoleucine, leucine, lysine, phenylalanine, and threonine) in lower amount	(Barba et al., 2017)
Chemical treatment	Mung bean	1mM ATP solution for 5 min, and stored at 20 °C	3 days	Maintained the quality, delayed browning and softening	(Chen et al., 2018)
	Mung bean	20 mM ascorbic acid for 2 h, and stored at 4 °C	7 days	Reduced enzymatic browning of sprouts via PPO inhibition	(Sikora and Świeca, 2018)
	Mung bean	Ethanol vapor for 1 h and stored at 7±1 °C	120 h	Suppressed the sprout length and weight, reduced the non-enzymatic browning, and maintained overall acceptability	(Goyal and Siddiqui, 2014)

	Broccoli	10 mL of 10 mM $CaSO_4$ every 12 h of cultivation period and after harvest stored at 4 °C	15 days	Enhanced antioxidant activities	(Guo et al., 2018)
	Sunflower	Before harvest, watered with 500 mL of 1% chitosan solution and after harvest stored at 4±1 °C	9 days	Induced ferric reducing antioxidant potential, DPPH free radical scavenging activity, and all bioactive compounds	(Supapvanich et al., 2018)

III. MATERIALS AND METHODS

3.1 Plant materials (Soybean sprouts cultivation)

Soybean seeds (Glycine max, cultivar “BS5012”) were used for cultivating sprouts according to the manufacturer's instructions (Saladacosmo Co., Ltd., Japan). The cultivation practices were carried out in darkness. Briefly, approx. 130 g dry soybean seeds used for each cultivation were cleaned and rinsed with clean water. Then, the seeds were soaked in tap water for 8 h (from 10:00 to 18:00) at 25 °C to induce germination. After soaking, all soaked seeds were transferred to a cylindrical plastic container used as a cultivation chamber. The seeds were cultivated with tap water by top showering at least 7 times per day to maintain relative humidity at 80–90% of the cultivation environment. Relative humidity was monitored by a Thermo recorder (TR-72 wb-S, T & D corporation, Japan). The cultivation was conducted in an incubator set at 25 °C for 4 days (approx. 96 h), where time counting was started from the seed soaking procedure, to produce a hypocotyl length of 8±2 cm. Four-day-old sprouts were then harvested and used for gene expression and quality assessment. The cultivated sprouts used for experiments were selected according to uniform hypocotyl length, lack of bruises, discoloration, breakage, etc.

3.2 Sample preparation and storage conditions

The harvested samples set in a basket with small holes were gently soaked into 200 ppm sodium hypochlorite solution (v/v) made from 5% sodium hypochlorite for 1 min, where the pH range was maintained at 5.5–6.5 for disinfection. For rinsing, the basket with sprouts was transferred to a container that was continuously filled with tap water. During this step, the samples were left in water for 1 min and then rinsed twice. Then, the sprouts were gently covered using clean kitchen towel paper and kept for 10 min to dry the surface of the sprouts. After finishing the minimum surface drying using a towel paper, a total of approx. 1700 harvested sprouts were divided into four groups (425 sprouts each) and placed in chambers (10 mm thick acrylic cylinders, 4.8 L) that were used for the following storage treatments: control (20 °C-air), low O_2 (20 °C-5% O_2), high CO_2 (20 °C-15% CO_2 + 20% O_2), and low temperature (10 °C-air) with 80 ± 5% relative humidity. All the sprouts were stored under constant dark conditions because light can have adverse effects on soybean sprout quality. The experiments were conducted for 24 h in all treatments. The gas compositions of all treatments were controlled using a flow-through system through a mass flow controller (CU-2130, HORIBA STEC, Kyoto, Japan), and the percentage of O_2 and CO_2 was monitored by O_2 and CO_2 sensors (TR-V550, KEYENCE CORPORATION, Japan). The gas flow rate through the chamber was 100 mL min^{-1}.

3.3 Evaluation of quality change

3.3.1 Respiration rate

During the 24 h storage period of soybean sprouts, the rates of respiratory CO_2 production were measured by a flow-through method using gas chromatography (GC) as described in (Fahmy and Nakano, 2014) with some modifications. Briefly, approx. 150 g of soybean sprouts were placed into the acrylic chamber (4.8 L) equipped with gas inlet and outlet tubes at a flow rate of 100 mL min^{-1} (mass flow controller CU-2130, HORIBA STEC, Kyoto, Japan). The closed chambers were placed in incubators (PHC Corporation, MIR-154-PJ, Tokyo, Japan) and were connected to an air compressor (KAPSEL-CON YC-8-F, Yaezaki Pneumatic Co., Ltd., Japan) to produce airflow through the inlet tubes. The gas mixture of O_2 with N_2 that went through a gas blender (MU-3411, HORIBA STEC, Kyoto, Japan) connected with automatic gas composition-controlled software (GasMixer software, HORIBA STEC, Kyoto, Japan) was used to produce 5% O_2 flow into the chambers. Similarly, a mixture of CO_2 and O_2 with N_2 was used to produce 15% CO_2 + 20% O_2. The chromatograms were analyzed using an integrator based on the CO_2 standard curve (GC solution software, Shimadzu, Kyoto, Japan) to determine CO_2 emission and O_2 consumption. The CO_2 production rate was calculated from the differences in the gas concentration between the inlet and outlet using Equation (1) (Fonseca et al., 2002).

$$r_{CO_2} = (Y_{co_2}^{out} - Y_{co_2}^{in}) / 100 \times F / W \times P / \mathrm{R}T \times 10^3 \tag{1}$$

Where r_{CO_2} is the respiration rate for CO_2 production (mmol kg^{-1} h^{-1}), $y_{co_2}^{out}$ and $y_{CO_2}^{in}$ are the volumetric concentration (%) of CO_2 in the outlet and inlet gas samples, respectively. W is the weight of the sample (kg), F is the flow rate (L h^{-1}), P is the atmospheric pressure (= 101.3 kPa), R is the universal gas constant (= 8.314 L kPa K^{-1} mol^{-1}), and T is the absolute temperature (K).

3.3.2 Weight loss

Weight loss was assessed by measuring the weights of soybean sprouts before and after 24 h of storage under various conditions. Then, the weight loss was calculated as a percentage of the initial weight using Equation (2):

$$Weight\ Loss\ \% = ((Wi - Ws)/Wi) \times 100 \tag{2}$$

Where Wi = initial weight and Ws = weight at sampling period.

3.3.3 Browning Index

Assessment of browning was conducted using the color change properties of soybean sprouts. For each measurement, twenty soybean sprout cotyledons were arranged on a plastic petri dish with as little gap as possible, and their lightness (L*), redness (a*), and yellowness (b*) values were obtained using an RM200QC spectrocolorimeter (X-Rite, Grand Rapids, Michigan, USA). The above measurement was performed three times randomly on the surface of cotyledons for each replication, and five replications were conducted for each treatment. The browning index was calculated according to Equations (3), (4) (Kasim and Kasim, 2015):

$$x = (a* + 1.75L*)/(5.645L* + a* - 0.3012b*) \tag{3}$$

$$BI = [100(x - 0.31)]/0.17 \tag{4}$$

The browning index of soybean sprouts was also evaluated by 12 panelists. Samples were visually evaluated using the following scale: 1 = no browning, 2 = slight browning (1–2 spots of browning), 3 = moderate browning (<25% browning), 4 = moderately severe (25–50% browning) and 5 = severe (>50% browning) (Amin et al., 2017; Sommano et al., 2011). Browning was assessed from twenty sprouts per treatment and expressed as a score by Equation (5):

$$\text{Browning index} = \Sigma\ (\text{scale of browning} \times \text{percent of corresponding sprouts in each class}) \tag{5}$$

3.4 Sampling method and gene expression analysis by quantitative real-time PCR (qPCR)

The soybean sprout samples used for gene expression analysis were collected at 4 h intervals from the beginning of storage treatments starting at 0 h (10:00 am of harvesting day) to the end of the storage 24 h (10:00 am of the next day). The cotyledon and hypocotyl parts (total length of 3 cm) of five replicates (1 replication = 5 sprouts) were frozen in liquid N_2 and then kept at −80 °C until analysis.

The frozen samples were individually ground to a fine powder in liquid nitrogen with a prechilled mortar and pestle for gene expression analysis. Total RNA was isolated using an RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA, with a 1.8–2.0 ratio of absorbance at 260 nm and 280 nm, was used to synthesize cDNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). The cDNA was then used for qPCR analysis. The reference gene (membrane-binding domain (Fab1/YOTB/Vac1/EEA1)-encoding genes, *FYVE*), and circadian clock genes (*CCA1, LHY, PRR7, GI, TOC1*, and *LUX*) were used in this study. Clock output genes related to postharvest physiology including fumarate hydratase 1

(*FUM1*), citrate synthase (*CS*), and 2-oxoglutarate dehydrogenase (*2-OGDH*), polyphenol oxidase 1 (*PPO1*), phenylalanine ammonia-lyase (*PAL*), and dehydration-responsive element-binding 5 (*DREB5*) were also used. Set of the primers for all genes are indicated in Table 4. For clock genes, it is difficult to differentiate soybean *LHY* from *CCA1*, as all four LHY-CCA1-LIKE orthologs of soybean resemble the Arabidopsis *CCA1* and *LHY* genes. Thus, this study specifically mentioned *LHY*/*CCA1* as a LHY-CCA1-Like 1 (*LCL1*) gene according to Marcolino-Gomes et al. (2014). The qPCR analyses were conducted using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Lithuania) and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. The target gene expression levels were normalized to the expression of the internal standard *GmFYVE* gene based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.5 Analysis of circadian rhythm behavior during the storage period

To quantitatively analyze the circadian rhythm in stored soybean sprouts, clock gene expression data were used to fit the following general cosine curve Equation (6).

$$Y = b \times cos(2\pi(t - c)/24) + d \tag{6}$$

Where *Y* represents the expression variable for each gene at time *t* (h); estimated oscillation parameters *b, c,* and *d* value refer to the relative amplitude, phase, and midline of the cosine curve, respectively. Phase is the time at which the regression curve is at its maximum. Amplitude is the curved height of the midline at its phase. Midline is the average level of the curve. The estimated parameters were obtained through nonlinear regression. To reduce the confounding effect of period differences in clock gene expression, the period was assumed to be 24 h. The default outputs (b, c, and d value) from the nonlinear regression were set to Equation (6) to obtain the regression cosine curve. Five replications were used to define the parameters of the cosine curve. To evaluate the goodness of fit of the cosine curve generated from the cosine curve equation, among the five replications, the coefficient of determination (R^2) was calculated. The significance of the rhythm (R^2 value) was tested using a P value obtained from the slope test.

