1 **Employing phytosulfokine α (PSK α) for delaying broccoli florets yellowing**
2 **during cold storage**

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Abstract

During postharvest life, florets yellowing confines broccoli's economic and nutritional value. We investigated the mechanisms employed by 150 nM phytosulfokine α (PSK α) for delaying florets yellowing in broccoli during cold storage. Our results showed that SUMO E3 ligase (*SIZ1*) gene expression was higher in florets treated with PSK α , which may prevent endogenous H₂O₂ accumulation, resulting from the higher activity of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. Besides, higher expression of *methionine sulfoxide reductase* and *cysteine peroxiredoxin* concomitant with lower expression of *heat shock proteins 70/90* genes may arise from higher expression of *SIZ1* gene. Following, lower expression and activity of *phospholipase D* and *lipxygenase* may be liable for membrane integrity protection featured by lower malondialdehyde accumulation in florets treated with PSK α . Additionally, florets treated with PSK α exhibited higher endogenous cytokinin accumulation which may arise from higher expression of *isopentenyl transferase* concomitant with lower expression of *cytokinin oxidase* genes.

Keywords: Heat shock proteins, Methionine sulfoxide reductase, Broccoli yellowing, SUMO E3 ligase SIZ1, Isopentenyl transferase, Cytokinin oxidase

1. Introduction

Because of its higher health-promoting phytochemicals, broccoli (*Brassica oleracea* var. *Italica*) consumption is beneficial for human health because of the high concentrations of bioactive phytochemicals found in the different varieties (Duarte-Sierra, Nadeau, Angers, Michaud, & Arul, 2019). Broccoli is cultivated for its inflorescences that are harvested before sepal aperture, i.e., while they are still immature. In commercially broccoli cultivation, premature harvesting of inflorescences accelerates broccoli senescence, which leads to floret yellowing, limiting shelf-life and commercial value (Aghdam & Alikhani-Koupaei, 2021; Aghdam & Luo, 2021; Aghdam, Sayyari, & Luo, 2020; Cai et al., 2019; Li et al., 2017; Zheng et al., 2019).

During postharvest life, higher ethylene biosynthesis due to induction of ACC synthase (*ACS*) and ACC oxidase (*ACO*) genes expression and enzymes activity may be pivotal for triggering floret yellowing by promoting membrane phospholipids degradation by induction of phospholipase D (*PLD*) gene expression and enzyme activity, leading to free unsaturated fatty acids production, being the substrate for peroxidation by induced lipoxygenase (*LOX*) gene expression and enzyme activity (Cai et al., 2019; Gomez-Lobato, Civello, & Martínez, 2012). *LOX* enzyme activity is not only responsible for membrane unsaturated fatty acids peroxidation, but it also is responsible for O_2^- production during senescence and stresses. Besides, electron leakage from electron transport systems activity in mitochondria and chloroplast or induction of NADPH oxidase enzyme activity in the plasma membrane may be responsible for intracellular reactive oxygen species (ROS) overproduction (Aghdam, Jannatizadeh, Luo, & Paliyath, 2018; Luo, Li, Du, & Mou, 2015; Wang, Li, Li, Li, & Luo, 2021). Membrane free unsaturated fatty acids non-enzymatically peroxidation by ROS accumulation or enzymatically peroxidation by *LOX* activity is responsible for the deterioration of membrane fluidity and integrity reflected by a higher malondialdehyde (MDA) accumulation (Gomez-Lobato et al., 2012; Shi, Gao, Zuo, Wang, Wang, & Fan, 2016). Also, higher *LOX* activity is pivotal for endogenous jasmonic acid accumulation causing the acceleration of broccoli floret yellowing by chlorophylls degradation via triggering pheophorbide a oxygenase (*PaO*) pathway activity (Fang et al., 2020; Gomez-Lobato et al., 2012).

Recently, exogenous cytokinin application and endogenous cytokinin accumulation in transgenic broccoli harboring an isopentenyl transferase (*IPT*) gene have proved that cytokinin serves as a promising anti-senescence molecule in broccoli by preventing chlorophyll degradation, inducing

ROS scavenging systems, suppressing ethylene biosynthesis, promoting heat shock proteins (*HSPs*) genes expression and proteins accumulation and finally inhibiting membrane deterioration because of lower *PLD* and *LOX* genes expression and proteins accumulation (Liu, Li, Lai, Lo, & Chen, 2013). Therefore, making use of strategies for suppression of ethylene biosynthesis along with the promotion of cytokinin biosynthesis as anti-senescence molecules, hampering *PLD* and *LOX* genes expression and enzymes activity, promoting ROS avoiding and scavenging systems activity, concurrent with *HSPs* genes expression and proteins accumulation may be beneficial for the suppression of floret yellowing in broccoli during postharvest life. In recent years, promising efforts have been done to suppress floret yellowing in broccoli by recruiting brassinolide (Cai et al., 2019), putrescine (Zheng et al., 2019), and cytokinins (Reyes Jara, Gómez-Lobato, Civello, & Martínez, 2019) to suppress chlorophyll degradation through PaO pathway inhibition, suppress ethylene biosynthesis alongside promoting cytokinin biosynthesis, inducing ROS scavenging systems and assuring sufficient intracellular ATP availability for maintenance of membrane integrity featured by reduced electrolyte leakage and MDA accumulation.

Recently, it has been observed that activation of cysteine sumoylation by the promotion of SUMO E3 ligase (*SIZ1*) gene expression and enzyme activity may be beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation, by inducing ROS avoiding alternative oxidase and ROS scavenging superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*) and glutathione reductase (*GR*) genes expression and enzymes activity concurrent with promoting methionine sulfoxide reductase (*MSR*) gene expression, suppression of *PLD* and *LOX* genes expression and enzymes activity and promoting endogenous γ -aminobutyric acid and proline accumulation (Jiao, Chai, & Duan, 2019; Jiao & Duan, 2019).

Phytosulfokine α (PSK α ; Tyr(SO₃H)-Ile-Tyr(SO₃H)-Thr-Gln) has been recognized as a signaling pentapeptide implicated in plant development and response to stresses (Sauter 2015). By employing exogenous PSK α application, cytosolic second messenger guanosine 3',5'-cyclic monophosphate (cGMP) accumulation arising from endogenous PSK α signaling may be responsible for triggering friendly extracellular ATP and intracellular SnRK1 signaling pathways, triggering SIZ1 system activity accompanying by suppressing NAD⁺ dissipating poly(ADP-Ribose) polymerase 1 (PARP1) system activity for ensuring sufficient intracellular

ATP and NADPH supplying, triggering ROS avoiding and scavenging systems activity, promoting phenylpropanoid pathway activity, promoting endogenous melatonin accumulation, and improving DPPH, FRAP and DPPH scavenging capacity arising from higher phenols, flavonoids, anthocyanins and ascorbic acid accumulation may be responsible for attenuating chilling injury and fungal decay, delaying senescence and maintaining nutritional quality of fruits and vegetables during cold storage (Aghdam & Alikhani-Koupaei, 2021; Aghdam, Flores, & Sedaghati, 2021; Aghdam & Luo, 2021; Aghdam et al., 2020; Aghdam, Sayyari, & Luo, 2021; Song et al., 2017; Zhang et al., 2018).

