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Effect of Elicitation on Polyphenol and Carotenoid Metabolism in Butterhead Lettuce (*Lactuca sativa* var. capitata)

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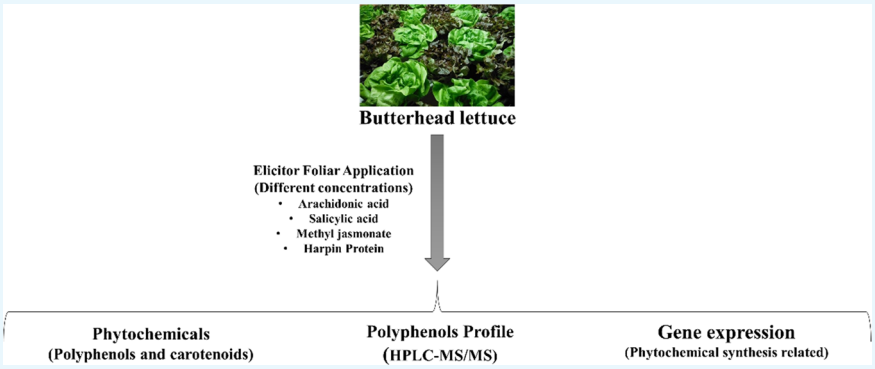

ABSTRACT: The effect of elicitation in butterhead lettuce on carotenoid and polyphenol metabolism was evaluated. Different concentrations of arachidonic acid (AA), salicylic acid (SA), methyl jasmonate (MJ) (15, 45, and 90 μM) and Harpin protein (HP) (30, 60, and 120 mg/L) were applied on red and green butterhead lettuces. Total phenolic and flavonoid content were incremented by MJ (90 μM) in green and red lettuce. Carotenoids were increased in red lettuce (AA; 45 μM). Green lettuce modifies their phenolic acid profile after elicitation with AA and MJ; meanwhile, red lettuce incremented mainly in hydroxycinnamic acids and flavonols, MJ being the elicitor with the highest effect. There was an impact on secondary metabolite enzyme gene transcript concentration. Phenylalanine ammonia-lyase (PAL) and lycopene beta cyclase (LBC) increased in both varieties after elicitation. A relationship between phytochemical increase and the activation of the metabolic pathways after elicitation in butterhead lettuce was observed.

1. INTRODUCTION

Nowadays, the use of a eustressor as preharvest treatment of fruits and vegetables to trigger resistance to different biotic stresses has grown worldwide. A eustressor is defined as a factor with a biological, chemical, or physical origin that could increase plant improvement due to the activation of responses that could include defense responses among them, leading to an increment of functional quality in several fruits and vegetables.[1−3] The source could be from abiotic or biotic origin, and they have been used in several fruits and vegetables due to their ability to activate the biosynthesis of secondary metabolites, which help the plant to adapt to different stress factors, as well as potentially improve human health when those vegetables are consumed in a regular diet.[4] Recently, different authors have reported that the use of elicitors promote the synthesis of several components of the secondary metabolism, such as polyphenols and carotenoids among others, due to their ability to act as scavengers of several reactive oxygen species (ROS), therefore providing the treated fruit or vegetable with characteristics of functional foods.[2,5,6] In the last few years, the use of elicitors on lettuce (*Lactuca sativa* L.) has taken high relevance.[7] There are several varieties of lettuce, but the most consumed are romaine, leaf type, iceberg, stem, and butterhead.[2,8−11] The concentration of phytochemicals between varieties shows a huge variation. Butterhead and iceberg are among those with the lowest phytochemical content, and for this reason, the use of elicitors have been studied with these two varieties[12] due to their ability to increase their bioactive molecules. Nonetheless, with respect to sensory acceptance, butterhead lettuce has better acceptance than iceberg among consumers. Molecules such as methyl

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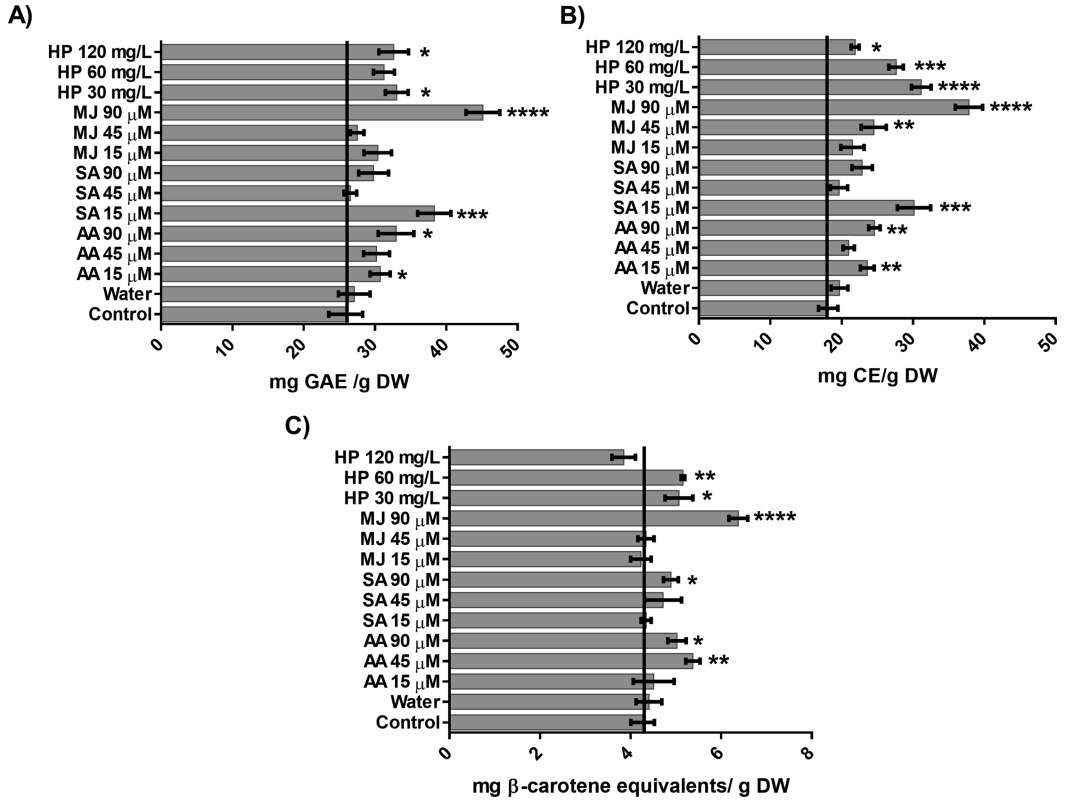

