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ORIGINAL PAPER

Inhibition of wound-induced ethylene does not prevent red discoloration in fresh-cut endive (*Cichorium intybus* L.)

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Abstract Consumer demand for lightly processed ready-to-eat fruits and vegetables increases consistently as convenience and easiness of preparation become decision factors in food purchases. However, red discoloration of cut leafy vegetables limit storage life and the flexibility of offering specific products. This is the case of fresh-cut endive (*Cichorium intybus* L.), that turn red quickly and thus have a limited shelf life either alone or in mixed vegetables products. Heat shock was shown to block red discoloration in cut endive, through significantly reducing the level of phenylalanine ammonia-lyase (*PAL1*) transcripts and stimulating peak levels of heat shock protein (*HSP90*) transcript accumulation shortly after cut. With the aim of evaluating to what extent the effect of heat shock was related to the blockage of wound-induced ethylene, we applied [S]-*trans*-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (AVG) to block ethylene synthesis and 1-methylcyclopropene (1-MCP) to block the response to ethylene in fresh-cut endive, and analysed wound induced ethylene emission as well as *HSP90* and *PAL* expression patterns as compared to samples treated by heat shock (46 °C/120 s). Ethylene was measured continuously during 48 h with a laser-based ethylene detector, and real-time quantitative PCR (qPCR) was performed for *PAL1* and *PAL2* as well as for degenerated *HSP90* primers. Red discoloration limited the shelf life of 1-MCP and AVG treated endives to 48 h, whereas samples treated with heat shock maintained good quality for 8 days. Wound-induced ethylene was blocked by AVG, inhibited by 1-MCP but not by heat shock, which was shown to delay ethylene emission by at least 8 h after cutting, after which the rates increased to higher levels than in the control and took longer to decrease. It was concluded that ethylene is not the main factor in the processes that lead to tissue discoloration in fresh-cut endive.

Keywords AVG · 1-MCP · *HSP90* · *PAL1* · *PAL2*

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Introduction

Processing and distribution of packaged fresh-cut leafy vegetables has increased dramatically in recent years due to the convenience these products provide to end-users. Unfortunately red discoloration, which is a typical disorder in this type of product, is more evident, more difficult to control and therefore more limiting to shelf life in endives than in other leafy vegetables.

Current biochemical model for endive's discoloration considers the metabolism of phenylpropanoids. In response to cutting, phenylalanine ammonia lyase (PAL), the committed enzyme in the phenylpropanoid pathway, catalises the production of phenols, these compounds are then oxidised by the action of polyphenol oxidases (PPO) to quinones, which in turn spontaneously polymerise to red or brown pigments [1].

Salman et al. [2] demonstrated that heat shock (46 °C/120 s) blocks the discoloration of fresh-cut endive by blocking the transcription of PAL. The blockage of *PAL1* was related to the expression of *HSP90*, which increased

significantly after the heat shock. In that same study, it was shown that an atmosphere depleted in O_2 and enriched in CO_2 did not affect significantly the discoloration of fresh-cut endive.

Nevertheless, the observation that wound-stressed produce emit more ethylene suggests that ethylene might play a role in increased PAL activity, as it happens in intact healthy plants for which this idea is also supported by reports that ethylene can regulate protein synthesis and PAL activity in sweet potato roots [3]. Therefore, inhibition of ethylene biosynthesis or action might have a role in delaying the red discoloration and extending storage life of cut endive.

Even though inhibiting ethylene perception in intact strawberry fruits with 1-MCP resulted in lower PAL activity [4], so far no reports were found on the effects of ethylene inhibitors on postharvest quality of fresh-cut vegetables such as endive as regarded to the expression of *PAL* and *HSP90* genes.

1-MCP has been shown to compete with ethylene for the binding site in plant tissue, which prevents ethylene from exerting its physiological action [5], a non-toxic mode of action, with negligible residue and active at very low concentrations [6]. Recently, 1-MCP was registered in several countries as a new post harvest tool (Smartfresh™, Agro fresh Inc., Philadelphia) for extending the shelf life and quality of plant products, for reducing or inhibiting ethylene action. Furthermore, 1-MCP has been shown to provide benefits in maintaining the quality of fresh-cut fruits [6].