This study was conducted to determine the link between exogenous PSK α application and broccoli floret yellowing and to elucidate the mechanisms from the perspective of *SIZ1* gene expression associated with ROS scavenging genes expression and enzymes activity, membrane deteriorating *PLD* and *LOX* genes expression, and enzymes activity, *HSP70* and *HSP90* genes expression, and endogenous cytokinin accumulation correlated with *IPT* and cytokinin oxidase (*CKO*) genes expression during storage at 4 °C for 28 days.

2. Materials and methods

2.1. Broccoli florets and PSK α treatment

Fresh broccoli (*Brassica oleracea* var. Italica) heads were harvested at commercial maturity when individual floret was still closed and dark green, and the inflorescence was compact, from a local vegetable farm in Karaj, Iran, packaged in polystyrene boxes covered with ice and immediately transported to the laboratory within three hours. Then, disease- and mechanical damage-free broccoli heads with uniform size (15–20 cm diameter), color, and stage of maturity, were selected for PSK α treatment. PSK α (soluble in sterile water; 1 mg/mL) was provided by Pepmic Co., Ltd, Suzhou, China. For PSK α treatment, 240 broccoli heads were allocated into 2 groups of 120 (40 heads per replicate) for the experimental treatments, applied by spraying heads with 25 mL of PSK α at 0 (control; double distilled water; ddH₂O) or 150 nM according to Aghdam et al. (2020). After drying overnight, the product was packaged in polyethylene bags (50 × 80 cm, 0.04 mm) and stored at 4 ± 0.5 °C and 85 % relative humidity for 28 days. Floret yellowing was assessed every 7 days during storage at 4 °C, then the floret of 10 broccolis was excised, immediately frozen in liquid nitrogen, powdered, and stored at –80 °C for biochemical and gene expression analysis. For biochemical and gene expression analysis, three technical

replications were carried out by three extractions from each biological replication to avoid instrumental error. The means of three technical replications are considered as one biological replication.

2.2. Broccoli floret yellowing

Broccoli floret yellowing (%) was assessed by an arbitrary scale from 0 to 9 according to Shi et al. (2016), where value 0 indicates 100 % surface was dark green, value 1 25 % yellowing, value 5 50 %, value 7 75 %, and value 9 indicates 100 % yellowing of broccoli floret.

2.3. Membrane integrity assay by MDA accumulation

For MDA accumulation analysis the thiobarbituric acid (TBA) assay was used as described by Hodges, DeLong, Forney, and Prange (1999), one gram of frozen powder was homogenized with 15 mL of 5 % (w/v) trichloroacetic acid (TCA). MDA accumulation was expressed in mmol kg^{-1} on a fresh weight basis.

2.4. ROS scavenging enzymes activity and endogenous H_2O_2 accumulation

For SOD, CAT, APX, and GR enzymes activity determination, one gram of frozen powder was homogenized with 20 mL of 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA and 2 % PVP. SOD, CAT, and APX enzymes activity were analysed according to Giannopolitis and Ries (1977), Beers and Sizer (1952), and Nakano and Asada (1981), respectively. GR enzyme activity was analysed according to Sofo, Tuzio, Dichio, and Xiloyannis (2005). SOD, CAT, APX, and GR enzymes activity were expressed in units of enzymatic activity per kg of protein, U kg^{-1} . For the analysis of endogenous H_2O_2 accumulation, the titanium (IV) methodology was used, according to Patterson, MacRae, and Ferguson (1984), one gram of frozen powder was homogenized with 5 mL of acetone at 0 °C. Endogenous H_2O_2 accumulation was expressed in $\mu\text{mol kg}^{-1}$ on a fresh weight basis.

2.5. Membrane deteriorating PLD and LOX enzymes activity

PLD enzyme activity was assayed by the choline Reinecke salt precipitation technique, according to Suttle and Kende (1980), one gram of frozen powder was homogenized with 10 mL of 50 mM Tris-HCl (pH 8.0) containing 10 mM KCl, 200 mM sucrose, 0.5 mM PMSF and 2 %

PVP (w/v). For substrate emulsion preparation 1,3-phosphatidylcholine was used according to Sajdok, Jandus, Valentová, Novotná, Káš, and Daussant (1995). LOX enzyme activity was analysed according to Phetsirikoon, Ketsa, and van Doorn (2012), one gram of frozen powder was homogenized with 10 ml of 100 mM phosphate buffer (pH 8.0) containing 2 % PVP (w/v). After centrifugation at 10,000 ×g for 30 min at 4 °C, the supernatant was used for LOX assay. PLD and LOX enzymes activity were expressed in units of enzymatic activity per kg of protein, U kg⁻¹.

2.6. Endogenous cytokinin accumulation

For endogenous cytokinin accumulation analysis, one gram of frozen powder was homogenized in 10 mL of phosphate-buffered saline (pH 7.2). After centrifugation at 12,000 × g for 20 min at 4 °C, the supernatant was collected for measurement of endogenous cytokinin at 450 nm by an ELISA Kit (Shanghai YuDuoBio, Shanghai, China) following the manufacturer's protocol. Endogenous cytokinin accumulation was expressed as zeatin riboside (ZR) in nmol kg⁻¹ on a fresh weight basis.

2.7. Genes expression assay by RT-qPCR

Total RNA extraction and cDNA synthesis were performed as described by Shi et al. (2016). Gene expression was measured by StepOne Real-Time PCR System. Primers used for the amplification of the genes sequences are shown in Table 1. The genes relative expression was quantified by normalization of the threshold cycle (Ct) value to Actin Ct value and calculated by the formula $2^{-\Delta\Delta C_t}$ according to Livak and Schmittgen (2001).

2.8. Statistical analysis

The experiment was planned using split plots in a time model based on a completely randomized design (CRD). For biochemical and genes expression analysis, three technical replications were carried out by three extractions from each biological replication to avoid instrumental error. The means of three technical replications are considered as one biological replication. All data were expressed as means ± standard errors (SE) from three biological replications. ANOVA was carried out, and means were compared using Tukey's test at the significance level of 0.01 with SPSS software (Version 19.0).

3. Results

3.1. Broccoli floret yellowing, MDA, and H₂O₂ accumulation

As shown in Supplementary Fig. 1, broccoli treated with PSK α at 150 nM exhibited remarkably lower floret yellowing, reaching 65 % of yellowing inhibition concerning control (non-treated broccoli), after 28 days of cold storage ($P < 0.01$). During cold storage, broccoli floret yellowing was correlated with higher MDA and H₂O₂ accumulation (Fig. 1A, B). However, broccoli floret treated with PSK α at 150 nM exhibited significantly lower MDA and H₂O₂ accumulation ($P < 0.01$; Fig. 1A, B) during cold storage compared with non-treated broccoli floret.

3.2. ROS enzymatic scavenging systems activity and endogenous H₂O₂ accumulation

As depicted in Fig. 2A-D, broccoli floret exhibited significantly higher SOD, CAT, APX, and GR ROS-scavenging enzymes activity ($P < 0.01$), which may be responsible for lower H₂O₂ accumulation in broccoli floret treated with PSK α at 150 nM during cold storage (Fig. 1B). Concurrent with higher SOD, CAT, APX, and GR activities, broccoli floret treated with PSK α at 150 nM exhibited higher *MSR* and cysteine peroxiredoxin (*CysPrx*) genes expression ($P < 0.01$; Fig. 2E, F) during cold storage compared with non-treated broccoli floret, which may contribute to lower H₂O₂ accumulation (Fig. 1B).