Figure 1. Content of phytochemicals in green butterhead lettuce samples in response to elicitors. Content of (A) total phenolics, (B) total flavonoids, and (C) total carotenoids. AA, arachidonic acid; SA, salicylic acid; MJ, methyl jasmonate; HP, Harpin protein; GAE, gallic acid equivalents; CE, catechin equivalents.

jasmonate (MJ), arachidonic acid (AA), salicylic acid (SA), and Harpin protein (HP) have been used as elicitors on recent investigations.[2,13−15] MJ used in romaine and butterhead lettuce increased polyphenol and carotenoid content.[8] AA and SA used in butterhead lettuce incremented polyphenol and carotenoid content, as well as antioxidant activity; meanwhile, HP had a bigger effect on carotenoids in butterhead lettuce.[2,9] Recent research, carried out by our research group, in butterhead lettuce showed that the elicitation effect depended on several factors, among them are the time of application and type of elicitor, and that the main effect was observed on polyphenols and carotenoid concentration.[2] There is little investigation that informs on the effect of concentration and type of elicitor on the phytochemical content in butterhead lettuce, as well as on the possible metabolic pathway by which lettuces increase these metabolites after elicitation has been applied.[16,17] For this reason, it is important to evaluate the effect of elicitation on polyphenols and carotenoids, and hence, it was evaluated in green and red butterhead lettuce, as well as the gene expression of the enzyme related to its synthesis.

2. RESULTS AND DISCUSSION

2.1. Phytochemical Content in Green and Red Lettuce. The effect of elicitors on the phytochemical content is conditioned by different factors such as the elicitor used, its concentration, time of application, and cultivar to which it is applied.[18] In our previous research, the effect of the day of application of the elicitor on the phytochemical content on green and red butterhead lettuces was evaluated, observing that for green and red lettuces were on preharvest at day 7 and 15, respectively.[2] For this reason, in order to evaluate the effect of different elicitor concentrations on the phenolic, carotenoid, and anthocyanin (only in red lettuce samples) content on green and red butterhead lettuces, these two days of application were used. To rule out an effect due to sample manipulation, two additional groups were added, lettuce without manipulation and lettuce with only water. No significant differences on phytochemical content were observed between both controls, indicating that manipulation of the samples did not affect the phytochemical content. The concentration of phytochemicals in green lettuce is shown in Figure 1. The content of total phenolic compounds (TPC) increased in all treatments compared to controls (Figure 1A). At all concentrations, AA and HP elicitors showed higher TPC compared to the control; however, no significant differences were observed between the values obtained in the elicitor's concentrations. SA presented the highest effect that was observed at the lowest concentration (15 μM), while no effect, compared to the control, was observed at the highest concentration. Contrary to SA, with MJ, the highest effect was observed at the highest MJ concentration (90 μM). Even though elicitors produced a slight effect on TPC on green lettuces, Figure 1B shows that the highest effect was observed in flavonoid content (FC). HP and SA showed the highest effect that was observed at the lowest elicitor's concentration. While the significant effect on FC was observed at the highest and lowest concentration (15 and 90 μM), no significant effect was observed at 45 μM. The effect of elicitors on carotenoid content (CAR) is shown in Figure 1C. MJ (90 μM), again, was the treatment with the highest effect on CAR, following the same pattern than those observed for TPC and FC. Lower MJ concentrations had no effect on CAR compared with the

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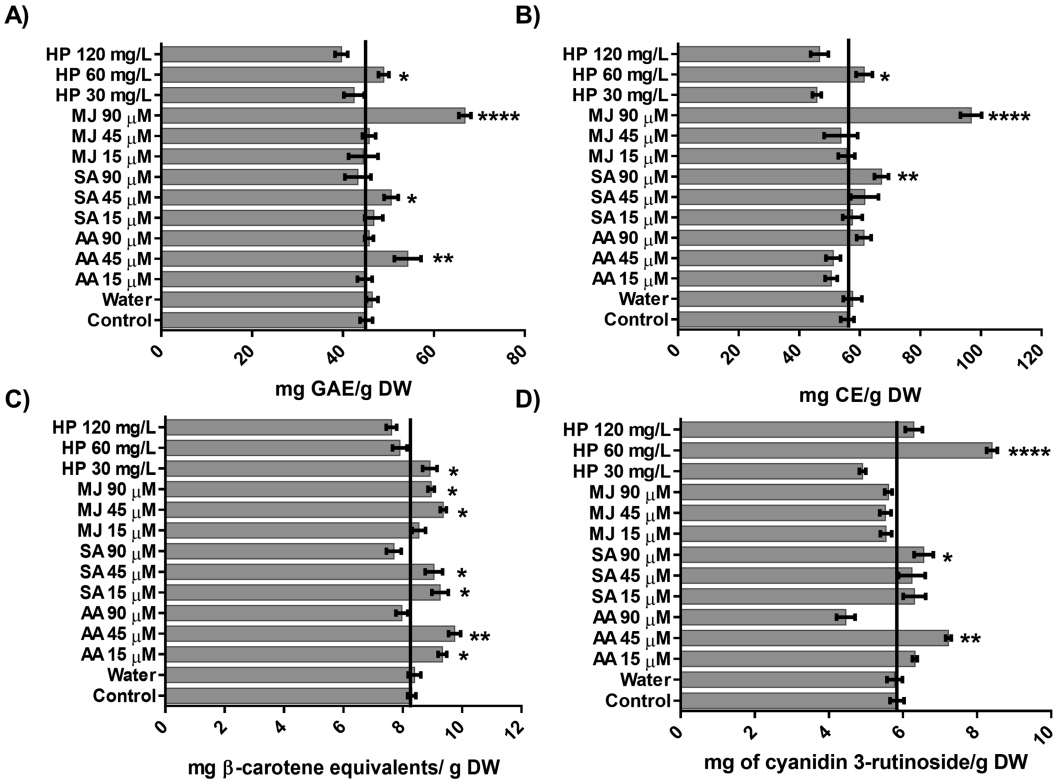