AVG is frequently used as a specific inhibitor of ethylene biosynthesis [7], and applied as foliar sprays (AVG 0.5 mM) reduce stress-induced ethylene. One possible mode of action of AVG could be through its effect on the synthesis of functional proteins.

The objective of the research reported here was to investigate the effects of wound-induced ethylene in the regulation of red discoloration in fresh-cut endive, through blockage of its synthesis by AVG, or action by 1-MCP.

Materials and methods

This project was carried out at Radboud University, Nijmegen, The Netherlands, within the Life Science Trace Gas Facility. The ethylene monitoring was performed at the Department of Molecular and Laser Physics, and the molecular analysis at the Laboratory of Microbiology, respectively.

Plant material

Fresh endives were bought from local market in Nijmegen, packed in plastic bag to avoid dehydration and stored at 6 or 8 °C. For the experiments, the endives were trimmed to remove the tip, the bottom and damaged leaves. A sharp stainless steel knife was used to cut the vegetables into 1 cm slices.

Experimental conditions

The samples were taken from a pool of fives endives, all treatments were repeated at least four times and applied as follows, (a typical pattern is shown in Fig. 2):

Control Trimmed and cut endives were rinsed in cold tap water and drained immediately after cutting, excess water was removed with paper towel, without rubbing.

1-MCP A whole endive was placed inside a 1 L jar with gas tight lid in which a rubber septum was inserted. An aliquot of a powder preparation containing 0.14% active 1-MCP was diluted in water inside a rubber-stoppered flask, a sample of headspace corresponding to 1.0×10^{-6} V 1-MCP was withdrawn and injected through the septum in the lid of the jar containing the endive. The jar was wrapped in aluminium to avoid light exposure and kept at 10 °C for 24 h.

AVG A measure of 4 ml of 0.5 mM solution of AVG was sprayed onto 50 g of cut, rinsed and drained endive slices before placing them in the cuvette for gas measurements.

Heat shock Heat shock was applied as in previous experiments Salman et al. [2]. Endive slices were put inside a bag made of a plastic net to facilitate hot water to penetrate among the pieces and immersed a thermostat-controlled water bath for 2 min at 46 ± 0.2 °C, followed by immediate cooling in refrigerated water. The pieces were drained and excess water removed with paper towel.

1-MCP + heat shock The endive was left in contact with 1-MCP overnight, cut, and submitted to heat shock as described above. The inverse order of treatments was tried, with no difference in response.

Heat shock + AVG After heat shock as described above, samples were sprayed with 0.5 mM AVG solution.

Photoacoustic detection of ethylene emission

Ethylene emission was monitored in real time by using a commercial laser-based ethylene detector (type ETD-300, Sense B·V., Nijmegen, The Netherlands) as in combination with a gas handling system. In brief, the detector consists of a CO_2 laser and a photoacoustic cell, in which the gas is detected. A detailed description of the method is given by Harren and Reuss [8]. The detector is able to detect on-line about 300 parts per trillion by volume of ethylene within a 5-s time scale.

The gas handling is performed by a valve control box (type VC-6, Sensor Sense B·V.). The gas emission from up to six samples per experiment is transported to the

ETD-300 alternately and at controlled flow rates, preventing accumulation induced effects. Both the laser-based ethylene detector and the valve control box are operated fully automatically by a computer program, and can perform continuous measurements for periods of up to several weeks.

Samples of control and treated endives (about 50 g fresh weight) were placed into 1 L volume closed glass cuvettes and continuously flushed with air at constant flow of 3 L h^{-1}. To avoid desiccation of the plant material, wet paper towels were put on the bottom of each cuvette. One cuvette containing only the wet paper towel was used to monitor the baseline. During the ethylene measurements the cuvettes were kept into an environmental chamber in dark and constant temperature of 10 °C. Ethylene emission from each sample was alternatively monitored by the ETD-300 for 10 min (5 s per acquisition point). For a better overview, we displayed the average value of ethylene production from the last 5 min of the 10 min of sampling, which represents the ethylene emission rate every 1 h.