3.6 Statistical analysis

All clock gene expression data (means of five replicates) used for nonlinear cosine curve fitting were analyzed using R studio software (version - 3.6.2, R Foundation for Statistical Computing). When applying the nonlinear function ‘nls’ in R, the default output provides a table of the t-statistics, which includes estimated values of the cosine curve parameters b, c, and d. To examine the correlation between predicted values and measured values, a slope test was performed using R studio software. The default output provides the coefficient of determination (R^2) and the P value of R^2, which indicates the goodness of fit and is considered an existing rhythmicity in clock gene expression.

Analysis of variance (ANOVA) was performed to test the significant difference between the means of respiration, weight loss, browning index and quality attribute-related genes under all storage conditions. Comparison of means between the control (20 °C air) and each treatment was individually analyzed by Dunnett's test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) using R studio software.

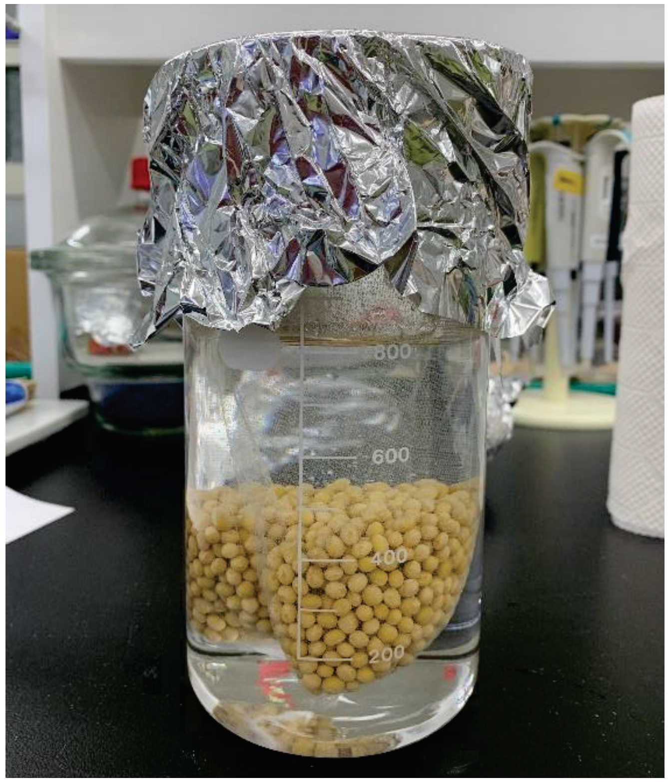


Figure 2: Soaked soybean seeds

Figure 3: Cultivated sprouts

Figure 4: Harvested sprouts (hypocotyl length 8±2 cm)

Table 4 Primer sequences used in the quantitative real-time PCR (qPCR) analysis

Gene name	Soybean ortholog gene or accession no.	Soybean name	Forward primer (5'-3')	Reverse primer (5'-3')	Ref.
FYVE	Glyma13g17500	*GmFYVE*	TTCTGTCTTCTGCAAGTGGTG	GATCCCTCATCCATACATTTCAG	(Marcolino-Gomes et al., 2015)
CCA1	Glyma07g05410	*GmLCL1*	ACCATAGGGCTTGGACAAGGAAAG	ACCTTGATTGTTGCTCGCTCCAAC	(Marcolino-Gomes et al., 2014)
LHY	Glyma07g05410	*GmLCL1*	ACCATAGGGCTTGGACAAGGAAAG	ACCTTGATTGTTGCTCGCTCCAAC	
PRR7	Glyma10g05520	*GmPRR7*	GGCAACAATTCTGGCACCACCTAA	GCAGCTGATGCTTCATGTTGTCAG	
GI	Glyma20g30980	*GmGI*	GTGGCAGATGGCCTTTCAAACCTT	CGGACATGTGCACTTGGATGAGAA	
TOC1	Glyma04g33110	*GmTOC1*	TGACATAAGGATGAAGGGCCAACC	TGAGGGCGCATATTGGATCAACAC	
LUX	Glyma12g06410	*GmLUX*	GAACCTAAGGTCAGCAGCAATCAC	TCAATTCGATCTCCTGCCAAATGC	
FUM1	XM_003518904.3	*GmFUM1*	TGCGCTGCTAAGGTGAACAT	TTGTGCCGCTTGCATAATCG	Primer-BLAST (NCBI)
CS	XM_003546810	*GmCS*	AAGTCAGTTAGCAGAATGGGAG	ACAGAAAGAGCACTCATGGC	(Vengavasi et al., 2016)
2-OGDH	XM_006602965.3	*Gm2-OGDH*	TGGTTGTCGACTTGGTGTGT	TGGATGGCTTCGGATTACCTTG	Primer-BLAST (NCBI)
PPO1	Glyma15g07710	*GmPPO1*	TCTATCCTTCGTGCCCACAGTC	AAATGGATGCAACGGAGAAGGG	(Jia et al., 2015)
PAL		*GmPAL*	GGACTTGCTGAAGGTTGTTGATAG	TGGAAGATTGATGTGCTTGTGTTC	(Jiao et al., 2016)
DREB5	Glyma12g33020	*GmDREB5*	TTGCCTACTACTACTCCTATATTCATTTCC	CCTTGAAATACACGGAGCCTTAG	(Marcolino-Gomes et al., 2015)

IV. RESULTS AND DISCUSSION

4.1 Changes in quality attributes during storage of soybean sprouts

4.1.1 Respiration rate

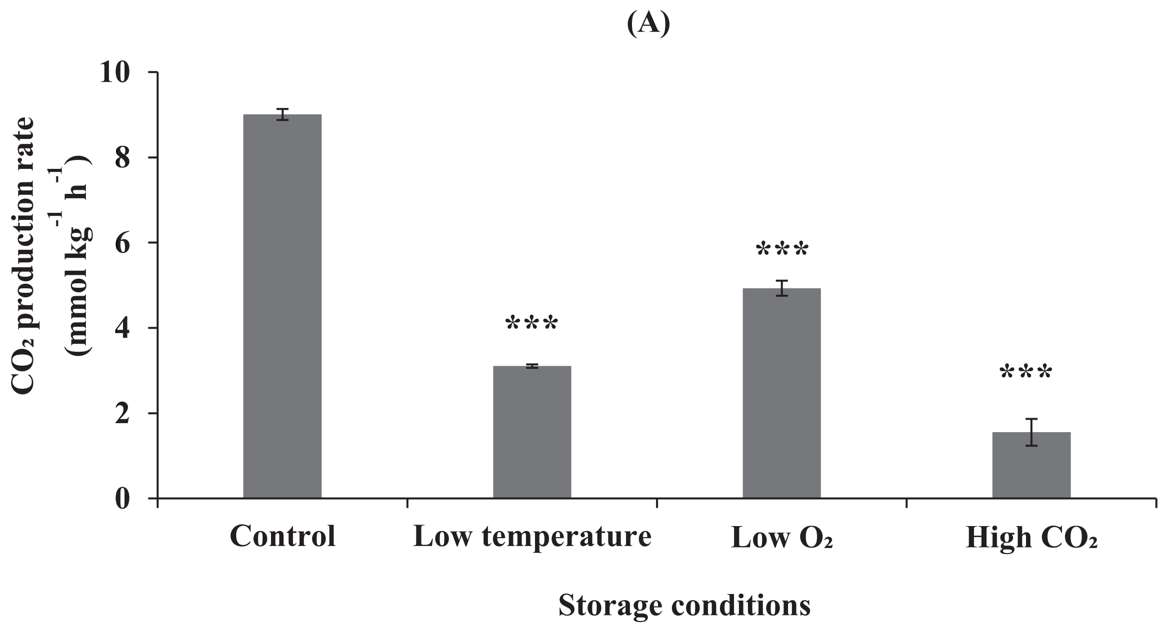
Respiration is the most important metabolic phenomenon that influences the postharvest quality and shelf life of fruits and vegetables, especially microscale vegetables, which includes sprouts. Figure 5 (A) represents the CO_2 production rate of soybean sprouts stored at control, low temperature, low O_2, and high CO_2 in constant dark conditions. After 24 h of storage, the CO_2 production rate of soybean sprouts stored under low temperature, low O_2, and high CO_2 was significantly lower than that of the control (***$P < 0.001$).

Low temperature and CA (low O_2 and high CO_2) treatments are essential approaches to prolong the postharvest life of fresh produce by controlling the rate of respiration (Toivonen, 2010), and have been widely used in commercial treatments. In this study, all treatments suppressed the respiration rate, which subsequently alleviated the quality deterioration of soybean sprouts compared to ambient air storage. To confirm this fact, changes in postharvest quality aspects of soybean sprouts under various storage conditions were then investigated by monitoring the weight loss and browning index.