Figure 2. Content of phytochemicals in red butterhead lettuce samples in response to elicitors. Content of (A) total phenolics, (B) total flavonoids, (C) total carotenoids, and (D) total anthocyanins. AA, arachidonic acid; SA, salicylic acid; MJ, methyl jasmonate; HP, Harpin protein; GAE, gallic acid equivalents; CE, catechin equivalents.

control. HP (30 and 60 mg/L) enhanced the production of CAR in comparison with the control. However, HP at the highest concentration (120 mg/L) had a negative effect on CAR. SA and AA treatments, except for AA (45 μM), did not highly affect CAR in green lettuce.

The effect of elicitors in red butterhead lettuce samples is shown in Figure 2. An increase in the production of TPC was observed in response to all treatments where again MJ (90 μM) was the elicitor with the highest response. AA (45 μM) promoted the second-best response for TPC with increments of 48 and 20%, respectively (Figure 2A). It is interesting to notice that the effect of elicitors on TPC was less evident in red lettuce as compared to green lettuce. No significant effect on TPC was observed with any HP and SA treatments, and only slight differences were observed at some AA and MJ concentrations. No significant effect was observed for FC (Figure 2B) in red lettuce, except for MJ (90 μM), and in a lower degree for 90 μM SA and 60mg/L HP. As in the case of TPC and FC, no significant effect on CAR content was observed after elicitation (Figure 2C). AA (15 and 45 μM) significantly increased CAR compared to the control; meanwhile, AA (90 μM) decreased the phytochemical content. The same behavior was observed with HP and SA, where the lower concentrations of HP (30 mg/L) and SA (15 and 45 μM) were the treatments with a significant effect on CAR while the highest concentration induced a decrease in the CAR level. MJ (90 μM), as well as in the other parameters, was the only elicitor that showed an effect at its higher concentration. Anthocyanin content (ANT) was only quantified in red lettuce because it was not detected in green samples. HP (60 mg/L), AA (45 μM), and SA (90 μM) were the only treatments with a significant effect on ANT in red lettuce.

Our result on the effect of elicitors on the phytochemical content in green lettuces agrees with previously published results. MJ solutions were used in romaine lettuce samples, observing a 35% increase in TPC, with respect to the control.[19] Several studies have shown that MJ increases TPC in different vegetables;[20−22] however, the mechanism by which these compounds increase in the vegetables remains unclear. Złotek et al.[9] reported that AA (100 μM) significantly increased the content of carotenoids while no effect on TFC was observed in butterhead lettuce. These results are in agreement with those reported in the present study, where AA increased the content of CAR in red lettuce. SA, as well as MJ, has shown an upward effect on phytochemical content in moringa and romaine lettuce.[6,19] The concentration used of each elicitor in both red and green butterhead lettuce had a direct effect on the phytochemical content with an increase or a decrease in their levels. At high concentration of HP, there was a decrease in the concentration of carotenoids for green and red butterhead lettuce and in total phenolic compounds and total flavonoids for red lettuce only. Studies suggest that higher concentrations of stress response inducers have an adverse effect on the cellular stability of the vegetable or fruit to which it is applied,[23] leading to a decrease of these bioactive molecules. At high concentrations, there is a higher incidence of ROS, generated in response to the presence of the elicitor, which induces greater cell death, which may result in a decrease in phytochemicals. On the other hand, there are circumstances in which the lower concentration does not induce the increase of concentration of bioactive compounds, such as in the case of HP (30 mg/L), where even a decrease in FC and CAR was observed, suggesting that at low elicitor concentration, the

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Table 1. Phenolic Compounds Detected in Aqueous Methanolic Extracts from Green Butterhead Lettuce by HPLC-ESI-QTOF/MS−MS[a]