Before entering the cuvettes, the air passed through a platinum-based catalyser Sensor Sense B·V.) to remove any traces of external ethylene or other hydrocarbons. A scrubber with KOH and $CaCl_2$ were placed before the ETD-300 to reduce the CO_2 and the water content in the gas flow, respectively.

Data from the empty cuvette (baseline) were subtracted from the emission rates obtained.

Weight loss

Three replicates from each of the treatment combinations 1-MCP + HS and AVG + HS were weighed at the beginning of the experiment and after 4 and 8 days in storage. The weight loss was calculated (%) in reference to the initial weight of the cut endive.

Molecular analysis

Samples 10 g were taken during ethylene measurement at 3; 6; 18; 24 and 48 h for molecular analysis. These samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Total RNA was obtained by the method of Chomczynski and Sacchi [9], and the analysis was performed as described in our previous report [2]. Masterscript™ RT-PCR system (5 PRIME) was used for cDNA synthesis from 400 ng of total RNA. Primers for *PALl1* and *PAL2* genes were developed from *Daucus carota* and *Lactuca sativa* sequences: (*DcPAL1Fw*: 5′-CAAACAGGATCAAAGAATGCAGGTC-3′, *DcPAL1Rev*: 5′-ACCTTGTCAAACTCTTCTCCAGGC-3′, *LsPAL2Fw:* 5′-CACCCTCCTTCAAGGTTACTCCGG-3′, *LsPAL2Rev*: 5′-GGGACGAGATCGCCGGAGGCGG-3′). Degenerated primers for *Hsp90* and primer's control actin gene were designated. (*DegHSP90.1Fw*: 5′-GGAWRTKCTMGGDGACAAGGTTG-3′, *DegHSP90.1Rev*: 5′-GCYTGTGCCTTCATRATYCTCTCC-3′), and *CiACTINFw*: 5′-TGAATGCCGGCAGCTTCCATTCCG-3′, *CiACTINRev*: 5′-CTTCGAACAAGAGCTCGAAACCGCC-3′), respectively.

Amplified fragments of PCR product were purified and sequenced using *Genome Express* services. The homologies of qPCR amplified fragments to sequences in the databases are displayed in Table 1. The quantitative assessment of mRNA levels was performed using the Manual RealMasterMix SYBR ROX (5 PRIME) and the iCycler iQv3 (BIO-RAD). Quantitative real-time PCR based on the fluorescence emitted by the amplification products in the presence of SYBR Green, allowed quantification of the accumulation of the target transcript relative to the *actin* transcript taken as reference for the experimental conditions.

Results

In this study, only heat treatment inhibited red discolouration and the heated samples were similar in appearance to the fresh-cut endive (Fig. 1). Images displayed in Fig. 1, taken during 8 days in storage at 10 °C, show that red discolouration was similar in samples treated with 1-MCP, AVG and the control. However, no discolouration was seen

Table 1 Homologies of qPCR amplified fragments to sequences in the databases

PCR fragment	Length[a] (bp)	Accession number	Homology[b]	BLAST score
ACTIN	121	EF528575	*Helianthus annuus ACTIN* mRNA (AF282624.1)	6e−28
HSP90.1	83	EF528574	*Nicotiana tabacum HSP90* mRNA (AB264546.1)	3e−22
PAL1	78	EF528573	*D. carota DcPAL1* gene (D85850.1)	3e−04
PAL2	91	EF528572	*L. sativa PAL2* mRNA (AF411134.1)	4e−25

Source Salman et al. [2]

[a] Sequence provided by Genome express

[b] GenBank accession numbers of sequences homologous to qPCR fragments are in parentheses