4.1.2 Weight loss

The effect of various storage treatments on the weight loss of soybean sprouts is presented in Figure 5 (B). After 24 h of storage, 4.08% weight loss was found in the control samples, whereas lower levels of weight loss at 2.08%, 2.82% and 2.54% were recorded under low temperature, low O_2 and high CO_2 storage conditions, respectively. This is likely the result of a lower respiration rate of soybean sprouts observed in the low temperature and CA treatments compared to the control soybean sprouts.

Postharvest weight loss has a large impact on fruit and vegetable quality. Weight loss is mainly due to the loss of water caused by transpiration and respiration processes and is a major cause of quality deterioration in fresh produce after harvest. Studies in several sprouts, such as mung bean, artichoke, and peanut sprouts, have shown that low temperature and packaging treatment with high CO_2 and low O_2 can reduce weight loss. Additionally, suppression of metabolic processes such as respiration and transpiration is considered a reason for weight loss reduction during storage (Amin et al., 2017; Guo et al., 2022; Zhang et al., 2018). Similarly, our study also revealed that a reduced weight loss occurred coincidently with a decrease in the respiration rate of soybean sprouts under low temperature and CA storage.



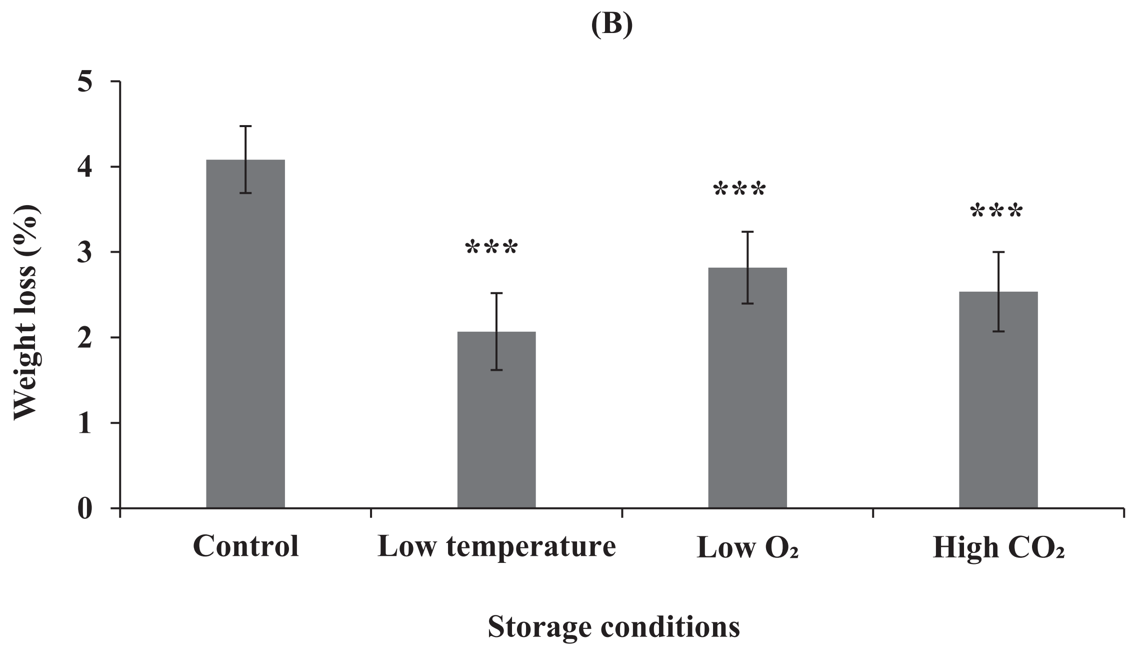


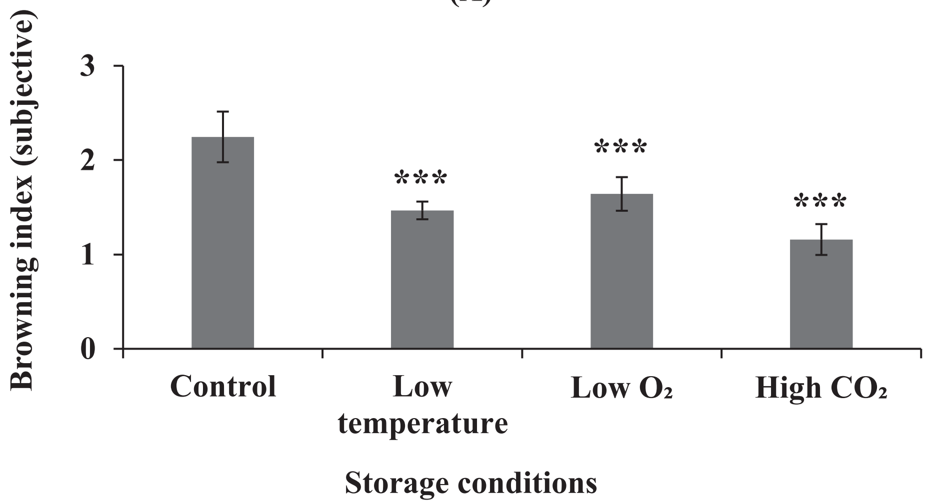
Figure 5: Effect of low temperature, low O_2, and high CO_2 on (A) CO_2 production rate (n = 3), (B) weight loss (n = 10) of soybean sprouts after 24 h storage in constant dark conditions. Bars for each group with an asterisk indicate statistically significant differences using the Dunnett test (*P < 0.05, **P < 0.01, ***P < 0.001). Data presented are the mean ± SD.

4.1.3 Browning Index

The browning index (discoloration) is commonly tested to evaluate visual appearance quality, which is one of the most important factors in the postharvest storage quality of sprouts (Amin et al., 2017; Singh et al., 2014). As the major discolored part of soybean sprouts is cotyledons, the browning of cotyledons was mainly estimated to assess the quality of soybean sprouts during storage in this study. The estimation of browning degree by the methods described in Section 3.3.3 and the correlation of subjective and objective browning indices during storage are shown in Figure 6. Although slight browning symptoms were observed in soybean sprout cotyledons under ambient air temperature storage (control) after 24 h, a lower browning index (subjectively assessed) was detected in low temperature, low O_2 and high CO_2 treated sprouts than in the control (Fig. 6A). Similarly, the browning index derived from the L*, a*, and b* values also showed significant decreases in all treatments, which was concomitant with less browning (Fig. 6B). Since the results obtained from both measurement methods were highly correlated ($R^2 = 0.87$) (Fig. 6C), evaluation of soybean sprout browning by the visual scoring method could be used along with objective assessment. The results also indicated that low temperature, low O_2, and high CO_2 storage maintained the visual quality of soybean sprouts by suppressing discoloration. This reduction in browning of soybean sprouts was also likely due to a decrease in respiration.

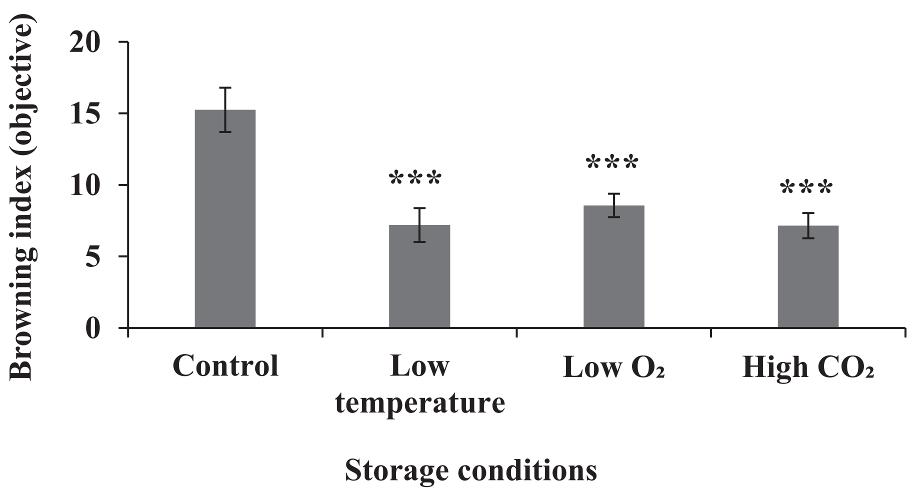
Browning index (objective) measurement using colorimetric parameters (L*, a*, b*) has been extensively used to indicate browning. An earlier study reported that an increase in browning index values has been associated with browning development in green beans (Kasim and Kasim, 2015). Similarly, the browning development of soybean sprouts in this study was observed in all storage treatments, with the highest increase in the browning index value in the soybean sprout samples of the control group. This finding supported the results of visual quality that the browning of soybean sprouts was reduced by low temperature, low O_2, and high CO_2 storage treatments. In a previous study, Amin et al. (2017) suggested that active packaging under high CO_2 and low O_2 is effective in reducing the incidence of discoloration in artichoke sprouts. However, a correlation with visual quality and respiration rate has been reported in microgreens, where low temperature had a lower respiration rate and higher visual quality ratings (Berba and Uchanski, 2012).