peak	Rt (min)	mass expected [H-]	mass obtanied	formula	proposed compound	class	W	AA 15 μM	AA 45 μM	AA 90 μM	SA 15 μM	SA 45 μM	SA 90 μM	MJ 15 μM	MJ 45 μM	MJ 90 μM	PH 30 mg/L	PH 60 mg/L	PH 120mg/L
1	1.54	331.0665	331.0661	C22H18O1	Galloyl-hexose	Hydroxybenzoic		+	++	++	+++	++	++	+++	++	+++	+++	++	++
2	2.29	262.0147	261.0062	C9H10O7S	Dihydrocaffeic acid sulfate	Hydroxycinnamic		-	-	+	-	+	-	+	++	+	-	+	-	-	+
3	2.70	315.0716	315.0712	C13H15O9	Dihydroxybenzoic acid hexose	Hydroxybenzoic		+	-	-	-	+	-	+	+	+	+	-	-	-	-
4	4.02	312.0481	311.0296	C13H12O9	Caffeoyltartaric acid (caftaric acid) 2	Hydroxycinnamic		-	+	+	+	+	++	++	+++	++	++	++	++	++	+
5	4.28	315.0716	315.0712	C13H15O9	Dihydroxybenzoic acid hexose	Hydroxybenzoic		+	-	-	-	+	-	+	++	+	-	+	+	-	+
6	4.43	342.0951	341.0880	C15H18O9	Caffeoyl-hexose	Hydroxycinnamic		-	+	+	+	+	++	++	+++	++	+	++	++	++	++
7	4.89	579.2078	579.0975	C28H35O13	Syringic acid hexose	Hydroxybenzoic		-	+	+	+	+	++	++	+++	+	+	+	+	+	+
8	5.61	326.0638	325.0548	C14H14O9	Fertaric acid	Hydroxycinnamic		+++	+++	+++	+++	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
9	5.64	192.0634	191.0544	C7H12O6	Quinic acid	Hydroxybenzoic		+	+	+	+	+	-	+	+	-	-	-	-	-	-
10	5.74	353.1358	353.0864	C16H18O9	Caffeoylquinic acid	Hydroxycinnamic		+	+	+	+	+	+	++	+	+	+	+	+	+	+
11	5.83	339.0716	339.0711	C15H15O9	Esculetin 6-O-glucoside	Coumarin		+	+	+	-	-	+	-	+	+	++	+	+	+	+
12	6.03	725.1201	725.1198	C30H29O21	Quercetin 3-O-(6″-O-malonyl)-glucoside 7-O-glucuronide	Flavonol		+++	+	+	+	+++	++	+	+++	+++	+++	+++	+++	+++	+++
13	6.06	180.0423	179.0333	C9H8O4	Caffeic acid	Hydroxycinnamic		-	+	+	+	-	+	++	+	+	-	+	+	-	-
14	6.13	224.0685	223.0608	C11H12O5	Sinapic acid	Hydroxycinnamic		-	-	+++	-	-	++	++	-	+++	-	-	-	-	+++
15	6.35	296.0532	295.0447	C13H12O8	Caffeoylmalic acid	Hydroxycinnamic		++	+++	++	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
16	6.81	474.0798	473.0723	C22H18O12	Dicaffeoyltartaric acid	Hydroxycinnamic		-	+	+	+	+	+	+	+	+	+	+	+	+	+
17	6.85	452.0955	451.0876	C20H19O12	2-O-p-hydroxybenzoyl-6-O-galloyl glucoside	Hydroxybenzoic		-	+	-	-	+	-	++	+	+	-	+	+	-	+
18	6.92	490.1111	489.1034	C23H22O12	Kaempferol 3-O-6-acetylglucoside	Flavonol		+++	+++	+++	+++	-	+++	+++	-	+++	+++	+++	+++	+++	-
19	6.97	312.0481	311.0296	C13H12O9	Caffeoyltartaric acid (caftaric acid) 1	Hydroxycinnamic		+	+	+	+	+	+	+	+	+	-	-	-	-	+
20	7.30	477.0669	477.0672	C21H17O13	Quercetin 3-glucuronide	Flavonol		+	++	-	+	++	+	++	+++	+	-	-	-	-	+
21	7.35	463.0877	463.0871	C21H19O12	Quercetin 3-glucoside	Flavonol		++	+	++	+++	+++	-	+	+++	++	+++	+++	+++	+++	+++
22	7.37	516.1268	515.1188	C25H24O12	Dicaffeoylquinic acid	Hydroxycinnamic		-	-	-	-	+	-	-	+	+	-	-	-	-	+
23	7.45	457.0771	457.0808	C22H17O11	Caffeoyltartaric-p-coumaroyl acid	Hydroxycinnamic		+	-	-	-	+	-	+	+	-	+	-	-	-	+
24	7.48	296.0532	295.0447	C13H12O8	Caffeoylmalic acid	Hydroxycinnamic		+	-	-	-	-	-	-	-	-	+	+	-	-	+
25	7.56	550.0959	549.0884	C24H22O15	Quercetin malonylglucoside	Flavonol		++	-	+	+	++	-	+	++	++	++	+	++	-	-
26	7.79	461.1006	461.0719	C21H18O12	Kaempherol-3-O-glucuronide	Flavonol		-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	7.84	499.1240	499.1253	C16H17O8	p-Coumaroyl-caffeoylquinic acid	Hydroxycinnamic		+	-	-	-	+	-	-	-	-	+	+	+	+	-
28	8.35	581.1659	581.1681	C30H29O12	Tri-4-hydroxyphenylacetyl glucoside	Hydrolyzable tannins		+++	+++	+++	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
29	8.86	300.0845	300.1567	C13H16O8	Hydroxybenzoyl hexose	Hydroxybenzoic		+	-	+	-	-	+	-	-	-	++	+	-	+	+
30	10.83	474.0798	473.0723	C22H18O12	Dicaffeoyltartaric acid 2	Hydroxycinnamic		+	-	+	+	+	-	-	+	+	+	+	+	+	+++
31	11.72	353.1358	353.0867	C16H18O9	Caffeoylquinic acid 2	Hydroxycinnamic		-	+++	+++	-	-	-	+	-	-	-	-	-	-	+++

-	No change or diminution
+	Increase in 0-25%
++	Increased 26-50%
+++	Increased 51-100%
++++	Increased >100%

[a]AA, arachidonic acid; HP, Harpin protein; MJ, methyl jasmonate; SA, salicylic acid; W, water.

plant defense mechanisms, which will induce the synthesis of phytochemicals, is not activated.

2.2. Effect of Elicitors on the Polyphenol Profile. To evaluate the effect of stress response mediated by elicitors on the profile of phenolic compounds found in green and red butterhead lettuce, a qualitative high-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF/MS−MS) analysis was carried out. The compounds were elucidated based on the available data on the literature using the 4 digit mass obtained compared with mass expected, as well as the isotopic profile including fragment ions.[24,25] The identities, retention times, formula, and masses from lettuce cultivars by HPLC-QTOF-MS are presented in Tables 1 and 2. The qualitative analysis showed that a total of 46 phenolic compounds were identified in green and red butterhead lettuce, according to their exact mass and isotopic profile. These compounds were classified according to their polyphenol family (flavonoids, hydroxybenzoic acids, and hydroxycinnamic acids). The response of each polyphenol family varied according to the type of lettuce as well as the applied treatment (elicitor and concentration). Qualitative analysis was carried out by comparing the relative abundance of each phenolic molecule present in each sample against its control (green or red control). The effect of elicitors on individual phenolic compounds in green lettuce is shown in Table 1. In this table, it is possible to observe that almost all elicitors increased the content of hydroxybenzoic and hydroxycinnamic acids, while practically no effect was observed for flavonoids. Hydroxybenzoic acids, such as galloyl hexose, showed an increase with most treatments, where MJ (15 and 90 μM), AS (15 μM), and HP (30 mg/L) were the elicitors with the highest effect on this polyphenol family. However, in general terms, the change observed in the content of hydroxybenzoic acids, except for the samples treated with SA (15 μM), which increased the abundance of all hydroxybenzoic acids identified, was not that higher compared with the effect on hydroxycinnamic acids. Hydroxycinnamic acids were the polyphenols that had a higher increase in green butterhead lettuce. MJ, in all concentrations, was the elicitor with the greatest effect on this