3.3. HSPs genes expression governed by *SIZ1* gene expression

As shown in Fig. 3A, broccoli floret treated with PSK α at 150 nM exhibited a significantly higher level of *SIZ1* expression ($P < 0.01$) during cold storage. Concurrent with this higher expression, PSK α -treated broccoli floret also exhibited significantly higher *HSP70* and *HSP90* genes expression ($P < 0.01$; Fig. 3B, C) during cold storage.

3.4. Membrane deteriorating *PLD* and *LOX* enzymes activity

During cold storage, higher MDA accumulation in broccoli floret (Fig. 1A) was associated with higher *PLD* and *LOX* genes expression (Fig. 5B, D) and enzymes activity (Fig. 4A, C). But, as depicted in Fig. 5, broccoli floret treated with 150 nM PSK α exhibited significantly lower *PLD* and *LOX* genes expression and enzymes activity ($P < 0.01$; Fig. 4A-D) during cold storage when compared with non-treated broccoli floret.

3.5. Endogenous cytokinin accumulation

As depicted in Fig. 5A, falling endogenous zeatin riboside cytokinin accumulation in broccoli floret during cold storage might be ascribed to the diminution of *IPT* expression (Fig. 5B) concurrent with rising *CKO* expression (Fig. 5C). Significantly higher endogenous cytokinin accumulation ($P < 0.01$; Fig. 5A) in broccoli floret treated with PSK α at 150 nM may result from significantly higher *IPT* expression levels concurrent with significantly lower *CKO* expression levels ($P < 0.01$; Fig. 5B, C) during cold storage compared with non-treated broccoli floret.

4. Discussion

Recently, Aghdam et al. (2020) reported that employing PSK α at 0, 75, 150, 225, and 300 nM retarded the yellowing of broccoli florets by 0, 25, 65, 32, and 14 %, respectively, during cold storage. These results indicated that the broccoli treated with PSK α at 150 nM exhibited the lowest florets yellowing during cold storage. However, the retarding effects of PSK α on florets yellowing in broccoli decreased by increasing PSK α concentration from 150 to 300 nM. However, broccoli treated with PSK α at 250 and 300 nM exhibited significantly lower florets yellowing in comparison with control florets during cold storage. As regards to PSK α function as bioactive signaling peptide hormone in plants, promoting florets yellowing in broccoli florets by increasing PSK α concentration from 150 to 300 nM may be ascribed to disruption endogenous PSK α signaling by PSK α receptor (PSKR1) oversaturation. But, future investigation is required for discovering the endogenous PSK α signaling in florets in detail. In the present study, higher MDA accumulation in broccoli floret arising from higher H₂O₂ accumulation (Figs. 1A, 1B) during storage at 4 °C for 28 days indicated that broccoli floret suffered from oxidative stress which may be ascribed to ineffective ROS scavenging activity. Broccoli floret during cold storage at 4 °C for 28 days induced *SIZ1* gene expression as a protective mechanism for overcoming oxidative stress (Fig. 3A). However, higher H₂O₂ and MDA accumulation in broccoli floret denoted insufficient efficiency of *SIZ1* gene expression to overcome oxidative stress. In broccoli floret treated with PSK α at 150 nM lower H₂O₂ and MDA accumulation was concomitant with higher *SIZ1* gene expression during cold storage (Fig. 1A, 1B and 3A). Therefore, we can assume that exogenous PSK α applied at 150 nM promoted the efficiency of *SIZ1* gene expression to overcome oxidative stress. SUMO E3 ligase SIZ1 is pivotal for proteins

SUMOylation as a post-translational mechanism recruited by plants during their response to senescence and stresses. Cysteine sumoylation by SUMO E3 ligase SIZ1 enzyme is pivotal for protein stability and functionality (Morrell & Sadanandom, 2019). Zhang et al. (2018) reported that overexpression of *SIZ1* gene conferred thermotolerance in tomato plant by activation of HSFA1 leading to *HSP70* and *HSP90* genes expression induction and proteins accumulation concurrent with higher *APX* gene expression and enzyme activity, all these processes being beneficial for the maintenance of membrane integrity featured by lower MDA accumulation due to lower O_2^- and H_2O_2 accumulation.

Also, conferring chilling tolerance in peach fruit by exogenous nitric oxide treatment might be ascribed to endogenous signaling molecule IP_3 accumulation leading to induction of *SIZ1* expression, which in its turn it is involved in the induction of SOD, CAT, APX, and GR genes expression and enzymes activity concurrent with the promotion of *MSR* gene expression, and suppression of *PLD* and *LOX* genes expression and enzymes activity, all of these processes being beneficial also for maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation (Jiao et al., 2019; Jiao & Duan, 2019). Hence, overcoming oxidative stress by higher *SIZ1* gene expression in broccoli floret treated with $PSK\alpha$ at 150 nM featured by lower H_2O_2 and MDA accumulation may arise from activation of ROS scavenging enzymes activity (SOD, CAT, APX, and GR), promotion of *HSP70*, and *HSP90* genes expression, and hampering membrane deteriorating *PLD* and *LOX* genes expression and enzymes activity (Figs. 1A, 1B, 2A-D, 3A-C, 4A-D).

In plants, chloroplast, mitochondria, peroxisome, and plasma membrane are responsible for ROS production during senescence and stresses. By an array of antioxidant enzymes such as SOD, CAT, APX, and GR, plants exhibit their capability to overcome oxidative stress (Aghdam et al., 2018). By O_2^- dismutation to H_2O_2 , SOD serves as the first-line in ROS scavenging. Next, APX, CAT, and GR are responsible for H_2O_2 scavenging (Aghdam et al., 2018). Suppressing broccoli floret yellowing by application of exogenous putrescine (Zheng et al., 2019) and 6-benzylaminopurine (Xu, Yang, Chen, Jin, Wang, & Zheng, 2012) might be ascribed to higher *SOD*, *CAT*, *APX*, and *GR* genes expression and enzymes activity along with lower *LOX* gene expression and enzyme activity, leading to lower O_2^- and H_2O_2 accumulation, which is beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation. In addition to ascorbate/glutathione (AA/GSH) cycle activity, *MSR* and *CysPrx*

genes expression and enzymes activity may be beneficial to surmount oxidative stress. During senescence and stresses, methionine (Met) oxidation to methionine sulfoxide (MetO) due to ROS accumulation entails deterioration of protein stability and functionality. In plants, MSR activity is pivotal to maintain redox stability of methionine-rich proteins in response to ROS accumulation by Met recovery from MetO. In addition to MSR, CysPrx activity in the cytoplasm, chloroplast and mitochondria are beneficial for H₂O₂ scavenging during senescence and stresses. NADPH-dependent thioredoxin reductase (NTR) activity is responsible for providing functional thioredoxin for the promotion of MSR and CysPrx activity during senescence and stresses (Rey & Tarrago, 2018). Chumyam, Faiyue, and Saengnil (2019) reported that browning alleviation in fresh-cut guava fruit treated with H₂O₂ during storage at 25 °C for 2 days is due to the promotion of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes, which is pivotal for the supply of sufficient intracellular NADPH to trigger NTR activity leading to higher CysPrx activity, both being beneficial for lowering H₂O₂ accumulation alongside with maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation. Therefore, we can assume that the higher SOD, CAT, APX, and GR activities alongside with higher *MSR* and *CysPrx* genes expression in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *SIZ1* gene expression during cold storage, which is pivotal for reduction of H₂O₂ accumulation and maintenance of membrane integrity featured by lower MDA accumulation during cold storage (Figs. 1A, 1B, 2A-F, 3A).