(A)



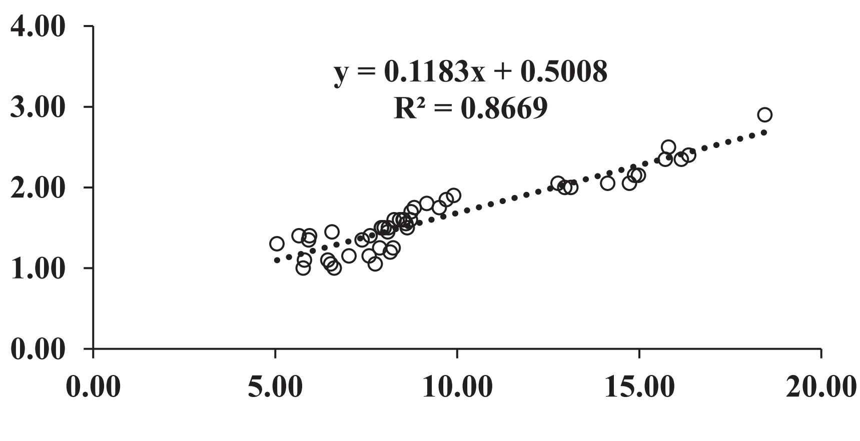
Browning index (subjective)
3
2
1
0
***
***
***
Control
Low
temperature
Low O_2
High CO_2
Storage conditions

(B)



Browning index (objective)
20
15
10
5
0
***
***
***
Control
Low
temperature
Low O_2
High CO_2
Storage conditions

(C)



4.00
3.00
2.00
1.00
0.00
y = 0.1183x + 0.5008
R^2 = 0.8669
0.00
5.00
10.00
15.00
20.00

(D)

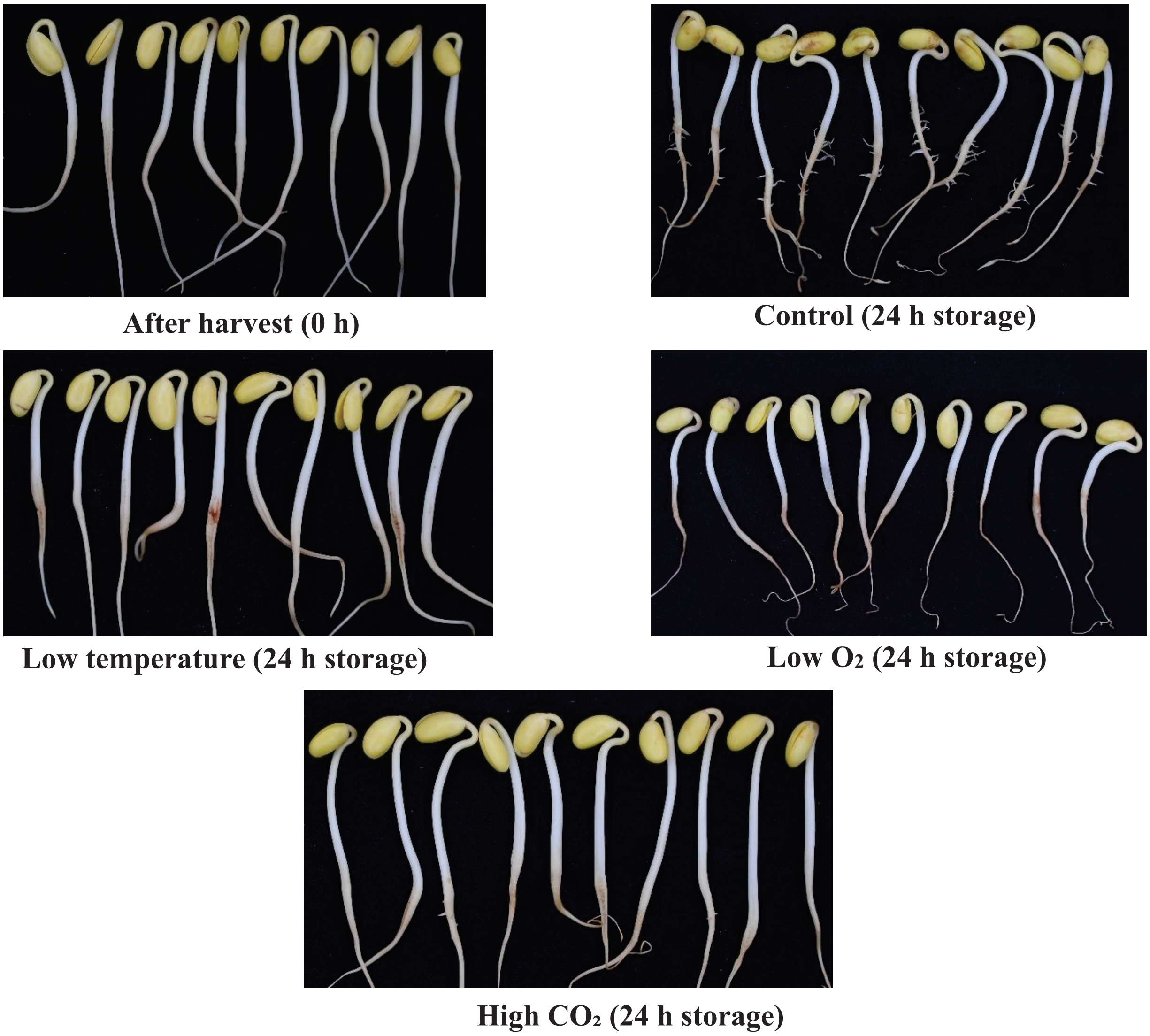


Figure 6: Effect of low temperature, low O_2, and high CO_2 on (A) browning index (subjective) (n = 12) (after 24 h storage), (B) browning index (objective) (n = 5) (after 24 h storage), and (C) relationship between subjective and objective data of browning index, and (D) appearance of soybean sprouts in constant dark conditions. Bars for each group with an asterisk indicate statistically significant differences using the Dunnett's test (*P < 0.05, **P < 0.01, ***P < 0.001).

4.2 Circadian clock analysis in postharvest dark storage conditions

4.2.1 Evaluation of rhythmic pattern of clock gene expression

To investigate the individual rhythmic behavior of soybean clock genes stored under ambient temperature (control), low temperature, low O_2, and high CO_2 in constant dark conditions, the measured values of each gene expression data were fit to a general cosine curve Equation (6). Regression cosine curves were determined by a cosinor analysis, and the R^2 values are shown in Figure 7. The significance of the rhythm was tested using P values obtained from the slope test.

According to Figure 7 (A, C, D), the expression levels of clock genes (*GmLCL1, GmPRR7, GmGI*, and *GmLUX*) showed rhythmic waves with R^2 values of 0.30-0.70 ($P < 0.001$) when soybean sprouts were stored under control and CA (low O_2 and high CO_2) conditions. On the other hand, the significant wave form was unnoticeable for genes under low temperature conditions except *GmGI* and *GmLUX*. As a result, both genes (*GmGI, GmLUX*) maintained the corresponding behavior ($R^2 > 0.3$, $P < 0.001$), and other genes showed feeble rhythmicity with an R^2 value of ~0.20 ($P < 0.05$) at low temperatures (Fig. 7B). However, *GmTOC1* expression under low O_2 conditions displayed arrhythmicity similar to that of the control, with the lowest R^2 value of 0.04 ($P > 0.05$). Overall, this section describes how fitting of a cosine curve can be used to visualize circadian data in each condition that would be appropriate for rhythmicity assessment.

This study aimed to examine the existence of circadian rhythm in soybean sprouts that have never been exposed to the cycling of light from germination to storage. Thus, a cosine curve was employed to analyze the circadian oscillation of clock genes and to detect the 24 h circadian rhythm in soybean sprouts. It is well documented that cosinor analysis is a classic approach for estimating the pattern of smooth rhythm even though the data points are relatively short (Cornelissen, 2014). The cosinor-based model assumes that the expression level of a gene is a sine or cosine function of the circadian time. Circadian profiles are considered rhythmic when a significant cosine fit (R^2) is confirmed. Recently, statistical support for the presence of significant circadian rhythmicity was evaluated by the cosinor-based method at a P value less than 0.01 (Ding et al., 2022; Parsons et al., 2020). In addition, $P < 0.001$ was also applied for testing circadian rhythmicity in urinary bile acid of rats using cosinor analysis (Kawai et al., 2020). To confirm the presence of circadian rhythmicity in soybean sprouts during storage, $P < 0.01$ was used as a statistical significance cutoff to declare circadian rhythmicity. Interestingly, the obtained R^2 values in this study remained statistically significant at $P < 0.001$, which is higher than the conservative threshold. Moreover, JTK_CYCLE, another efficient algorithm (Hughes et al., 2010) was applied to confirm 24 h rhythmicity at threshold $P < 0.01$ in soybean sprout clock gene expression (Data available in the supplementary data Table S1). Results of JTK_CYCLE displayed a significantly rhythmic (e.g., *GmLCL1, GmPRR7, GmGI, GmLUX*, $P < 0.01$ in control, low O_2, high CO_2) and non-rhythmic (e.g., *GmLCL1*, *GmPRR7*, *GmTOC1*, $P > 0.01$ in low temperature) transcripts that were consistent with the results of the cosinor analysis. Since, both methods showed nearly similar outputs, only the results of cosinor analysis were used to discuss the influence of postharvest stresses on the soybean sprout circadian clock in the present study. To the best of our knowledge, this analysis has not yet been applied in postharvest storage conditions. This is the first report that statistically confirms the significant rhythm of clock gene expression in postharvest storage of soybean sprouts even under constant dark conditions.