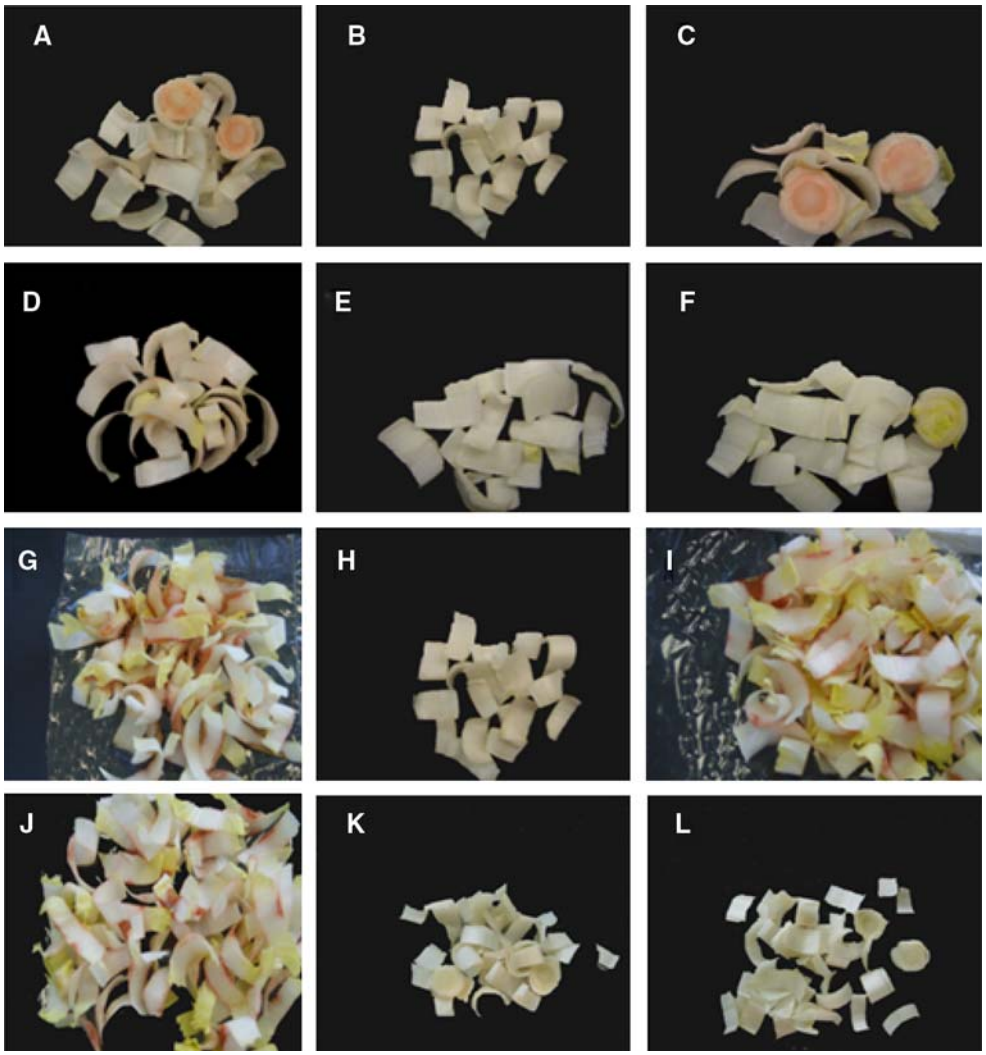

Fig. 1 Images of endive cuts after 2 (**a**–**f**) and 8 (**g**–**l**) days at 10 °C. Control unheated samples (**a** and **g**); heated samples (**b** and **h**); unheated samples treated with 1-MCP (**c** and **i**); unheated samples treated with AVG (**d** and **j**); samples treated with combinations of 1-MCP and heat shock (**e** and **k**) and heat shock plus AVG (**f** and **l**). Heated samples were treated in a water bath at 46 °C/120 s immediately after slicing; 1-MCP was applied overnight before slicing; AVG was applied by spray onto cut endive, and whole endives sliced at the moment of evaluation were used as control

in samples treated with heat shock alone or in combination with either 1-MCP or AVG until 8 days.