In addition to ROS scavenging systems activity, HSPs serve as molecular chaperones rendering evident beneficial effects on protein synthesis, renaturalization, and stabilization. In addition to serving as molecular chaperones, HSPs exhibit ROS scavenging capacity, which is beneficial for the maintenance of membrane fluidity and integrity. Also, HSPs binding to membrane phospholipids and galactolipids is beneficial for the maintenance of membrane fluidity and integrity (Aghdam, Sevillano, Flores, & Bodbodak, 2013). During postharvest senescence and stresses, PLD is responsible for the hydrolysis of phosphatidylcholine and phosphatidylethanolamine releasing choline and ethanolamine, respectively, and producing phosphatidic acid. Phosphatidic acid phosphatase enzyme activity is responsible for diacylglycerol production from phosphatidic acid for lipolytic acyl hydrolase enzyme activity providing free fatty acids for enzymatic peroxidation by LOX or non-enzymatically by ROS giving rise to MDA production (Aghdam et al., 2018). Hence, it can be assumed that higher

HSP70 and *HSP90* genes expression accompanied by lower *PLD* and *LOX* genes expression and enzymes activity in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *SIZ1* gene expression during cold storage, which is pivotal for maintenance of membrane integrity featured by lower MDA accumulation during cold storage (Fig. 1A, 3A-C, 4A-D).

Chloroplastic methylerythritol 4- phosphate (MEP) pathway is pivotal for isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) production, the substrates of IPT, the enzyme involved in cytokinins biosynthesis in the chloroplast (Sakakibara 2006). In addition to cytokinin biosynthesis by IPT activity, cytokinin deactivation by CKO is pivotal for the maintenance of intracellular cytokinin homeostasis. According to Gapper (2003), ethylene and cytokinin antagonistically regulate broccoli floret senescence during postharvest life. Suppressing postharvest senescence in broccoli floret may arise from higher endogenous cytokinin accumulation due to higher *IPT* gene expression concurrent with lower *CKO* gene expression, which may have a beneficial effect in blocking ethylene biosynthesis and signaling. On the other hand, higher *CKO* gene expression combined with lower *IPT* gene expression and therefore lower endogenous cytokinin accumulation in broccoli floret accelerates postharvest senescence by triggering ethylene biosynthesis and action (Gapper, Coupe, McKenzie, Sinclair, Lill, & Jameson, 2005). Exogenous cytokinin has been applied to inhibit senescence in broccoli shown up by floret yellowing. This strategy suppresses chlorophyll degradation by chlorophyllase and Mg²⁺ dechelataase, promoting SOD, CAT, and APX activities leading to lower O₂⁻ and H₂O₂ accumulation, which is beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation, concurrent with higher phenols accumulation leading to higher DPPH scavenging capacity (Xu et al., 2012). According to our results and considering the findings from other authors described above, higher endogenous cytokinin zeatin riboside accumulation in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *IPT* gene expression concurrent with lower *CKO* gene expression during cold storage (Fig. 5A-C). Therefore, endogenous cytokinin zeatin riboside accumulation in broccoli floret treated with PSK α at 150 nM may be pivotal in delaying senescence shown up by floret yellowing during cold storage.

5. Conclusion

In summary, higher *SIZ1* expression in broccoli floret treated with 150 nM PSK α may be pivotal for higher SOD, CAT, APX, and GR activities concurrent with higher *MSR* and *CysPrx* expressions leading to lower H₂O₂ accumulation. Lower H₂O₂ accumulation due to higher activity of ROS scavenging systems concurrent with lower PLD and LOX genes expression and enzymes activity alongside higher *HSP70* and *HSP90* genes expression in broccoli floret treated with 150 nM PSK α may be beneficial for the maintenance of membrane integrity featured by lower MDA accumulation. In addition to oxidative stress alleviation by the promotion of ROS scavenging systems, higher endogenous cytokinin zeatin riboside accumulation due to higher *IPT*/lower *CKO* genes expression may be beneficial in senescence delay manifested by lower floret yellowing in broccoli treated with 150 nM PSK α . Our findings may permit the horticultural industry to take advantage of a promising postharvest procedure for the preservation of broccoli remarkably extending its postharvest life.

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Conflict of interest: The authors declare that they have no conflict of interest.

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474 **Table caption:**

475 **Table. 1.** Primers used for qRT-PCR.

Gene name	Functional annotations	Primer sequences (5'-3')
<i>SIZ1</i>	Proteins sumoylation	F: GCGAGGCTACTTCTGCAAAC R: CCGAGGTCTTACAGAACGCT
<i>PLD</i>	Membrane deterioration	F: CGCATGTCACTCTGGTACGAA R: TCGCTCGAGTATAAGTCCCAA
<i>LOX</i>	Membrane deterioration	F: TGTCCAGAACGACACTGAGC R: CGCTGCATGTAGAGCAGAAG
<i>HSP70</i>	Chaperoning activity	F: GTGATCGACGCTGACTTCAC R: TGGACAGCTTCACACATTGC
<i>HSP90</i>	Chaperoning activity	F: GGACCGGGAGAACCATAAGC R: GAATCGTAGCAGGAGAGGGC
<i>MSR</i>	Proteins stability	F: TACAGAGCGGAAAACCTACCAC R: ACCTGACTAGTTACTTAGCCAT
<i>CysPrx</i>	H ₂ O ₂ scavenging	F: ACTTCCGTCCAAGAAAGGCT R: CCTCCAGAGTACACACACGA
<i>IPT</i>	Cytokinin accumulation	F: CTCCTTCCGGGTAGACATGC R: TGCAAGATCGACTGAGAGGC
<i>CKO</i>	Cytokinin accumulation	F: GATGTCCACAATGCGTCCAA R: TCCTTGAAGCGAGTGACCAT
<i>Actin</i>	Housekeeping gene	F: CCAGAGGTCTTGTTCCAGCCATC R: TTCCACCACTGAGCACAATGTTAC

Figures caption:

Fig. 1. MDA (A) and H₂O₂ (B) accumulation in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at *P* = 0.01 level.

Fig. 2. SOD (A), CAT (B), APX (C) and GR (D) enzymes activity accompanying by *MSR* (E) and *CysPrx* (F) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n=3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P=0.01$ level.