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peak	Rt (min)	mass expected [H-]	mass obtanied	formula	proposed compound	class	W	AA 15 μM	AA 45 μM	AA 90 μM	SA 15 μM	SA 45 μM	SA 90 μM	MJ 15 μM	MJ 45 μM	MJ 90 μM	PH 30 mg/L	PH 60 mg/L	PH 120 mg/L
1	1.54	331.0665	331.0661	C22H18O1	Galloyl-hexose	Hydroxybenzoic		+	-	-	-	-	-	-	-	-	-	+	++++
2	2.28	300.0845	299.0787	C13H16O8	Salicylic acid-O-hexoside	Hydroxybenzoic		++++	++++	-	++++	++++	++++	++++	++++	++++	-	-	++++
3	2.29	262.0147	261.0062	C9H10O7S	Dihydrocaffeic acid sulfate	Hydroxycinnamic		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4	2.70	315.0716	315.0712	C13H15O9	Dihydroxybenzoic acid hexose	Hydroxybenzoic		+	-	-	-	-	-	-	-	-	-	+	+
5	2.85	154.0266	153.9876	C7H6O4	Dihydroxybenzoic acid	Hydroxybenzoic		-	-	++++	++++	++++	-	-	++++	++++	-	++++	-	++++
6	4.02	312.0481	311.0296	C13H12O9	Caffeoyltartaric acid (caftaric acid) 2	Hydroxycinnamic		-	+	+	+	+	+	+	+	++	+	+	+	+
7	4.28	315.0716	315.0712	C13H15O9	Dihydroxybenzoic acid hexose	Hydroxybenzoic		++	-	-	-	-	+	-	-	-	-	++	+	++++
8	4.43	342.0951	341.0880	C15H18O9	Caffeoyl-hexose	Hydroxycinnamic		+	++	-	-	-	+	+	+	+	++	+	+	+
9	4.89	579.2078	579.0975	C28H35O13	Syringic acid hexose	Hydroxybenzoic		+	-	++	+	+	+	+	+	+	+	++	+	+
10	5.32	343.1029	343.1023	C15H19O9	Dihydrocaffeic acid hexose	Hydroxycinnamic		++	-	++	++	-	++	++	-	-	-	++	++	+
11	5.61	326.0638	325.0548	C14H14O9	Fertaric acid	Hydroxycinnamic		-	-	-	-	++++	-	++++	-	-	-	++++	-	-
12	5.64	192.0634	191.0544	C7H12O6	Quinic acid	Hydroxybenzoic		-	+	++++	++++	-	-	-	++++	++++	++++	++++	++++	+
13	5.73	385.1135	385.1131	C17H21O10	Sinapoyl glucoside	Hydroxycinnamic		+	+	-	+	+	-	+	+	+	-	-	-	-
14	5.74	353.1358	353.0864	C16H18O9	Caffeoylquinic acid	Hydroxycinnamic		-	+	+	++	+	+	+	+	+	+	+	+	+
15	5.83	339.0716	339.0711	C15H15O9	Esculetin 6-O-glucoside	Coumarin		+	+	+	+	+	+	-	+	+	+	+	+	-
16	5.93	325.0923	325.0913	C15H17O8	p-Coumaroyl glucoside	Hydroxycinnamic		-	+	-	-	-	-	+	-	-	-	+	-	-
17	6.03	725.1201	725.1198	C30H29O21	Quercetin 3-O-(6″-O-malonyl)-glucoside 7-O-glucuronide	Flavonol		+	+	++	++	-	-	+	-	-	+	-	-	-
18	6.06	180.0423	179.0333	C9H8O4	Caffeic acid	Hydroxycinnamic		+	+	+	+	++	++	+	+	+	-	++	++	++
19	6.13	224.0685	223.0608	C11H12O5	Sinapic acid	Hydroxycinnamic		++	++	-	-	-	++	+	++	++	++	+	-	-
20	6.35	296.0532	295.0447	C13H12O8	Caffeoylmalic acid	Hydroxycinnamic		-	+	+	+	+	+	++++	-	-	-	+	+	-
21	6.65	533.0931	533.0925	C24H21O14	Luteolin malonylhexose	Flavonol		+	++++	-	++++	++++	+	-	-	-	++++	-	-	++++
22	6.72	508.1217	507.1139	C23H24O13	Syringetin 3-O-glucoside	Flavonol		-	-	+	+	+	++	-	+	+	+	+	+	+
23	6.78	168.0423	167.9356	C8H8O4	Vanillic acid	Hydroxybenzoic		-	++++	++++	++++	-	-	-	++++	++++	-	++++	++++	++++
24	6.81	474.0798	473.0723	C22H18O12	Dicaffeoyltartaric acid	Hydroxycinnamic		+	+	+	+	+	+	-	-	-	+	+	+	-
25	6.85	452.0955	451.0876	C20H19O12	2-O-p-hydroxybenzoyl-6-O-galloyl glucoside	Hydroxybenzoic		+	+	++	+	+	+	+	+	+	+	++	+	+
26	6.92	490.1111	489.1034	C23H22O12	Kaempferol 3-O-6-acetylglucoside	Flavonol		-	+	-	+	-	+	-	-	-	+	+	+	+
27	6.97	312.0481	311.0296	C13H12O9	Caffeoyltartaric acid (caftaric acid) 1	Hydroxycinnamic		+	+	+	++	+	+	+	+	+	+	+	+	+
28	7.01	180.0423	179.0333	C9H8O4	Caffeic acid	Hydroxycinnamic		-	-	++	-	-	-	++++	++++	++++	-	++++	++++	++++
29	7.06	522.1373	521.1299	C24H26O13	Rosmarinyl glucoside	Hydroxycinnamic		-	+	+	+	+	+	+	-	-	+	+	+	+
30	7.20	448.1006	447.0944	C21H20O11	Kaempferol-O-dihexoside	Flavonol		++	-	++	-	-	++	-	++++	++++	++++	-	-	++++
31	7.24	302.0427	301.0334	C15H10O7	Quercetin	Flavonol		+	++++	++++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
32	7.30	477.0669	477.0672	C21H17O13	Quercetin 3-glucuronide	Flavonol		+	++++	+	++++	++++	++++	+	+	+	++	++	++	++
33	7.35	463.0877	463.0871	C21H19O12	Quercetin 3-glucoside	Flavonol		+	+	+	+	+	+	+	+	+	+	-	-	-
34	7.37	516.1268	515.1188	C25H24O12	Dicaffeoylquinic acid	Hydroxycinnamic		-	-	-	+	-	-	-	-	-	-	-	-	-
35	7.42	500.0722	499.0631	C21H21ClO12	Delphinidin 3-galactoside	Anthocyanin		++++	++++	-	-	-	-	++++	++++	++++	-	-	-	-
36	7.45	457.0771	457.0808	C22H17O11	Caffeoyltartaric-p-coumaroyl acid	Hydroxycinnamic		+	+	+	++	+	+	-	-	+	+	-	-	-
37	7.48	296.0532	295.0447	C13H12O8	Caffeoylmalic acid	Hydroxycinnamic		-	-	-	-	-	-	-	+	+		-	-	-
38	7.56	550.0959	549.0884	C24H22O15	Quercetin malonylglucoside	Flavonol		+	+	+	+	+	+	+	+	+	+	+	+	+
39	7.73	448.1006	447.0925	C21H19O11	Kaempherol 3-O-galactoside	Flavonol		-	-	-	++	-	-	-	-	-	-	-	-	-
40	7.79	461.1006	461.0719	C21H18O12	Kaempherol-3-O-glucuronide	Flavonol		+	+	+	+	+	-	+	-	-	-	+	-	-
41	7.84	499.1240	499.1253	C16H17O8	p-Coumaroyl-caffeoylquinic acid	Hydroxycinnamic		-	-	-	-	-	-	-	-	-	-	-	-	-
42	8.35	581.1659	581.1681	C30H29O12	Tri-4-hydroxyphenylacetyl glucoside	Hydrolyzable tannins		-		-	-	-	-	-	-	-	+	-	-	-
43	8.60	285.0399	285.0456	C15H9O6	Kaempferol	Flavonol		++	+	+	++	+	++	+	-	-	+	+	+	-
44	8.86	300.0845	300.1567	C13H16O8	Hydroxybenzoyl hexose	Hydroxybenzoic		-	-	-	-	-	-	-	-	-	-	-	+	++
45	10.83	474.0798	473.0723	C22H18O12	Dicaffeoyltartaric acid 2	Hydroxycinnamic		++	++++	++++	+	++++	++++	-	+	+	++++	++++	++++	++++
46	11.72	353.1358	353.0867	C16H18O9	Caffeoylquinic acid 2	Hydroxycinnamic		+	++++	++++	++++	+	++++	+	+	+	++++	+	+	++++