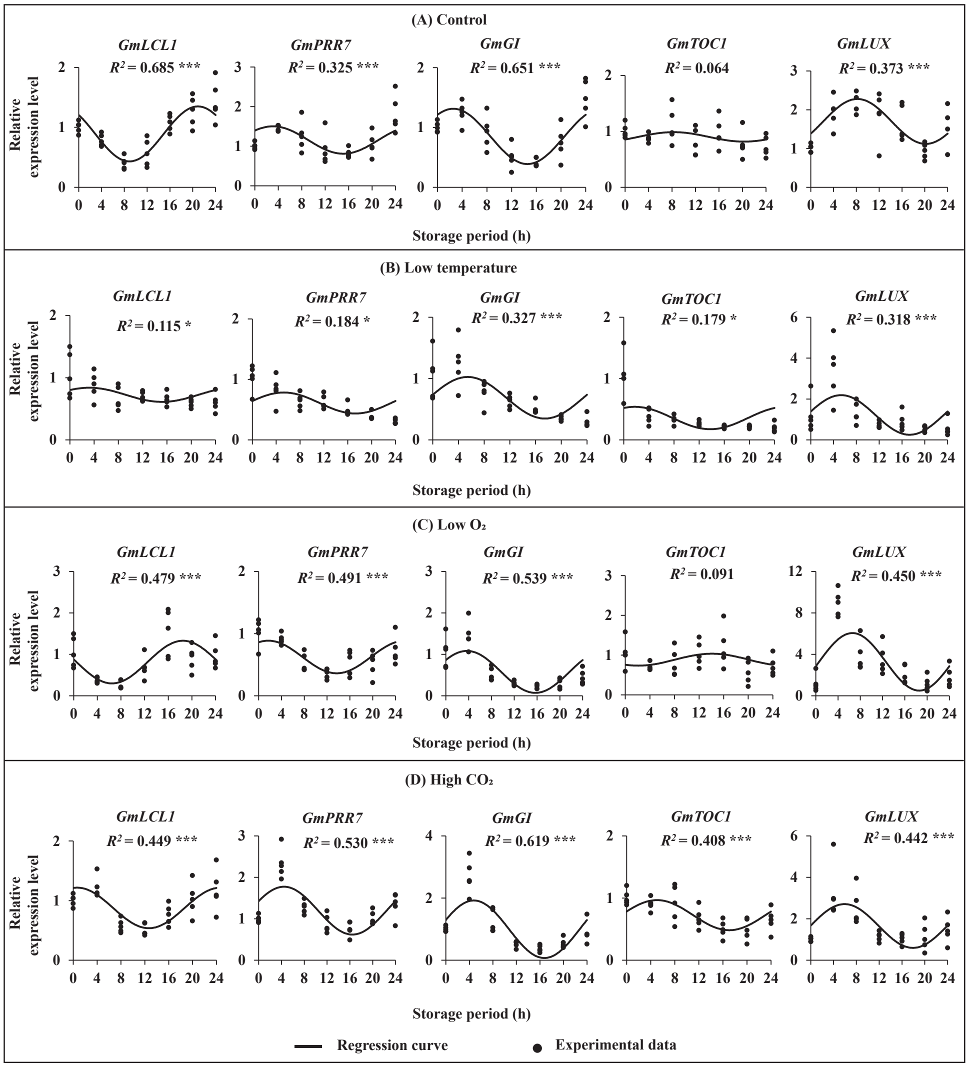


Figure 7: Fitting curve of relative clock gene expression with the cosine curve equation under different storage conditions (A) 20 °C-air, (B) 10 °C-air, (C) 20 °C-5 % O_2, and (D) 20 °C-15 % CO_2 + 20 % O_2. The target genes of soybean sprouts under different storage conditions were *GmCCA1*, *GmLHY*, *GmPRR7*, *GmGI*, *GmTOC1*, and *GmLUX*. R^2 stands for determination of coefficient calculated by cosine curve fitting and significancy of rhythm tested using *P*-value by slope test (**P* <0.05, ***P* <0.01, ****P* <0.001). The solid line indicates the regression cosine curve obtained from non-linear regression. Various plots indicate relative gene expression values measured using qPCR from five biological replicates.

4.2.2 Quantitative evaluation of the circadian rhythm under ambient air temperature

To quantify the conserved rhythmic expression of clock genes under ambient temperature (control), the key oscillation parameter (phase) was considered for the evaluation (Table 5).

Based on the calculated data, the phase of the *GmLCL1* gene of soybean sprouts showed a storage period of 20.90 h under the control (sampling time at ~6:00 am, see materials and methods section 3.4 for details) (Table 5, Fig. 7A). The nearly dawn time of the cycle could be considered when *GmLCL1* has an abundance of expression levels. This circumstance can be explained by assuming that due to the loosely coupled nature of the oscillator, the initial phase of the soybean clock started independent of its own time to coordinate with environmental signals (Thain et al., 2000; Webb et al., 2019). Subsequently, other clock genes are expressed depending on the specific time point of the cycle. For example, during a time series of a 24 h continuous dark storage period, *GmPRR7* and another essential gene, *GmGI,* started their accumulation at approx. 3.09 h and 2.56 h, respectively, which is considered daytime (sampling time at ~13:00). Finally, *GmLUX* displayed its maximum at 8.18 h, which is considered evening (sampling time at ~18:00) (Table 5, Fig. 7A). As a result, the sequence interval of *GmPRR7* and *GmLUX* was maintained at approx. ~6 h and ~12 h after dawn to complete one cycle. It is evident in Arabidopsis plants that the *PRR7* and *TOC1* genes accumulated to their maximum at ~6 h and ~12 h after dawn, respectively, without depending on the light/dark cycle (Matsushika et al., 2000). However, although *GmTOC1* was not rhythmic ($R^2 = 0.06$) (Fig. 7A) enough to maintain the negative feedback loop, *GmGI* and *GmLUX* might act as positive regulators for the morning genes *GmLCL1* instead of *GmTOC1* (McClung, 2006). These data support the findings of a significant rhythm in clock gene expression except *TOC1* in cowpea legumes (Weiss et al., 2018). Overall, the above results suggest that ambient air temperature storage maintains circadian rhythm in clock gene expression under dark conditions.

To assess the rhythmic performance of clock gene expression, a phase (c) of the 24 h period was derived through nonlinear regression. Noticeably, the phases of all clock gene expression under constant dark and ambient temperatures are compatible with the circadian oscillation described for soybean plants grown under normal environmental conditions with both light/dark and temperature (Marcolino-Gomes et al., 2014; Vezza et al., 2020). They also found the peak expression of *GmLCL1* and *GmLUX* genes during dawn and dusk, while expression of *GmPRR9,* and *GmGI* mainly peaked during the daytime of a cycle. This allowed us to conclude that soybean sprout clock genes under ambient temperature storage showed circadian oscillation without the absence of light. An explanation for the initiation of the soybean sprout clock may be the hydration or soaking of dry seeds, since imbibition acts as an environmental signal responsible for synchronizing the clocks among populations of seedlings (Zhong et al., 1998). Another possible explanation could be that the harvesting process might reset the clock of soybean sprouts; however, further clarification is needed to describe the onset time of the circadian oscillation of soybean sprouts.

Table 5 Circadian rhythm assessing parameters of clock genes determined by non-linear regression

	Clock gene name	Control	Low temperature	Low O_2	High CO_2
Relative amplitude, *b*	*GmLCL1*	0.46	-	0.51	0.34
	GmPRR7	0.35	-	0.26	0.58
	GmGI	0.35	0.34	0.51	0.92
	GmTOC1	-	-	-	0.24
	GmLUX	0.58	0.97	2.78	1.06
Phase, *c*	*GmLCL1*	20.90	-	18.48	0.67
	GmPRR7	3.09	-	1.68	4.43
	GmGI	2.56	5.44	3.69	4.74
	GmTOC1	-	-	-	5.05
	GmLUX	8.18	5.33	6.58	5.92

4.2.3 Effect of controlled storage atmosphere on the amplitude and phase of the circadian rhythm

CA storage induced distinct changes in circadian clock gene expression, which was revealed by alterations in amplitude (b) and phase (c). According to Table 5, the fluctuation of amplitude (b) can be seen in each clock gene under low O_2 and high CO_2 storage, indicating that the clock components are responsive to these signals. In low O_2, there was a high amplitude of *GmLCL1*, *GmGI*, and *GmLUX* and a low amplitude of *GmPRR7*, while high CO_2 produced a low amplitude of *GmLCL1* and a high amplitude of *GmPRR7, GmGI*, and *GmLUX.* This might be explained by the recent study of Vezza et al. (2020), which mentioned that fluctuations in clock gene expression were mainly caused by exogenic stress signals and are responsible for affecting clock gene expression by altering the amplitude.

Along with amplitude, high CO_2 also altered the phase of all genes compared with the control (Fig. 8A). Noticeably, while day oscillator genes tend to display a delayed phase, evening genes have the opposite phase transition under high CO_2 storage. The phase (c value) of the morning gene *GmLCL1* at high CO_2 was 0.67 h, while that of the control was 20.90 h. This phase of morning genes was shifted at an ~4 h delay (Table 5, Fig. 8A). *GmPRR7* and *GmGI* genes also exhibited a delayed phase-shifting compared to the control (Fig. 8A). Alternatively, an irregular early phase was observed in the evening genes *GmTOC1* (c = 5.05 h) and *GmLUX* (c = 5.92 h) under high CO_2 storage (Table 5). As a result, the timing intervals in the sequential expression of *GmTOC1* and *GmLUX* from *GmLCL1* were ~5 h differences. This was affected by high CO_2 storage. It has been reported that *TOC1* and *LUX* are direct regulators of *CCA1/LHY* in Arabidopsis (Hazen et al., 2005). Therefore, the phase advancements in *TOC1/LUX* might be the reason for the loss of normal expression of the morning-phased genes *CCA1/LHY* in soybean sprouts. This result is similar to that observed in the soybean circadian clock, where drought stress conditions altered *CCA1/LHY*-like expression by changing the evening expressed genes (Marcolino-Gomes et al., 2014).