Fig. 3. *SIZ1* gene expression (A) accompanying *HSP70* (B) and *HSP90* (C) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Fig. 4. PLD and LOX enzymes activity (A, B) and genes expression (C, D) in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Fig. 5. Endogenous cytokinin ZR accumulation (A) concurrent with *IPT* (B) and *CKO* (C) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

1 **Employing phytosulfokine α (PSK α) for delaying broccoli florets yellowing**
2 **during cold storage**

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Abstract

During postharvest life, florets yellowing confines broccoli's economic and nutritional value. We investigated the mechanisms employed by 150 nM phytosulfokine α (PSK α) for delaying florets yellowing in broccoli during cold storage. Our results showed that SUMO E3 ligase (*SIZ1*) gene expression was higher in florets treated with PSK α , which may prevent endogenous H₂O₂ accumulation, resulting from the higher activity of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. Besides, higher expression of *methionine sulfoxide reductase* and *cysteine peroxiredoxin* concomitant with lower expression of *heat shock proteins 70/90* genes may arise from higher expression of *SIZ1* gene. Following, lower expression and activity of *phospholipase D* and *lipxygenase* may be liable for membrane integrity protection featured by lower malondialdehyde accumulation in florets treated with PSK α . Additionally, florets treated with PSK α exhibited higher endogenous cytokinin accumulation which may arise from higher expression of *isopentenyl transferase* concomitant with lower expression of *cytokinin oxidase* genes.

Keywords: Heat shock proteins, Methionine sulfoxide reductase, Broccoli yellowing, SUMO E3 ligase SIZ1, Isopentenyl transferase, Cytokinin oxidase

1. Introduction

Because of its higher health-promoting phytochemicals, broccoli (*Brassica oleracea* var. *Italica*) consumption is beneficial for human health because of the high concentrations of bioactive phytochemicals found in the different varieties (Duarte-Sierra, Nadeau, Angers, Michaud, & Arul, 2019). Broccoli is cultivated for its inflorescences that are harvested before sepal aperture, i.e., while they are still immature. In commercially broccoli cultivation, premature harvesting of inflorescences accelerates broccoli senescence, which leads to floret yellowing, limiting shelf-life and commercial value (Aghdam & Alikhani-Koupaei, 2021; Aghdam & Luo, 2021; Aghdam, Sayyari, & Luo, 2020; Cai et al., 2019; Li et al., 2017; Zheng et al., 2019).

During postharvest life, higher ethylene biosynthesis due to induction of ACC synthase (ACS) and ACC oxidase (ACO) genes expression and enzymes activity may be pivotal for triggering floret yellowing by promoting membrane phospholipids degradation by induction of phospholipase D (PLD) gene expression and enzyme activity, leading to free unsaturated fatty acids production, being the substrate for peroxidation by induced lipoxygenase (LOX) gene expression and enzyme activity (Cai et al., 2019; Gomez-Lobato, Civello, & Martínez, 2012). LOX enzyme activity is not only responsible for membrane unsaturated fatty acids peroxidation, but it also is responsible for O_2^- production during senescence and stresses. Besides, electron leakage from electron transport systems activity in mitochondria and chloroplast or induction of NADPH oxidase enzyme activity in the plasma membrane may be responsible for intracellular reactive oxygen species (ROS) overproduction (Aghdam, Jannatizadeh, Luo, & Paliyath, 2018; Luo, Li, Du, & Mou, 2015; Wang, Li, Li, Li, & Luo, 2021). Membrane free unsaturated fatty acids non-enzymatically peroxidation by ROS accumulation or enzymatically peroxidation by LOX activity is responsible for the deterioration of membrane fluidity and integrity reflected by a higher malondialdehyde (MDA) accumulation (Gomez-Lobato et al., 2012; Shi, Gao, Zuo, Wang, Wang, & Fan, 2016). Also, higher LOX activity is pivotal for endogenous jasmonic acid accumulation causing the acceleration of broccoli floret yellowing by chlorophylls degradation via triggering pheophorbide a oxygenase (PaO) pathway activity (Fang et al., 2020; Gomez-Lobato et al., 2012).

Recently, exogenous cytokinin application and endogenous cytokinin accumulation in transgenic broccoli harboring an isopentenyl transferase (IPT) gene have proved that cytokinin serves as a promising anti-senescence molecule in broccoli by preventing chlorophyll degradation, inducing

ROS scavenging systems, suppressing ethylene biosynthesis, promoting **heat shock proteins (HSPs)** genes expression and proteins accumulation and finally inhibiting membrane deterioration because of lower *PLD* and *LOX* genes expression and proteins accumulation (Liu, Li, Lai, Lo, & Chen, 2013). Therefore, making use of strategies for suppression of ethylene biosynthesis along with the promotion of cytokinin biosynthesis as anti-senescence molecules, hampering *PLD* and *LOX* genes expression and enzymes activity, promoting ROS avoiding and scavenging systems activity, concurrent with *HSPs* genes expression and proteins accumulation may be beneficial for the suppression of floret yellowing in broccoli during postharvest life. In recent years, promising efforts have been done to suppress floret yellowing in broccoli by recruiting brassinolide (Cai et al., 2019), putrescine (Zheng et al., 2019), and cytokinins (Reyes Jara, Gómez-Lobato, Civello, & Martínez, 2019) to suppress chlorophyll degradation through PaO pathway inhibition, suppress ethylene biosynthesis alongside promoting cytokinin biosynthesis, inducing ROS scavenging systems and assuring sufficient intracellular ATP availability for maintenance of membrane integrity featured by reduced electrolyte leakage and MDA accumulation.

Recently, it has been observed that activation of cysteine sumoylation by the promotion of SUMO E3 ligase (*SIZ1*) gene expression and enzyme activity may be beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation, by inducing ROS avoiding alternative oxidase and ROS scavenging superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*) and glutathione reductase (*GR*) genes expression and enzymes activity concurrent with promoting methionine sulfoxide reductase (*MSR*) gene expression, suppression of *PLD* and *LOX* genes expression and enzymes activity **and promoting endogenous γ -aminobutyric acid and proline accumulation** (Jiao, Chai, & Duan, 2019; Jiao & Duan, 2019).

Phytosulfokine α (PSK α ; Tyr(SO₃H)-Ile-Tyr(SO₃H)-Thr-Gln) has been recognized as a signaling pentapeptide implicated in plant development and response to stresses (Sauter 2015). **By employing exogenous PSK α application, cytosolic second messenger guanosine 3',5'-cyclic monophosphate (cGMP) accumulation arising from endogenous PSK α signaling may be responsible for triggering friendly extracellular ATP and intracellular SnRK1 signaling pathways, triggering SIZ1 system activity accompanying by suppressing NAD⁺ dissipating poly(ADP-Ribose) polymerase 1 (PARP1) system activity for ensuring sufficient intracellular**

ATP and NADPH supplying, triggering ROS avoiding and scavenging systems activity, promoting phenylpropanoid pathway activity, promoting endogenous melatonin accumulation, and improving DPPH, FRAP and DPPH scavenging capacity arising from higher phenols, flavonoids, anthocyanins and ascorbic acid accumulation may be responsible for attenuating chilling injury and fungal decay, delaying senescence and maintaining nutritional quality of fruits and vegetables during cold storage (Aghdam & Alikhani-Koupaei, 2021; Aghdam, Flores, & Sedaghati, 2021; Aghdam & Luo, 2021; Aghdam et al., 2020; Aghdam, Sayyari, & Luo, 2021; Song et al., 2017; Zhang et al., 2018).

This study was conducted to determine the link between exogenous PSK α application and broccoli floret yellowing and to elucidate the mechanisms from the perspective of *SIZ1* gene expression associated with ROS scavenging genes expression and enzymes activity, membrane deteriorating *PLD* and *LOX* genes expression, and enzymes activity, *HSP70* and *HSP90* genes expression, and endogenous cytokinin accumulation correlated with *IPT* and cytokinin oxidase (*CKO*) genes expression during storage at 4 °C for 28 days.