The differences among treatments as regarded to ethylene emission rates in response to cutting are seen in Fig 2. In control samples, ethylene emission reached a peak shortly after 6 h and started to decrease after 12 h. As expected, AVG completely blocked ethylene synthesis, either alone or in combination with other treatments. On the other hand, the effect of 1-MCP was to reduce the intensity of ethylene emission, either alone or combined with heat shock, without affecting the shape of the curves. Heat-shock effect on ethylene synthesis was shown to be temporary, blocking for the first 6–8 h after cutting, after which the emission rate increased sharply and reached much higher levels than in the control. A time gap of about 12 h was observed between the peaks of ethylene emission from control and heat shocked cut endives.

The qPCR experiments allowed transcript accumulation analysis of Pal1, Pal2 and Hsp90 genes in endive tissues, as shown in Fig. 3a–c. PAL1, PAL2 transcripts were present in the plant material before wounding and increased significantly in the first 18 h of storage at 10 °C (Fig. 3a, b). Both *PAL1* and *PAL2* transcripts accumulation were affected only by heat shock, with no additional effect from the combination of this treatment with neither AVG nor 1-MCP. The results of qPCR showed relatively stable amounts of *HSP90* transcripts in control and 1-MCP or AVG-treated samples (Fig. 3c). In heat-treated samples, on the other hand, there was a marked increase, with a peak 6 h after cutting.

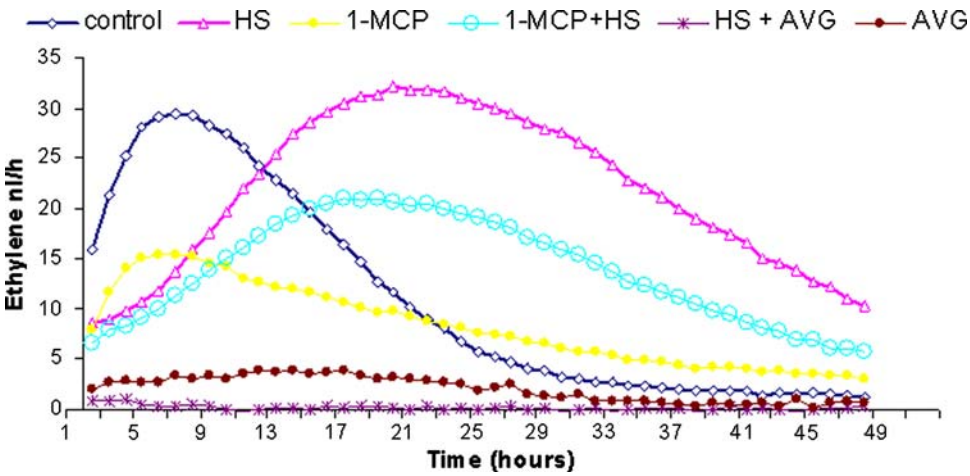

Fig. 2 On line monitoring of ethylene production in fresh-cut endive in response to HS; AVG; 1-MCP and HS + AVG; HS + 1-MCP. Wounding rapidly induced ethylene biosynthesis in control and reached its peak after 6 h at 10 °C. HS temporarily inhibited ethylene production during 10–12 h at 10 °C. Ethylene emission was completely blocked by AVG and reduced by 1-MCP. All samples had the same FW = 50 g. Samples were taken from a pool of five endives