Additionally, the phase of the *GmLCL1* gene under low O_2 storage was 18.48 h. This was an approx. 3 h difference compared to that of the control. Perhaps most noticeably, low O_2 storage had little effect on the phase of the *GmPRR7, GmGI*, and *GmLUX* genes, although it exhibited a variation in amplitude. In detail, the phase of *GmPRR7, GmGI*, and *GmLUX* under low O2 described only a 1–2 h phase difference compared with that of the control (Table 5, Fig. 8B). This indicated a negligible effect of low O_2 on the phase of clock gene expression. This result is likely due to the O_2 concentration used for storage treatment. In addition, previous studies reported that moderate drought stress affects the amplitude but has no strong influence on the phase of soybean clock components (Li et al., 2019; Marcolino-Gomes et al., 2014). Soybean sprouts kept under low O_2 stress might express cellular response mechanisms similar to those of soybean plants grown under moderate drought stress conditions. CA is an important storage method that maintains the quality of fresh produce. To our knowledge, this is the first report to discuss the impact of CA conditions on the circadian clock gene expression of fresh produce. The circadian clock in plant cells can synchronize under natural conditions. When other external stimuli, such as CA storage (low O_2 and high CO_2 stress), are applied; desynchronization in the circadian system could be induced because of phase and amplitude alterations in clock gene expression. However, the mechanisms by which clock gene alterations occur under CA storage are still unknown. A possible explanation is that postharvest low O_2 and high CO_2 stress-responsive genes may act as signaling molecules and thereby influence the change in clock-associated gene expression. To test this hypothesis, genetic analysis is necessary in the future to identify CA-responsive pathway genes and their biological significance on these modulations.

4.2.4 Effect of low temperature on the amplitude and phase of the circadian rhythm

The circadian clock of soybean sprouts seems to exhibit different behaviors in response to cold. According to the R^2 value (Fig. 7B), low temperature storage displayed rhythmicity only for two genes (*GmGI* and *GmLUX*), whereas the expression of another gene diminished over time. Interestingly, *GmLUX* expression dramatically increased with a peak amplitude (b) of 0.97 at almost the same phase (c = 5.33 h) as *GmGI* (c = 5.44 h). However, phase-shifting was detected compared to the control (Table 5, Fig. 8C). A similar result has also been observed in Arabidopsis, where low temperature stress dampened the circadian expression of oscillator genes except *LUX* with high amplitude cycles (Bieniawska et al., 2008). Additionally, it has been reported that *LUX* plays a positive role in maintaining the circadian clock by upregulating *CCA1/LHY* (Hazen et al., 2005). Since the normal expression of morning genes *CCA1/LHY* was not found under low temperature storage in this study, it could be said that low temperature storage possibly influenced a disruption in the overall circadian clock.

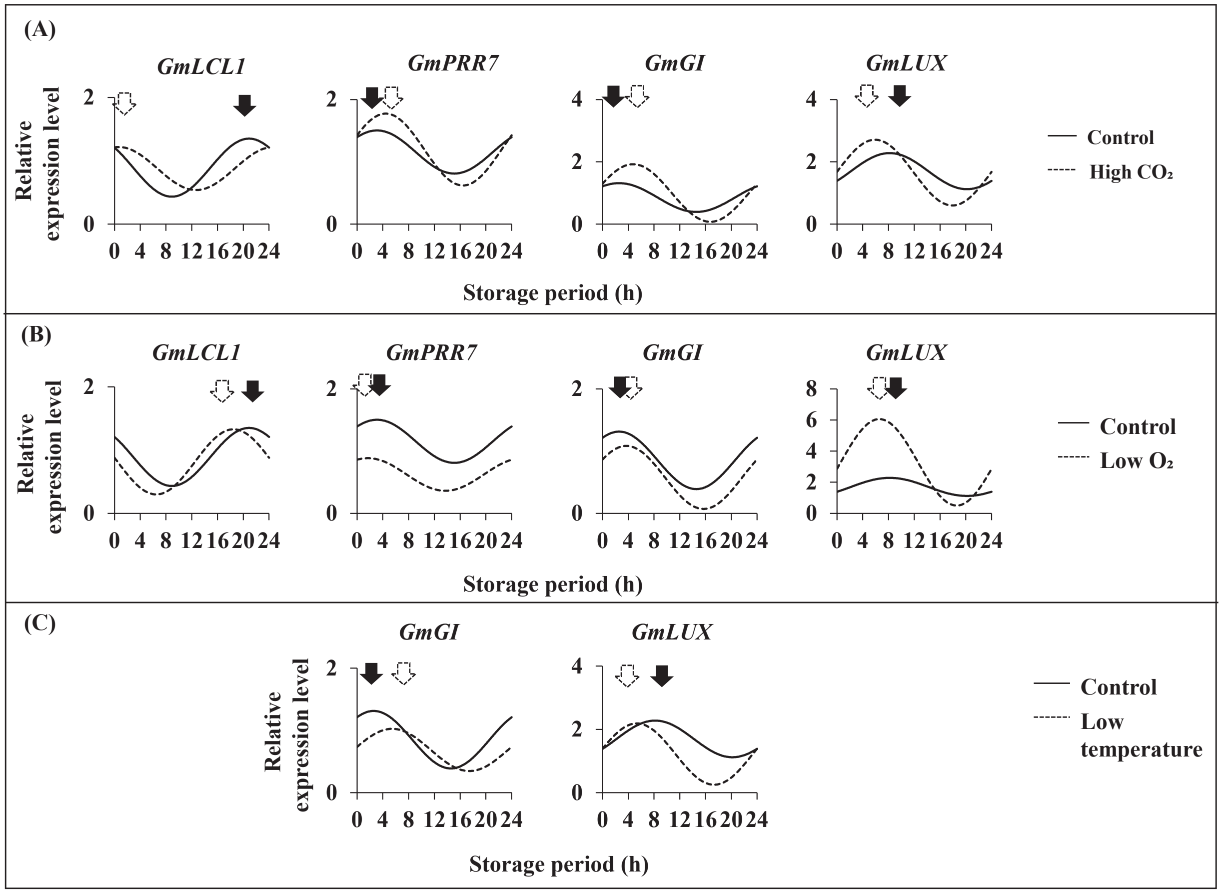


Figure 8: Phase-shifting comparison structure of rhythmic clock gene expression under different storage conditions. Graphs illustrating a phase-shifting difference between (A) control and high CO_2, (B) control and low O_2, (C) control and low temperature. Line graphs indicating the 24 h regression cosine curve obtained from the relative expression levels of each gene fitted with the cosine curve Equation (6). Black and dotted arrows indicate the phase of the expression under control and other storage conditions, respectively.

4.3 Behaviors of postharvest physiological attributes-related gene expression of soybean sprouts

Circadian output pathways provide a link between the circadian oscillator and various physiological and biological processes of plants (Srivastava et al., 2019). Since our results revealed that all the storage treatments altered the rhythmicity and expression level of circadian clock genes, the expression of target genes which relate to postharvest physiological attributes were also analyzed to prove a link between circadian clock and changes in postharvest quality. Gene relating to respiratory pathway (TCA cycle) intermediates (*GmFUM1, GmCS, Gm2-OGDH*), browning producing enzymes (*GmPPO1, GmPAL*), and drought or cold stress responsive factor (*GmDREB5*) of soybean sprouts were analyzed as clock output gene expression at 4 h intervals for 24 h storage. Under control condition, the expression of *GmFUM1, GmCS, Gm2-OGDH, GmPPO1* and *GmPAL* showed a wave fluctuation with a broad peak shape at 8 h–12 h of storage, which might indicate an active cellular function under ambient temperature storage (Fig. 9). In contrast, the expression levels of these genes were consistently suppressed by low temperature, low O_2, and high CO_2 storage treatments (Fig. 9). The expression level of *GmDREB5* under low temperature treatment was slightly higher than that of the control. Nevertheless, the peak of expression level of all storage treatments was not remarkably detected during 24 h storage (Fig. 9F). The results suggested that *DREB* gene of soybean sprouts might be either clock independent and/or not responsive during postharvest stress.

Previous study points towards a circadian regulation of TCA cycle intermediates, and reactive oxygen species (ROS) pathway genes as a clock related output, which is associated with cellular function (Fukushima et al., 2009; Lai et al., 2012). When plants undergo aerobic metabolism such as respiration, it generates toxic by-products of oxygen known as ROS. Browning substance PPO is mainly formed as a result of increased level of ROS promoting genes such as

PAL. It was previously observed that ROS pathway genes followed a circadian manner with a time-of-day-specific phase (Lai et al., 2012). Similarly, our results provided evidence that the expression of genes involved in the respiration and browning events increased rhythmically under ambient temperature storage (control). It was likely that ambient storage temperature (input signal) exerts a direct effect on clock output pathway when the clock oscillation is active (Lai et al., 2012). Alternatively, a cold and drought responsive *GmDREB5* expression suggested that circadian clock oscillation effect is not uniform in all output pathways of postharvest soybean sprouts.