2. Materials and methods

2.1. Broccoli florets and PSK α treatment

Fresh broccoli (*Brassica oleracea* var. *Italica*) heads were harvested at commercial maturity when individual floret was still closed and dark green, and the inflorescence was compact, from a local vegetable farm in Karaj, Iran, packaged in polystyrene boxes covered with ice and immediately transported to the laboratory within three hours. Then, disease- and mechanical damage-free broccoli heads with uniform size (15–20 cm diameter), color, and stage of maturity, were selected for PSK α treatment. PSK α (soluble in sterile water; 1 mg/mL) was provided by Pepmic Co., Ltd, Suzhou, China. For PSK α treatment, 240 broccoli heads were allocated into 2 groups of 120 (40 heads per replicate) for the experimental treatments, applied by spraying heads with 25 mL of PSK α at 0 (control; double distilled water; ddH₂O) or 150 nM according to Aghdam et al. (2020). After drying overnight, the product was packaged in polyethylene bags (50 × 80 cm, 0.04 mm) and stored at 4 ± 0.5 °C and 85 % relative humidity for 28 days. Floret yellowing was assessed every 7 days during storage at 4 °C, then the floret of 10 broccolis was excised, immediately frozen in liquid nitrogen, powdered, and stored at –80 °C for biochemical and gene expression analysis. For biochemical and gene expression analysis, three technical

replications were carried out by three extractions from each biological replication to avoid instrumental error. The means of three technical replications are considered as one biological replication.

2.2. Broccoli floret yellowing

Broccoli floret yellowing (%) was assessed by an arbitrary scale from 0 to 9 according to Shi et al. (2016), where value 0 indicates 100 % surface was dark green, value 1 25 % yellowing, value 5 50 %, value 7 75 %, and value 9 indicates 100 % yellowing of broccoli floret.

2.3. Membrane integrity assay by MDA accumulation

For MDA accumulation analysis the thiobarbituric acid (TBA) assay was used as described by Hodges, DeLong, Forney, and Prange (1999), one gram of frozen powder was homogenized with 15 mL of 5 % (w/v) trichloroacetic acid (TCA). MDA accumulation was expressed in mmol kg⁻¹ on a fresh weight basis.

2.4. ROS scavenging enzymes activity and endogenous H₂O₂ accumulation

For SOD, CAT, APX, and GR enzymes activity determination, one gram of frozen powder was homogenized with 20 mL of 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA and 2 % PVP. SOD, CAT, and APX enzymes activity were analysed according to Giannopolitis and Ries (1977), Beers and Sizer (1952), and Nakano and Asada (1981), respectively. GR enzyme activity was analysed according to Sofo, Tuzio, Dichio, and Xiloyannis (2005). SOD, CAT, APX, and GR enzymes activity were expressed in units of enzymatic activity per kg of protein, U kg⁻¹. For the analysis of endogenous H₂O₂ accumulation, the titanium (IV) methodology was used, according to Patterson, MacRae, and Ferguson (1984), one gram of frozen powder was homogenized with 5 mL of acetone at 0 °C. Endogenous H₂O₂ accumulation was expressed in μmol kg⁻¹ on a fresh weight basis.

2.5. Membrane deteriorating PLD and LOX enzymes activity

PLD enzyme activity was assayed by the choline Reinecke salt precipitation technique, according to Suttle and Kende (1980), one gram of frozen powder was homogenized with 10 mL of 50 mM Tris-HCl (pH 8.0) containing 10 mM KCl, 200 mM sucrose, 0.5 mM PMSF and 2 %

PVP (w/v). For substrate emulsion preparation 1,3-phosphatidylcholine was used according to Sajdok, Jandus, Valentová, Novotná, Káš, and Daussant (1995). LOX enzyme activity was analysed according to Phetsirikoon, Ketsa, and van Doorn (2012), one gram of frozen powder was homogenized with 10 ml of 100 mM phosphate buffer (pH 8.0) containing 2 % PVP (w/v). After centrifugation at 10,000 ×g for 30 min at 4 °C, the supernatant was used for LOX assay. PLD and LOX enzymes activity were expressed in units of enzymatic activity per kg of protein, U kg⁻¹.

2.6. Endogenous cytokinin accumulation

For endogenous cytokinin accumulation analysis, one gram of frozen powder was homogenized in 10 mL of phosphate-buffered saline (pH 7.2). After centrifugation at 12,000 × g for 20 min at 4 °C, the supernatant was collected for measurement of endogenous cytokinin at 450 nm by an ELISA Kit (Shanghai YuDuoBio, Shanghai, China) following the manufacturer's protocol. Endogenous cytokinin accumulation was expressed as zeatin riboside (ZR) in nmol kg⁻¹ on a fresh weight basis.

2.7. Genes expression assay by RT-qPCR

Total RNA extraction and cDNA synthesis were performed as described by Shi et al. (2016). Gene expression was measured by StepOne Real-Time PCR System. Primers used for the amplification of the genes sequences are shown in Table 1. The genes relative expression was quantified by normalization of the threshold cycle (Ct) value to Actin Ct value and calculated by the formula $2^{-\Delta\Delta C_t}$ according to Livak and Schmittgen (2001).

2.8. Statistical analysis

The experiment was planned using split plots in a time model based on a completely randomized design (CRD). For biochemical and genes expression analysis, three technical replications were carried out by three extractions from each biological replication to avoid instrumental error. The means of three technical replications are considered as one biological replication. All data were expressed as means ± standard errors (SE) from three biological replications. ANOVA was carried out, and means were compared using Tukey's test at the significance level of 0.01 with SPSS software (Version 19.0).

3. Results

3.1. Broccoli floret yellowing, MDA, and H₂O₂ accumulation

As shown in Supplementary Fig. 1, broccoli treated with PSK α at 150 nM exhibited remarkably lower floret yellowing, reaching 65 % of yellowing inhibition concerning control (non-treated broccoli), after 28 days of cold storage ($P < 0.01$). During cold storage, broccoli floret yellowing was correlated with higher MDA and H₂O₂ accumulation (Fig. 1A, B). However, broccoli floret treated with PSK α at 150 nM exhibited significantly lower MDA and H₂O₂ accumulation ($P < 0.01$; Fig. 1A, B) during cold storage compared with non-treated broccoli floret.

3.2. ROS enzymatic scavenging systems activity and endogenous H₂O₂ accumulation

As depicted in Fig. 2A-D, broccoli floret exhibited significantly higher SOD, CAT, APX, and GR ROS-scavenging enzymes activity ($P < 0.01$), which may be responsible for lower H₂O₂ accumulation in broccoli floret treated with PSK α at 150 nM during cold storage (Fig. 1B). Concurrent with higher SOD, CAT, APX, and GR activities, broccoli floret treated with PSK α at 150 nM exhibited higher *MSR* and *cysteine peroxiredoxin (CysPrx)* genes expression ($P < 0.01$; Fig. 2E, F) during cold storage compared with non-treated broccoli floret, which may contribute to lower H₂O₂ accumulation (Fig. 1B).