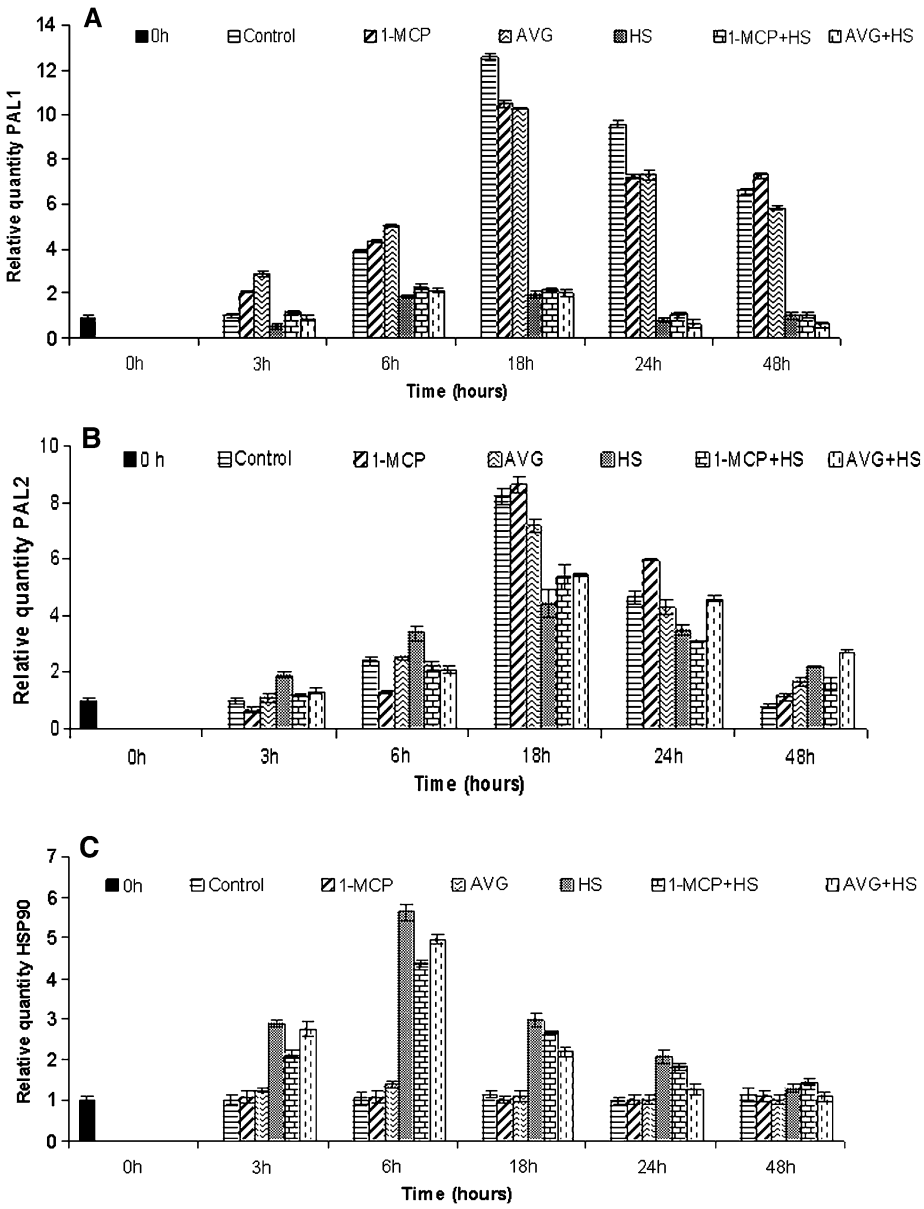

Fig. 3 Accumulation of PAL1 (**a**), PAL2 (**b**) and HSP90 (**c**) transcripts in sliced cut endive treated with 1-MCP, AVG, heat shock, 1-MCP + HS and AVG + HS stored at 10 °C for 0, 3, 6, 18, 24 and 48 h, in air. Samples were taken from a pool of five endives. Each value is the mean of three replicates (±SE)

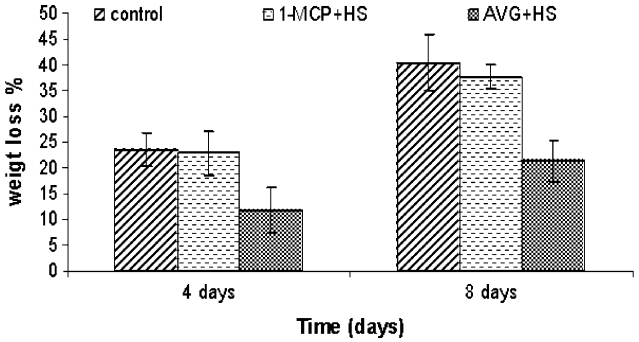

Fig. 4 Weight loss in heated sliced endives treated with 1-MCP and AVG, held for 8 days at 10 °C. Samples were taken from a pool of five endives. Each value is the mean of three replicates (±SE)

Despite the fact that neither AVG nor 1-MCP alone were effective to prevent red discolouration, it is interesting to remark that the pieces treated with heat shock combined with AVG lost less weight and remained unchanged in appearance during storage of 8 days at 10 °C, as compared to those that received the combination HS + 1-MCP (Fig. 4).