-	No change or diminution
+	Increase in 0-25%
++	Increased 26-50%
+++	Increased 51-100%
++++	Increased >100%

[a]AA, arachidonic acid; HP, Harpin protein; MJ, methyl jasmonate; SA, salicylic acid; W, water.

phenolic acid family. HP (30 and 120 mg/L) also showed an increase in hydroxycinnamic acid content; meanwhile, all AA treatments showed the lowest effect on hydroxycinnamic acid content. Compounds such as fertaric acid, caffeoylmalic acid, and caffeoylquinic acid showed a higher increase compared to the control. Similar phenolic profile variations due to elicitation in butterhead lettuce were recently reported by Viacava et al.[11] MJ (90 μM) showed the highest increase in dihydrocafeic acid-hexose, fertaric acid, and caffeoylmalic acid among others. MJ (45 and 90 μM) and HP (30 and 60 mg/L) showed the highest increase in flavonoids compared with the control. Among identified flavonoids in green lettuces,

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quercetin, kaempferol, and isoharmetin derivatives showed the highest increase after elicitation, while isoquercetin, quercetin malonylglucoside, and quercetin-3-glucuronide, remained practically unchanged after elicitation. As previously mentioned, the effect of elicitors on the phytochemical profile is related to several factors such as the time of application, concentration of elicitor, and lettuce variety to which the treatments are applied. These results agree that the type and concentration of elicitor are factors that must be considered before it is used as treatment, due to the effect that could be produced in phytochemicals such as flavonoids.[7]

Polyphenols identified in red lettuce are shown in Table 2. Hydroxybenzoic acids were not highly modified in this lettuce variety after elicitation. Only AA (45 and 90 μM) and HP (60 and 120 mg/L) showed a higher effect on the content of this polyphenol family; meanwhile, SA (45 and 90 μM) was the elicitor with the lowest effect on these compounds. Among hydroxybenzoic acids, galloyl hexose, salicylic acid-O-hexoside, and vinyl acid showed the highest increase compared to the control. HP (120 mg/L) highly modified the content of quinic acid and salicylic acid. Hydroxycinnamic acids in red lettuce were also modified after elicitation. This effect was observed mainly on caffeic acid derivatives, in agreement with results observed in green lettuce. Compounds derived from caffeic acid, which can be directed via tartaric acid or via quinic acid, one for the synthesis of chicoric acid and the other for the chlorogenic acid pathway, have been reported in different varieties of lettuce such as romaine and butterhead.[11,26] Identified flavonoids in red lettuce were flavonols and one anthocyanin. SA and MJ, at all concentrations, were the elicitors with the highest effect on this polyphenol family. In the case of red lettuce compounds, quercetin and kaempferol were the polyphenols with the highest response after treatment. Złotek et al.[9] reported that the use of 100 μM solutions of AA and jasmonic acid produced modifications in the polyphenolic profile, increasing the concentration of kaempferol, quercetin, and caffeic acid derivatives such as chlorogenic acid and ferulic acid when applied in green butterhead lettuce.