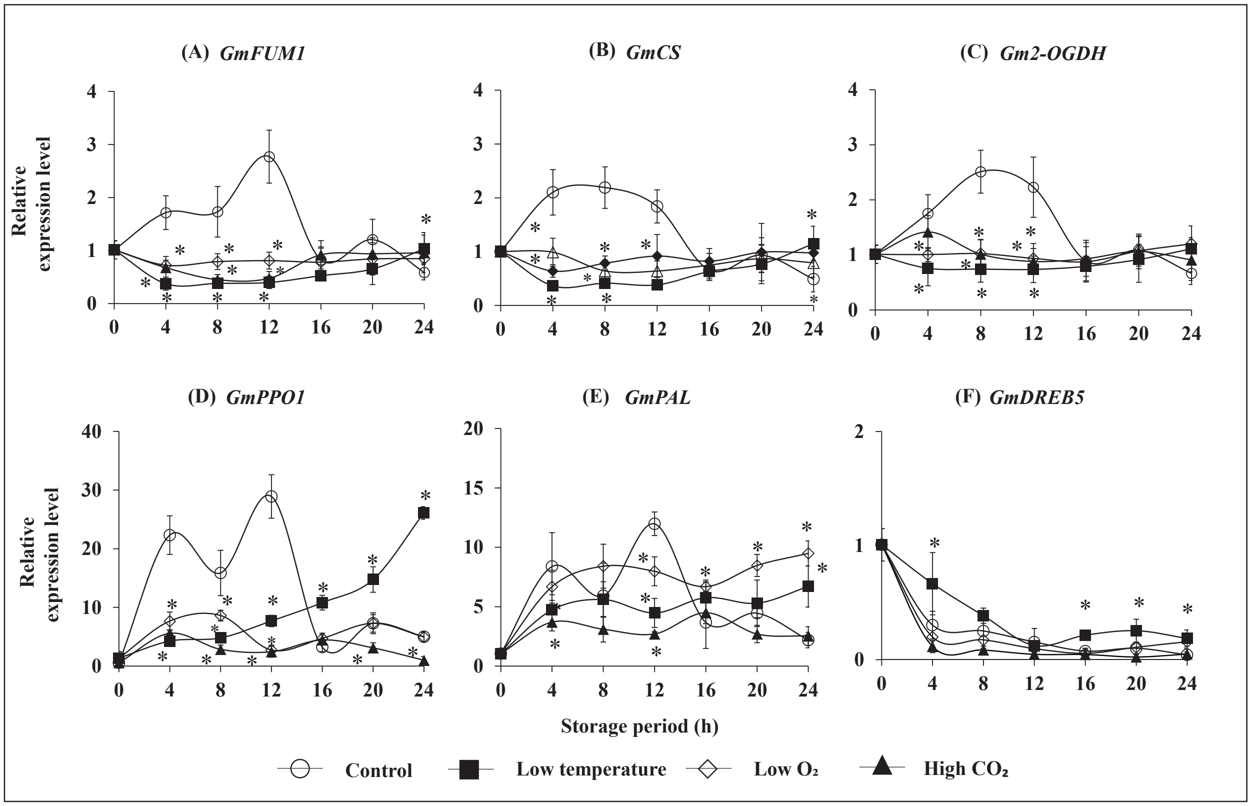


Figure 9 Effect of low temperature, low O_2, and high CO_2 on clock output genes related to different postharvest physiological attributes; (A) *GmFUM1*, (B) *GmCS*, (C) *Gm2-OGDH*, (D) *GmPPO1*, (E) *GmPAL*, and (F) *GmDREB5*. Data presented are the mean of five replicates ± SD. (*) indicates significant difference between control and treated samples in each time point (Dunnett's test, $P < 0.05$).

4.4 A possible link between the circadian clock and postharvest quality

Fig. 10 represents a diagram proposing a putative link between circadian clock oscillation and postharvest quality maintenance of soybean sprouts under various storage conditions. Generally, the model of the circadian clock system consists of three modules: (1) abiotic factors, mainly light and temperature, are attributed as input pathway signals, (2) central oscillator genes generate circadian rhythm using input signals, and finally, (3) the output pathways correspond to various physiological and developmental processes. Viewed in this way, the proposed diagram (Fig. 10) of the present study demonstrated that ambient air (control), low temperature, low O_2, and high CO_2 act as input signals and are responsible for creating different rhythms during central oscillator gene expression. Additionally, the respiration rate, weight loss, browning index, and quality attribute-related genes were measured to investigate the physiological and physical conditions of soybean sprouts as outputs. In short, under low temperature, low O_2, and high CO_2 storage, a restrained quality deterioration process (lower levels of quality attributes and their related gene expression) was detected simultaneously with a desynchronized rhythm during clock gene expression (diminishing rhythmic expression or altering phase and amplitude). Alternatively, opposite phenomena, such as quality degradation and circadian rhythm in clock gene expression, were found in the control.

The connection of the circadian clock with physiological processes such as respiration and photosynthesis is well clarified during plant growth and development stages. For example, disruption of the circadian clock can cause decreased transcript and metabolite levels involved in plant respiration (Fukushima et al., 2009). Consistent with this idea, our study suggests a possible link between perturbed circadian clock and postharvest quality maintenance of soybean sprouts. However, in contrast to our result, Liu et al., (2015) suggested that keeping the rhythm by 12 h light/dark cycle treatment improves color and maintains glucosinolate content in postharvest leafy vegetables. This might be because leafy vegetables can undergo light-dependent biological processes, including photosynthesis and phytochemical retention, and maintain light-responsive quality after harvest. However, transcriptional analysis of clock genes that generate clock rhythm under 12 h light/dark cycle treatment was not reported in Liu et al. (2015). Additionally, leaf organs may benefit from periodic light treatments even if they can have negative effects on soybean sprout quality, such as root elongation and initiation of green coloration (Ghani et al., 2016). Most importantly, the development of a novel postharvest technology for maintaining the quality and extending the shelf life of soybean sprouts is generally conducted without a combination of light irradiation. Postharvest research also considers the prevention of moisture loss of the fragile hypocotyl part and browning development of the bean part of soybean sprouts. Hence, it is difficult to compare the beneficial effects of maintaining the circadian rhythm (by light/dark cycle) on the quality of leafy vegetables and soybean sprouts. At this point, evaluating the link between circadian rhythm and quality control should be distinguished based on the morphology and phenotype of fresh commodities.

Taken together, the proposed interaction in Fig. 10 can be considered a fundamental concept in understanding the response behavior of clock gene expression altered by various storage conditions and their underlying interactions with changes in the quality attributes of soybean sprouts. This may provide clues to how we can genetically manipulate the circadian clock to maintain postharvest quality.

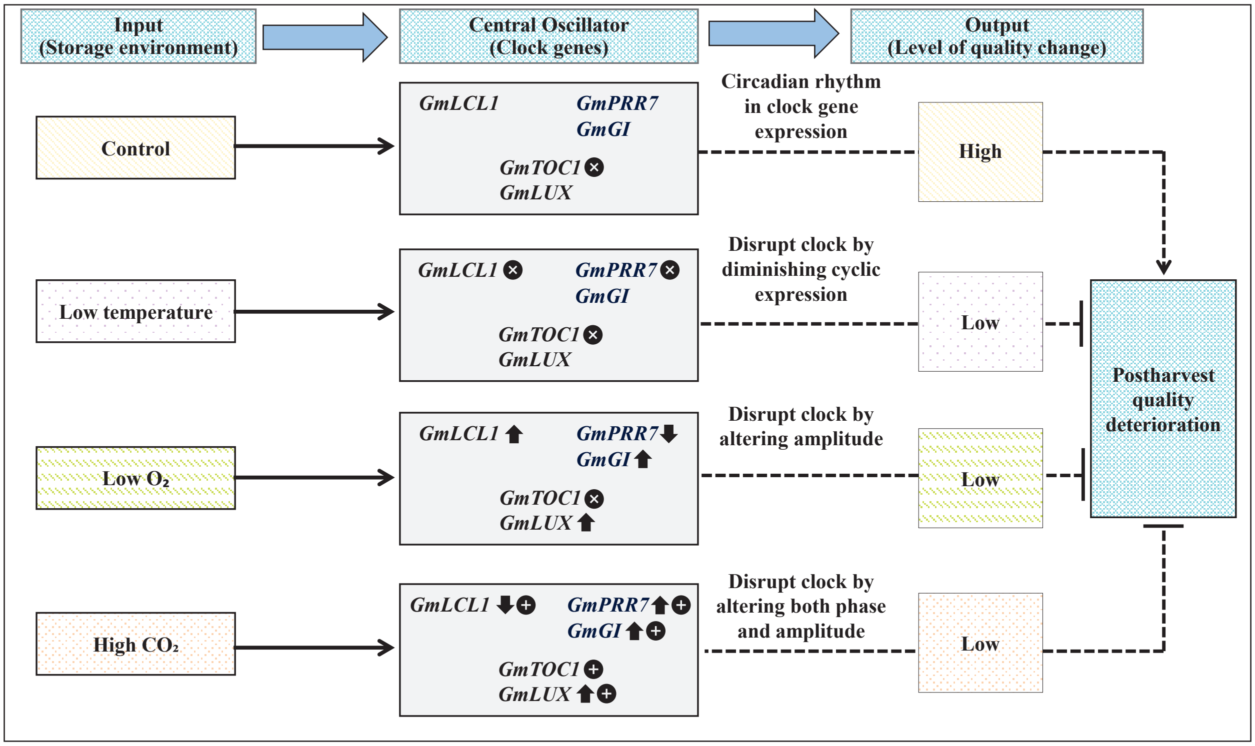


Figure 10 Schematic diagram proposing a relationship between circadian rhythm and postharvest quality of soybean sprouts stored under control, low temperature, low O_2, and high CO_2 conditions. Pointed arrows denote activation, and blunt-end arrows denote repression or inhibition. Putative links between disturbed circadian rhythm and postharvest quality maintenance are demonstrated as dashed lines. Up and down arrows indicate an increase and decrease in amplitude, respectively. The plus sign indicates the phase shift, and the cross sign indicates arrhythmic/noncycling expression.

V. CONCLUSION

Circadian rhythms permit living organisms to anticipate, adapt, and respond early to predictable daily environmental changes, optimizing their physiology, metabolism, behavior, and adjusting their biology. Circadian rhythms are driven internally, but the phase can be reset by external time cues such as light, dark and temperature cycles. Regulation of plant physiology by the circadian clock is widespread. Recent studies have shown that clock-regulated genes have direct involvement in several biotic and abiotic stresses that help plants to enhance the stress tolerance as well as plants fitness level. While a well-known relationship exists between environmental stress and the circadian clock in the vegetative stage of plants, little information is available for harvested fresh fruits and vegetables.

Postharvest begins after the separation of individual part from the plant that produced it by a deliberate human act. Fresh fruits and vegetables are living organs and quality deterioration occurs quickly after harvest due to the continuation of biological activity and metabolic processes. Thus, for fresh produce, it is an urgent need for understanding the behavior of clock gene expression and their underlying interactions with changes in quality which is vital for further improvement of postharvest handling. The application of postharvest abiotic conditions involving cold, and controlled atmosphere, has potential impact not only on preserving quality but also extending shelf life of perishables produces. Here, soybean sprouts have been selected as a sample which is considered as high perishable vegetables due to their high respiration rate and easy to deteriorate.