3.3. HSPs genes expression governed by *SIZ1* gene expression

As shown in Fig. 3A, broccoli floret treated with PSK α at 150 nM exhibited a significantly higher level of *SIZ1* expression ($P < 0.01$) during cold storage. Concurrent with this higher expression, PSK α -treated broccoli floret also exhibited significantly higher *HSP70* and *HSP90* genes expression ($P < 0.01$; Fig. 3B, C) during cold storage.

3.4. Membrane deteriorating *PLD* and *LOX* enzymes activity

During cold storage, higher MDA accumulation in broccoli floret (Fig. 1A) was associated with higher *PLD* and *LOX* genes expression (Fig. 5B, D) and enzymes activity (Fig. 4A, C). But, as depicted in Fig. 5, broccoli floret treated with 150 nM PSK α exhibited significantly lower *PLD* and *LOX* genes expression and enzymes activity ($P < 0.01$; Fig. 4A-D) during cold storage when compared with non-treated broccoli floret.

3.5. Endogenous cytokinin accumulation

As depicted in Fig. 5A, falling endogenous zeatin riboside cytokinin accumulation in broccoli floret during cold storage might be ascribed to the diminution of *IPT* expression (Fig. 5B) concurrent with rising *CKO* expression (Fig. 5C). Significantly higher endogenous cytokinin accumulation ($P < 0.01$; Fig. 5A) in broccoli floret treated with PSK α at 150 nM may result from significantly higher *IPT* expression levels concurrent with significantly lower *CKO* expression levels ($P < 0.01$; Fig. 5B, C) during cold storage compared with non-treated broccoli floret.

4. Discussion

Recently, Aghdam et al. (2020) reported that employing PSK α at 0, 75, 150, 225, and 300 nM retarded the yellowing of broccoli florets by 0, 25, 65, 32, and 14 %, respectively, during cold storage. These results indicated that the broccoli treated with PSK α at 150 nM exhibited the lowest florets yellowing during cold storage. However, the retarding effects of PSK α on florets yellowing in broccoli decreased by increasing PSK α concentration from 150 to 300 nM. However, broccoli treated with PSK α at 250 and 300 nM exhibited significantly lower florets yellowing in comparison with control florets during cold storage. As regards to PSK α function as bioactive signaling peptide hormone in plants, promoting florets yellowing in broccoli florets by increasing PSK α concentration from 150 to 300 nM may be ascribed to disruption endogenous PSK α signaling by PSK α receptor (PSKR1) oversaturation. But, future investigation is required for discovering the endogenous PSK α signaling in florets in detail. In the present study, higher MDA accumulation in broccoli floret arising from higher H₂O₂ accumulation (Figs. 1A, 1B) during storage at 4 °C for 28 days indicated that broccoli floret suffered from oxidative stress which may be ascribed to ineffective ROS scavenging activity. Broccoli floret during cold storage at 4 °C for 28 days induced *SIZ1* gene expression as a protective mechanism for overcoming oxidative stress (Fig. 3A). However, higher H₂O₂ and MDA accumulation in broccoli floret denoted insufficient efficiency of *SIZ1* gene expression to overcome oxidative stress. In broccoli floret treated with PSK α at 150 nM lower H₂O₂ and MDA accumulation was concomitant with higher *SIZ1* gene expression during cold storage (Fig. 1A, 1B and 3A). Therefore, we can assume that exogenous PSK α applied at 150 nM promoted the efficiency of *SIZ1* gene expression to overcome oxidative stress. SUMO E3 ligase SIZ1 is pivotal for proteins

SUMOylation as a post-translational mechanism recruited by plants during their response to senescence and stresses. Cysteine sumoylation by SUMO E3 ligase SIZ1 enzyme is pivotal for protein stability and functionality (Morrell & Sadanandom, 2019). Zhang et al. (2018) reported that overexpression of *SIZ1* gene conferred thermotolerance in tomato plant by activation of HSFA1 leading to *HSP70* and *HSP90* genes expression induction and proteins accumulation concurrent with higher *APX* gene expression and enzyme activity, all these processes being beneficial for the maintenance of membrane integrity featured by lower MDA accumulation due to lower O_2^- and H_2O_2 accumulation.

Also, conferring chilling tolerance in peach fruit by exogenous nitric oxide treatment might be ascribed to endogenous signaling molecule IP_3 accumulation leading to induction of *SIZ1* expression, which in its turn it is involved in the induction of SOD, CAT, APX, and GR genes expression and enzymes activity concurrent with the promotion of *MSR* gene expression, and suppression of *PLD* and *LOX* genes expression and enzymes activity, all of these processes being beneficial also for maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation (Jiao et al., 2019; Jiao & Duan, 2019). Hence, overcoming oxidative stress by higher *SIZ1* gene expression in broccoli floret treated with $PSK\alpha$ at 150 nM featured by lower H_2O_2 and MDA accumulation may arise from activation of ROS scavenging enzymes activity (SOD, CAT, APX, and GR), promotion of *HSP70*, and *HSP90* genes expression, and hampering membrane deteriorating *PLD* and *LOX* genes expression and enzymes activity (Figs. 1A, 1B, 2A-D, 3A-C, 4A-D).

In plants, chloroplast, mitochondria, peroxisome, and plasma membrane are responsible for ROS production during senescence and stresses. By an array of antioxidant enzymes such as SOD, CAT, APX, and GR, plants exhibit their capability to overcome oxidative stress (Aghdam et al., 2018). By O_2^- dismutation to H_2O_2 , SOD serves as the first-line in ROS scavenging. Next, APX, CAT, and GR are responsible for H_2O_2 scavenging (Aghdam et al., 2018). Suppressing broccoli floret yellowing by application of exogenous putrescine (Zheng et al., 2019) and 6-benzylaminopurine (Xu, Yang, Chen, Jin, Wang, & Zheng, 2012) might be ascribed to higher *SOD*, *CAT*, *APX*, and *GR* genes expression and enzymes activity along with lower *LOX* gene expression and enzyme activity, leading to lower O_2^- and H_2O_2 accumulation, which is beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation. In addition to ascorbate/glutathione (AA/GSH) cycle activity, *MSR* and *CysPrx*

genes expression and enzymes activity may be beneficial to surmount oxidative stress. During senescence and stresses, methionine (Met) oxidation to methionine sulfoxide (MetO) due to ROS accumulation entails deterioration of protein stability and functionality. In plants, MSR activity is pivotal to maintain redox stability of methionine-rich proteins in response to ROS accumulation by Met recovery from MetO. In addition to MSR, CysPrx activity in the cytoplasm, chloroplast and mitochondria are beneficial for H₂O₂ scavenging during senescence and stresses. NADPH-dependent thioredoxin reductase (NTR) activity is responsible for providing functional thioredoxin for the promotion of MSR and CysPrx activity during senescence and stresses (Rey & Tarrago, 2018). Chumyam, Faiyue, and Saengnil (2019) reported that browning alleviation in fresh-cut guava fruit treated with H₂O₂ during storage at 25 °C for 2 days is due to the promotion of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes, which is pivotal for the supply of sufficient intracellular NADPH to trigger NTR activity leading to higher CysPrx activity, both being beneficial for lowering H₂O₂ accumulation alongside with maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation. Therefore, we can assume that the higher SOD, CAT, APX, and GR activities alongside with higher *MSR* and *CysPrx* genes expression in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *SIZ1* gene expression during cold storage, which is pivotal for reduction of H₂O₂ accumulation and maintenance of membrane integrity featured by lower MDA accumulation during cold storage (Figs. 1A, 1B, 2A-F, 3A).