In red lettuce, the major effect was observed in flavonoid-type compounds, while in green lettuce, the main effect was observed in hydroxycinnamic acids. This effect on flavonoid content in red lettuces agrees with previously reported results. In Rutgers Scarlet Lettuce, and other red-colored lettuces, flavonoid-type polyphenols were higher than phenolic acids.[27] It has also been reported that flavonoid content in red varieties was higher than in green varieties.[26,28]

To elucidate the main effect of elicitors on the polyphenol profile, a principal component analysis (PCA) was carried out (Figure 1s, Supporting Information). A biplot of two main principal components (PCs) characterized the polyphenol families (phenolic acids and flavonoids) of green (Figure 1sA) and red (Figure 1sB) butterhead lettuces, with a cumulative explained total variance of 78.19 and 60.39%, respectively. For green lettuce, the first principal component (PC1) had the highest eigenvalue of 2.62 and accounted for 52.49% of the variability in the data set. The second PC (PC2) had an eigenvalue of 1.28 and accounted for 25.69% of the variance in the data. For red lettuce, PC1 and PC2 had eigenvalues of 2.21 and 1.41 and accounted for 36.86 and 23.52% of the variability in the data set, respectively. In green lettuce, all the treatments increased their polyphenol content compared with the control (Figure 1sA). Hydroxycinnamic acids were affected by all treatments, except for AA (90 μM), AA (15 μM) and MJ (15 μM) being the elicitors with the highest effect on this polyphenol family. All treatments showed an increase in flavonols and hydroxybenzoic acids in green lettuce. SA (15 μM) and MJ (15 μM) showed the highest increase in comparison with the control. Based on the PCA analysis, it is possible to assume that elicitation of green lettuces impacted phenolic acids, such as hydroxybenzoic acids as well as hydroxycinnamic acid content and in a lower degree in flavonols. In red lettuce (Figure 1sB), all treatments affected hydroxycinnamic acid content, AA (45 and 90 μM), MJ (90 μM), and HP (60 mg/L) being the elicitors with the greatest effect on this variety. HP (60 and 120 mg/L) highly increased the hydroxybenzoic acids in red lettuce, suggesting that this elicitor could be activating the pathway to their synthesis. This effect can be explained because HP is extracted from several bacteria such as *Pseudomonas*. So, this elicitor treatment has the characteristic that simulates a bacterial attack on the plant, which could trigger the increase of these compounds in response to the presence of potential bacterial infection to the plant.[29] Hydroxybenzoic acids have the ability to act as markers of many metabolic processes, being among them the plant signaling in the presence of the attack of a herbivore or pathogen.[30] Flavonoids, such as anthocyanins, and flavonols, in red lettuce, were modified in higher proportion by AA (15 μM) and SA (15 and 90 μM). In comparison with green lettuce, in red lettuce, there was an effect on the content of flavonoid-type molecules; however, these compounds' overall concentration was not highly incremented except for MJ (90 μM). The modifications on flavonoids in red lettuce could be related with their concentration; it has higher concentration than green lettuce, leading to an increase but in a lower impact than that observed in the green samples.[2,15]

2.3. Effect of Elicitors on Expression of Enzyme Transcripts. Quantitative polymerase chain reaction (qPCR) was used to evaluate the effect of elicitors on enzyme transcripts. The fold increase of transcripts compared to the control is shown in Table 3. All treatments influenced PAL gene expression, responsible for the synthesis of phenolic compounds. PAL gene transcripts significantly increased up to 9.96-fold (AA 90 μM) compared with the control in green lettuce. AA (15 μM), SA (45 and 90 μM), MJ (15 μM), and HP (30 mg/L) significantly increased 7.5-, 8.6-, 5-, 4.93-, and 9.07-fold, respectively, in PAL transcript expression in green lettuce. Interestingly, for some elicitor, such as AA, MJ, and HP, the highest effect was observed at lower elicitor concentration. CHS transcript, responsible for the synthesis of flavonoids, in green lettuce did not increase significantly as PAL. UFGT gene, responsible of the synthesis of anthocyanins, was not evaluated in green lettuce because anthocyanins were not detected in green lettuce. LBC gene expression, responsible for the synthesis of carotenoids, increased significantly by 2-fold with AA (45 μM) and HP (60 and 120 mg/L) with 1.6- and 2.8-fold at the lower and highest concentration, respectively. MJ also significantly increased the gene expression with a higher effect on the MJ 90 μM concentration (1.98-fold). Gene expression results correlated with those of total CAR content.

In the case of red lettuce, the observed effect of elicitors on the gene expression changes was lower compared to green lettuce. Green lettuces showed higher gene expression of the enzymes involved in the different syntheses of secondary metabolites compared with red lettuce. This agrees with

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Table 3. Enzyme Transcript Fold Increase in Butterhead Lettuce in Comparison with the Control[a]