In this dissertation, we investigated the effect of temperature and controlled atmosphere storage on the expression of clock genes (*GmCCA1*, *GmLHY*, *GmPRR7*, *GmGI*, *GmTOC1*, and *GmLUX*) and postharvest quality characteristics (respiration rate, weight loss and browning incidence) in soybean sprouts. By fitting clock gene expression data with the cosine curve equation, clock genes under different storage conditions prove their existence even in constant dark conditions. According to the amplitude and phase from the cosine curve fitting, quantified rhythm revealed that various storage conditions influenced the expression of circadian clock components to a different degree. Additionally, all the storage conditions showed a change in soybean sprouts' quality during storage. Overall, our results first suggest a biologically meaningful link between clock disruption and postharvest quality maintenance of soybean sprouts. We believe that in future the knowledge about the existence of the circadian clock system and its response behavior during varying storage could give a better understanding for elucidating/manipulating clock mechanism in fresh produce to control their postharvest quality and shelf life during postharvest handling.

SUPPLEMENTARY DATA TABLE

Table S1: Detection of circadian rhythm from clock gene expression data by JTK_CYCLE method

Storage conditions	Gene name	P value	BH. Q value	Phase	Amplitude
Control	*GmLCL1*	2.31E-07	1.54E-06	22	0.375
	GmPRR7	0.0002	0.0004	4	0.240
	GmGI	1.88E-08	1.88E-07	4	0.368
	GmTOC1	1	1	6	0.099
	GmLUX	9.88E-05	0.0002	10	0.679
Low temperature	*GmLCL1*	1	1	6	0.120
	GmPRR7	0.1959	0.2305	8	0.085
	GmGI	0.0071	0.0101	8	0.191
	GmTOC1	0.1506	0.1882	6	0.134
	GmLUX	0.0182	0.0242	6	0.559
Low O_2	*GmLCL1*	3.75E-06	1.25E-05	20	0.467
	GmPRR7	0.0003	0.0005	2	0.262
	GmGI	3.19E-07	1.58E-06	4	0.445
	GmTOC1	0.6284	0.6983	16	0.042
	GmLUX	0.0001	0.0003	8	2.326
High CO_2	*GmLCL1*	5.32E-05	0.0001	2	0.346
	GmPRR7	3.94E-07	1.58E-06	6	0.325
	GmGI	9.16E-11	1.83E-09	6	0.594
	GmTOC1	0.0067	0.0101	6	0.269
	GmLUX	8.49E-05	0.0002	6	0.686

PUBLICATION

Shomodder, A., Imaizumi T., Nagata, M., Kasai, E., Shiina T., Tsuta, M., Thammawong M., Nakano, K. (2023). Exploring the relationship between circadian rhythm and postharvest quality of soybean sprouts during storage. Journal of Plant Physiology, 281, 153906. (Published)

Shomodder, A., Thammawong M., Nakano, K. (2022). Postharvest Technologies for quality maintenance of sprouts. Reviews in Agricultural Science, 10, 239-256. (Published)

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisors Professor Kohei Nakano, and Associate Professor Manasikan Thammawong for all of their support, insights, guidance and encouragement. Sincere gratitude is extended to Associate Professor Teppei Imaizumi, Professor Masayasu Nagata, Professor Mizuki Tsuta for their useful advice, constructive feedback, and kind support during my PhD.

I am indebted to my lab members such as Matsuo, Ayaka Kato, Putri Wulandari Zainal, Cicih Sugianti, Fawzan Sigma for their help and pleasant company throughout the study period. I am also indebted to all students in Postharvest Engineering laboratory. Very special thanks to Nanami Sugiyama, and Hayashi Yuka for their involvement, and constant support in this research work.

Thanks to The United Graduate School of Agricultural Sciences, Gifu University for providing me the financial support as a Research Assistant to complete this research.

I am grateful to my mother, father, brother, and sister for their unconditional, unequivocal, and loving support. Last, but not least, my husband Bijoy Kumar Barman deserves endless gratitude for his constant love and understanding throughout the entire thesis process and every day. I am forever thankful for his unfailing support and continuous encouragement.

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DISSERTATION SUMMARY

Circadian clock is an endogenous timer that produce 24 h biological rhythm in plants to anticipate the light, dark and temperature cycles of a rotating planet. Coordination of circadian rhythms with the external environment provides numerous advantages to plants which are generally modulated by circadian clock genes. Regulation of plant physiology by the circadian clock is widespread. Recent studies have shown that clock-regulated genes have direct involvement in several biotic and abiotic stresses that help plants to enhance the stress tolerance as well as plants fitness level. While a well-known relationship exists between environmental stress and the circadian clock in the vegetative stage of plants, little information is available for harvested fresh fruits and vegetables. Fresh fruits and vegetables are living organs and quality deterioration occurs quickly after harvest due to the continuation of biological activity and metabolic processes. Thus, for fresh produce, understanding the behavior of clock gene expression and their underlying interactions with changes in quality are vital for further improvement of postharvest handling. The application of postharvest abiotic conditions involving cold, and controlled atmosphere, has potential impact not only on preserving quality but also extending shelf-life of perishables produces. Soybean sprouts are considered as highly perishable vegetables due to their high respiration rate and easy to deteriorate.

In this dissertation, the effect of temperature and controlled atmosphere storage on the expression of clock genes (*GmCCA1*, *GmLHY*, *GmPRR7*, *GmGI*, *GmTOC1*, and *GmLUX*) and postharvest quality characteristics and their related genes in soybean sprouts were investigated. By fitting the gene expression level obtained using the qPCR method with the cosine curve equation, it was successfully found that the circadian rhythm existed under constant dark storage conditions of soybean sprouts. A significant rhythm in clock gene expression was observed in control (20 °C-air) soybean sprouts. In contrast, low temperature (10 °C-air) storage diminished the cyclic expression of *GmLCL1*, *GmPRR7*, and *GmTOC1*, which also affected *GmGI* and *GmLUX* expression. Additionally, high CO_2 (20 °C-15% CO_2 + 20% O_2) concentrations during storage disturbed the circadian clock by affecting the phase and amplitude of each gene; for low O_2 (20 °C-5% O_2) concentrations, it was only affected by amplitude. Interestingly, low temperature, low O_2, and high CO_2 maintained postharvest quality, including reduced respiration, weight loss and browning incidence. The expression behaviors of postharvest quality attribute-related genes (*GmFUM1*, *GmCS, Gm2-OGDH, GmPPO1, GmPAL*) were also influenced by the storage treatments.

Overall, the findings first suggest a biologically meaningful link between clock disruption and postharvest quality maintenance of soybean sprouts. It is hoped that in future, the knowledge about the existence of the circadian clock system and its response behavior during storage will help to develop a new postharvest preservation technology by controlling circadian rhythm.

学位論文要旨

概日時計は、地球の自転による明暗と温度の周期に同調して生体リズムを作り出す内因性タイマーである。概日リズムを外部環境と同調させることは、植物にとって多くの利点をもたらすが、それは概日時計遺伝子によって調節されている。植物の生理反応の制御に概日時計は広く利用されている。最近の研究では、時計制御遺伝子がいくつかの生物的・非生物的ストレスに直接関与し、植物のストレス耐性や適応力を高めていることが明らかにされている。植物の生長段階における環境ストレスと概日時計の関係はよく知られているが、収穫後の野菜や果実についての知見はほとんどない。青果物は生命体であり、収穫後も生命活動や代謝過程が継続するため、すぐに品質が劣化する。従って、時計遺伝子の発現挙動やその背景にある品質変化との相互作用を理解することは、収穫後のハンドリングをさらに向上させるために不可欠である。低温や大気制御（Controlled Atmosphere, CA）などの非生物的ストレスの適用は、生鮮食品の品質を保持し、ひいては保存期間を延長する。特に、大豆モヤシは、呼吸速度が速く、劣化しやすいため、非常に腐敗しやすい野菜である。本論文では、大豆モヤシの時計遺伝子（*GmCCA1*, *GmLHY*, *GmPRR7*, *GmGI*, *GmTOC1*, *GmLUX*）および品質変化関連遺伝子（*GmFUM1, GmCS, Gm2-OGDH, GmPPO1, GmPAL, GmDREB5*）の発現、収穫後の品質特性（呼吸速度，重量減少，褐変発生率）に及ぼす温度および CA 貯蔵の影響について検討したものである。

リアルタイム PCR 法で得られた遺伝子発現量をコサイン曲線式でフィッティングしたところ、暗所貯蔵条件下における大豆モヤシにおいて概日リズムが存在することを見出すことに成功した。すなわち、20 °C-大気下で貯蔵した大豆モヤシでは、時計遺伝子発現に規則的な位相と振幅を持つ有意なリズムが観察された。一方、10 °C-air 貯蔵では、*GmCCA1*, *GmLHY*, *GmPRR7*, *GmTOC1* の周期的な発現が減少し、*GmGI* と *GmLUX* の発現にも影響した。さらに、20 °C-15%CO_2 + 20%O_2 貯蔵では、各遺伝子の位相と振幅に深刻な影響を与え、概日時計機能が乱された。しかし、20 °C-5%O_2 貯蔵では、時計遺伝子の振幅のみが影響を受けた。興味深いことに、すべての貯蔵処理が大

豆モヤシのポストハーベスト特性に影響を及ぼした。特に、10 °C-air，20 °C-5%O_2 および 20 °C-15 %CO_2 + 20%O_2 貯蔵では、呼吸速度が減少し、重量減少や褐変の発生などのポストハーベスト品質を維持した。

本論文では、大豆モヤシにおける収穫後の体内時計の変調が品質保持につながることが初めて示唆された。今後、これらの知見が概日リズムの調節による新しい収穫後の品質保持技術に貢献することが期待される。