Discussion

Fresh-cut endive has a short shelf life due to fast red discolouration upon wounding, therefore, this investigation was carried out to evaluate whether ethylene inhibition would be effective in reducing the discolouration and, extending the shelf life of this vegetable, so as to increase the possibilities of offering it to the market as a fresh cut produce itself or in mixed salad preparations.

Ethylene emission was blocked temporarily by heat shock (Fig. 2) before it increased after about 8 h. Within this time gap HSP90 transcripts increased sharply within the first 6 h, which could be evidence that the heat-shock proteins were at least in part regulated by ethylene.

On the other hand, *PAL1* and *PAL2* were strongly expressed in unheated cut tissues, with accumulation peaks of *PAL1* mRNA occurred 18 h after cutting, whereas in heated samples the lower mRNA accumulation of *PAL1* and *PAL2* were inversely related to *HSP90* (Fig. 3), an effect that was significantly more evident for *PAL1* and to which prevention of discolouration was related. Our data show that red discolouration of wounded endive tissues is more evidently associated with the *PAL1* isoform.

The intensity of discolouration of endive slices after 2 days, as seen in Fig. 1, clearly showed that heat-treated samples were not significantly different from the control. Image analysis reported by Salman et al. [2] demonstrated the differences in colour among samples treated similarly.

However, we found no definitive information on the involvement or requirement of ethylene in the regulation of heat-shock proteins.

We could also observe (Fig. 2) a predominant effect of heat shock over 1-MCP when these two treatments were combined. In addition, 1-MCP seemed to have no influence in *PAL1*, *PAL2* and *HSP90* transcripts levels.

Literature is richer in reports on the effects of 1-MCP on colour and phenolic-related changes in fruits than in vegetables, and the results found are variable according to the species and to the plant organ. Gong et al. [10] found no influence of 1-MCP on postharvest colour changes or stem browning in sweet cherries. Differently, Marcos et al. [11] observed that 1-MCP pre-treatment reduced the induction of *PAL* mRNA in orange, thus demonstrating that *PAL* expression changes are mediated by ethylene perception, similar results having been reported by Jiang et al. [4] who found that 1-MCP inhibited PAL activity and lowered anthocyanin production in strawberries. In another study, these last authors [5] they reported that exposure of fresh-cut apple to 1-MCP resulted in delayed colour changes.

On the other hand, studying the effect of AVG in the PAL activity in iceberg lettuce, Ke et al. [12], they found that the treatment with AVG-inhibited ethylene induced by the wounding but it did not affect the induction of PAL activity.

In the present study, we found that the regulation of *PAL* gene expression was also independent of wound-induced ethylene, since despite AVG inhibition of ethylene, no effect on red discolouration in fresh-cut endive was found and neither in reduction of *PAL1* and *PAL2* transcripts.

In other stress conditions, such as pathogenic inoculation, it has been also suggested that the increases in ethylene production and PAL activity are independent responses [13].

Conclusion

From the results described above, decreasing the level of wound-induced ethylene production by addition of inhibitors of enzymes involved in ethylene biosynthesis (AVG) or action (1-MCP) does not change *PAL1*, *PAL2* and *HSP90* transcription as compared to control. Therefore, we can conclude that the cutting process induced ethylene emission even in heat shocked samples; apparently ethylene regulates *HSP90* gene and has not a major role in regulating *PAL* gene expression in fresh-cut endive; and ethylene emission and red discolouration are independent processes in fresh-cut endive.

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