In addition to ROS scavenging systems activity, HSPs serve as molecular chaperones rendering evident beneficial effects on protein synthesis, renaturalization, and stabilization. In addition to serving as molecular chaperones, HSPs exhibit ROS scavenging capacity, which is beneficial for the maintenance of membrane fluidity and integrity. Also, HSPs binding to membrane phospholipids and galactolipids is beneficial for the maintenance of membrane fluidity and integrity (Aghdam, Sevillano, Flores, & Bodbodak, 2013). During postharvest senescence and stresses, PLD is responsible for the hydrolysis of phosphatidylcholine and phosphatidylethanolamine releasing choline and ethanolamine, respectively, and producing phosphatidic acid. Phosphatidic acid phosphatase enzyme activity is responsible for diacylglycerol production from phosphatidic acid for lipolytic acyl hydrolase enzyme activity providing free fatty acids for enzymatic peroxidation by LOX or non-enzymatically by ROS giving rise to MDA production (Aghdam et al., 2018). Hence, it can be assumed that higher

HSP70 and *HSP90* genes expression accompanied by lower *PLD* and *LOX* genes expression and enzymes activity in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *SIZ1* gene expression during cold storage, which is pivotal for maintenance of membrane integrity featured by lower MDA accumulation during cold storage (Fig. 1A, 3A-C, 4A-D).

Chloroplastic methylerythritol 4- phosphate (MEP) pathway is pivotal for isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) production, the substrates of IPT, the enzyme involved in cytokinins biosynthesis in the chloroplast (Sakakibara 2006). In addition to cytokinin biosynthesis by IPT activity, cytokinin deactivation by CKO is pivotal for the maintenance of intracellular cytokinin homeostasis. According to Gapper (2003), ethylene and cytokinin antagonistically regulate broccoli floret senescence during postharvest life. Suppressing postharvest senescence in broccoli floret may arise from higher endogenous cytokinin accumulation due to higher *IPT* gene expression concurrent with lower *CKO* gene expression, which may have a beneficial effect in blocking ethylene biosynthesis and signaling. On the other hand, higher *CKO* gene expression combined with lower *IPT* gene expression and therefore lower endogenous cytokinin accumulation in broccoli floret accelerates postharvest senescence by triggering ethylene biosynthesis and action (Gapper, Coupe, McKenzie, Sinclair, Lill, & Jameson, 2005). Exogenous cytokinin has been applied to inhibit senescence in broccoli shown up by floret yellowing. This strategy suppresses chlorophyll degradation by chlorophyllase and Mg²⁺ dechelataase, promoting SOD, CAT, and APX activities leading to lower O₂⁻ and H₂O₂ accumulation, which is beneficial for the maintenance of membrane integrity featured by lower electrolyte leakage and MDA accumulation, concurrent with higher phenols accumulation leading to higher DPPH scavenging capacity (Xu et al., 2012). According to our results and considering the findings from other authors described above, higher endogenous cytokinin zeatin riboside accumulation in broccoli floret treated with PSK α at 150 nM might be ascribed to higher *IPT* gene expression concurrent with lower *CKO* gene expression during cold storage (Fig. 5A-C). Therefore, endogenous cytokinin zeatin riboside accumulation in broccoli floret treated with PSK α at 150 nM may be pivotal in delaying senescence shown up by floret yellowing during cold storage.

5. Conclusion

In summary, higher *SIZ1* expression in broccoli floret treated with 150 nM PSK α may be pivotal for higher SOD, CAT, APX, and GR activities concurrent with higher *MSR* and *CysPrx* expressions leading to lower H₂O₂ accumulation. Lower H₂O₂ accumulation due to higher activity of ROS scavenging systems concurrent with lower PLD and LOX genes expression and enzymes activity alongside higher *HSP70* and *HSP90* genes expression in broccoli floret treated with 150 nM PSK α may be beneficial for the maintenance of membrane integrity featured by lower MDA accumulation. In addition to oxidative stress alleviation by the promotion of ROS scavenging systems, higher endogenous cytokinin zeatin riboside accumulation due to higher *IPT*/lower *CKO* genes expression may be beneficial in senescence delay manifested by lower floret yellowing in broccoli treated with 150 nM PSK α . Our findings may permit the horticultural industry to take advantage of a promising postharvest procedure for the preservation of broccoli remarkably extending its postharvest life.

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474 **Table caption:**

475 **Table. 1. Primers used for qRT-PCR.**

Gene name	Functional annotations	Primer sequences (5'-3')
<i>SIZ1</i>	Proteins sumoylation	F: GCGAGGCTACTTCTGCAAAC R: CCGAGGTCTTACAGAACGCT
<i>PLD</i>	Membrane deterioration	F: CGCATGTCACTCTGGTACGAA R: TCGCTCGAGTATAAGTCCCAA
<i>LOX</i>	Membrane deterioration	F: TGTCCAGAACGACACTGAGC R: CGCTGCATGTAGAGCAGAAG
<i>HSP70</i>	Chaperoning activity	F: GTGATCGACGCTGACTTCAC R: TGGACAGCTTCACACATTGC
<i>HSP90</i>	Chaperoning activity	F: GGACCGGGAGAACCATAAGC R: GAATCGTAGCAGGAGAGGGC
<i>MSR</i>	Proteins stability	F: TACAGAGCGGAAAACCTACCAC R: ACCTGACTAGTTACTTAGCCAT
<i>CysPrx</i>	H ₂ O ₂ scavenging	F: ACTTCCGTCCAAGAAAGGCT R: CCTCCAGAGTACACACACGA
<i>IPT</i>	Cytokinin accumulation	F: CTCCTTCCGGGTAGACATGC R: TGCAAGATCGACTGAGAGGC
<i>CKO</i>	Cytokinin accumulation	F: GATGTCCACAATGCGTCCAA R: TCCTTGAAGCGAGTGACCAT
<i>Actin</i>	Housekeeping gene	F: CCAGAGGTCTTGTTCCAGCCATC R: TTCCACCACTGAGCACAATGTTAC

Figures caption:

Fig. 1. MDA (A) and H₂O₂ (B) accumulation in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at *P* = 0.01 level.

Fig. 2. SOD (A), CAT (B), APX (C) and GR (D) enzymes activity accompanying by *MSR* (E) and *CysPrx* (F) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n=3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P=0.01$ level.

Fig. 3. *SIZ1* gene expression (A) accompanying *HSP70* (B) and *HSP90* (C) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Fig. 4. PLD and LOX enzymes activity (A, B) and genes expression (C, D) in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Fig. 5. Endogenous cytokinin ZR accumulation (A) concurrent with *IPT* (B) and *CKO* (C) genes expression in broccoli floret treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Supplementary Fig. 1. Floret yellowing in broccoli treated with PSK α at 0 and 150 nM during storage at 4 °C for 28 days. Data shown are mean values of n=3 and the error bars represent standard errors of the means. Mean values followed by different letters mean they are significantly different by Tukey's test at $P = 0.01$ level.

Exogenous PSK α application

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

*** Credit Author Statement**

Morteza Soleimani Aghdam conceived the idea and performed the experiments; **Morteza Soleimani Aghdam** and **Francisco Borja Flores** supported the data analysis, wrote and approved the manuscript.