		gene			
variety	treatment	PAL	CHS	UFGT	LBC
green lettuce	AA 15 μM	7.53 ± 0.73*	0.14 ± 0.05	N/A	1.26 ± 0.41
	AA 45 μM	1.21 ± 0.26	0.17 ± 0.113	N/A	2.53 ± 0.17*
	AA 90 μM	9.96 ± 1.56*	0.17 ± 0.07	N/A	1.23 ± 0.37
	SA 15 μM	2.44 ± 0.38	0.09 ± 0.02	N/A	0.29 ± 0.07
	SA 45 μM	8.64 ± 0.98*	0.23 ± 0.05	N/A	0.99 ± 0.06
	SA 90 μM	5.02 ± 0.79*	0.32 ± 0.08	N/A	0.5 ± 0.03
	MJ 15 μM	4.93 ± 0.58*	0.29 ± 0.10	N/A	1.60 ± 0.38*
	MJ 45 μM	3.21 ± 0.43	0.44 ± 0.12	N/A	0.56 ± 0.13
	MJ 90 μM	2.54 ± 0.77	0.35 ± 0.20	N/A	1.98 ± 0.42*
	HP 30 mg/L	9.07 ± 1.03*	0.33 ± 0.06	N/A	1.12 ± 0.12
	HP 60 mg/L	1.79 ± 0.01	0.13 ± 0.01	N/A	1.68 ± 0.29*
	HP 120 mg/L	2.13 ± 0.15	0.47 ± 0.155	N/A	2.84 ± 1.59*
red lettuce	AA 15 μM	0.41 ± 0.13	0.09 ± 0.02	8.79 ± 1.16*	0.32 ± 0.05
	AA 45 μM	1.61 ± 0.02*	0.15 ± 0.04	3.36 ± 0.38*	1.31 ± 0.16*
	AA 90 μM	0.71 ± 0.15*	0.17 ± 0.03	4.23 ± 1.36*	0.37 ± 0.12
	SA 15 μM	0.59 ± 0.10	0.09 ± 0.02	0.23 ± 0.09	0.26 ± 0.02
	SA 45 μM	0.37 ± 0.04	0.16 ± 0.04	0.38 ± 0.16	0.46 ± 0.06
	SA 90 μM	0.65 ± 0.10*	0.26 ± 0.07*	1.19 ± 0.42	0.52 ± 0.17
	MJ 15 μM	0.51 ± 0.15*	0.24 ± 0.04	0.38 ± 0.15	0.16 ± 0.05
	MJ 45 μM	0.64 ± 0.08*	0.29 ± 0.04*	0.41 ± 0.12	0.74 ± 0.15*
	MJ 90 μM	0.58 ± 0.04	0.26 ± 0.05*	0.45 ± 0.30	0.94 ± 0.38*
	HP 30 mg/L	2.48 ± 0.15*	0.79 ± 0.04*	0.97 ± 0.31	0.52 ± 0.09
	HP 60 mg/L	1.34 ± 0.26*	0.28 ± 0.10*	0.57 ± 0.04	0.4 ± 0.06
	HP 120 mg/L	0.4 ± 0.09	0.30 ± 0.01*	0.49 ± 0.16	0.3 ± 0.01

[a]AA, arachidonic acid; CHS, chalcone synthase; HP, Harpin protein; LBC, lycopene beta cyclase; MJ, methyl jasmonate; SA, salicylic acid; UFGT, UDP-glucose flavonoid 3-O-glucosyltransferase. Values represent the average (±SE) of three values of gene transcript fold increase. The asterisk (*) represents a significant fold increase compared with the control by ANOVA ($p \leq 0.05$). N/A represents gene expression not evaluated.

previous reported data where due to the high concentration of phytochemicals in red lettuce, the response after elicitor treatment was lower than in green lettuce.[2,15,26] PAL gene expression increased in several treatments, being the largest effect with HP (30 and 60 mg/L) with 2.4- and 1.3-fold compared with the control, respectively, followed by AA (45 and 90 μM) with 1.6- and 0.7-fold, respectively. CHS gene expression was not highly modified after elicitation; however, some treatments, such as MJ and HP, showed slightly significant increases compared to the control. UFGT gene expression was modified by most of the treatments, AA (15 μM) treatment being the one with the highest effect with an 8.7-fold increase in comparison with the control. LBC gene expression was only significantly increased with AA (45 μM) and MJ (45 and 90 μM).

qPCR analysis showed changes in the gene expression of genes involved in secondary metabolite synthesis. Elicitors' effect on the stress response in lettuce had an influence at a genetic level, modifying the relative expression of PAL, CHS, UFGT, and LBC genes. This modification could be related to the activation of these defense mechanisms, where the biosynthesis of secondary metabolites has an important role in plant defense. PAL is the key enzyme of the phenylpropanoid metabolism.[31] Jasmonates are known to activate transduction signals that trigger the synthesis of defense genes.[32] MJ applied exogenously has been effective for the production of secondary metabolites such as compounds derived from the phenylpropanoid pathway (phenolic compounds), alkaloids, and terpenes.[32] MJ has been reported to activate PAL gene expression in tobacco.[33] In grapes, MJ increased relative expression of PAL, CHS, and UFGT enzymes.[34] Jasmonates have also been reported to regulate transcriptional factors related to the synthesis of anthocyanins, such as the WD-repeat/bHLH/MYB transcriptional complexes.[35] HP is commonly used as an elicitor because it activates hypersensitivity response in the plant that later leads to acquired systemic response.[36] It is also known that it can modulate the expression of defense genes, especially those related to the lignification of cell walls and signal transduction.[37] In lettuce, the HP elicitor has been used although the studies carried out have evaluated the production of antioxidants, without considering the influence of genomic implications on the phytochemical concentration.[36] CHS and UFGT are involved in anthocyanin biosynthesis. CHS is the first enzyme in the flavonoid biosynthetic pathway responsible for catalyzing the condensation reaction of three units of malonyl-CoA acetate to produce 2′,4,6,4-tetrahydroxychalcone.[38] UFGT is involved in the anthocyanin biosynthesis by the addition of glucose to an anthocyanidin. AA is an eicosatetranoic acid commonly used in agriculture to protect crops against phytopathogens.[39] This elicitor induces a systemic response in the plants to produce resistance against the attack of the pathogens. AA can also induce a hypersensitivity response.[39] In potatoes infected with *Pseudomonas infestans*, AA applied at different concentrations resulted in an increased production of phytoalexins, which acted as an antibiotic in crops.[40] It has been observed in fungi, such as *Blakeslea trispora*, that the application of AA increased the expression of genes involved in the synthesis of carotenoids, such as LBCY, resulting in an increase of lutein and β-carotene content.

If we summarize all the obtained results, then a pathway, where elicitors may lead to an increase of phytochemicals, could be established. Figure 3 shows the synthetic pathway of phenolic compounds. According to results shown in Figure 1, MJ could activate the biosynthesis of hydroxycinnamic acids in green lettuce. This is in agreement with HPLC-MS results in which caffeic acid derivatives were the main phenolic acids produced after elicitation. Hydroxybenzoic acids increased to a lower degree compared to hydroxycinnamic acids. These results may suggest that elicitors activate the PAL gene pathway, the key enzyme of the phenylpropanoid metabolism,

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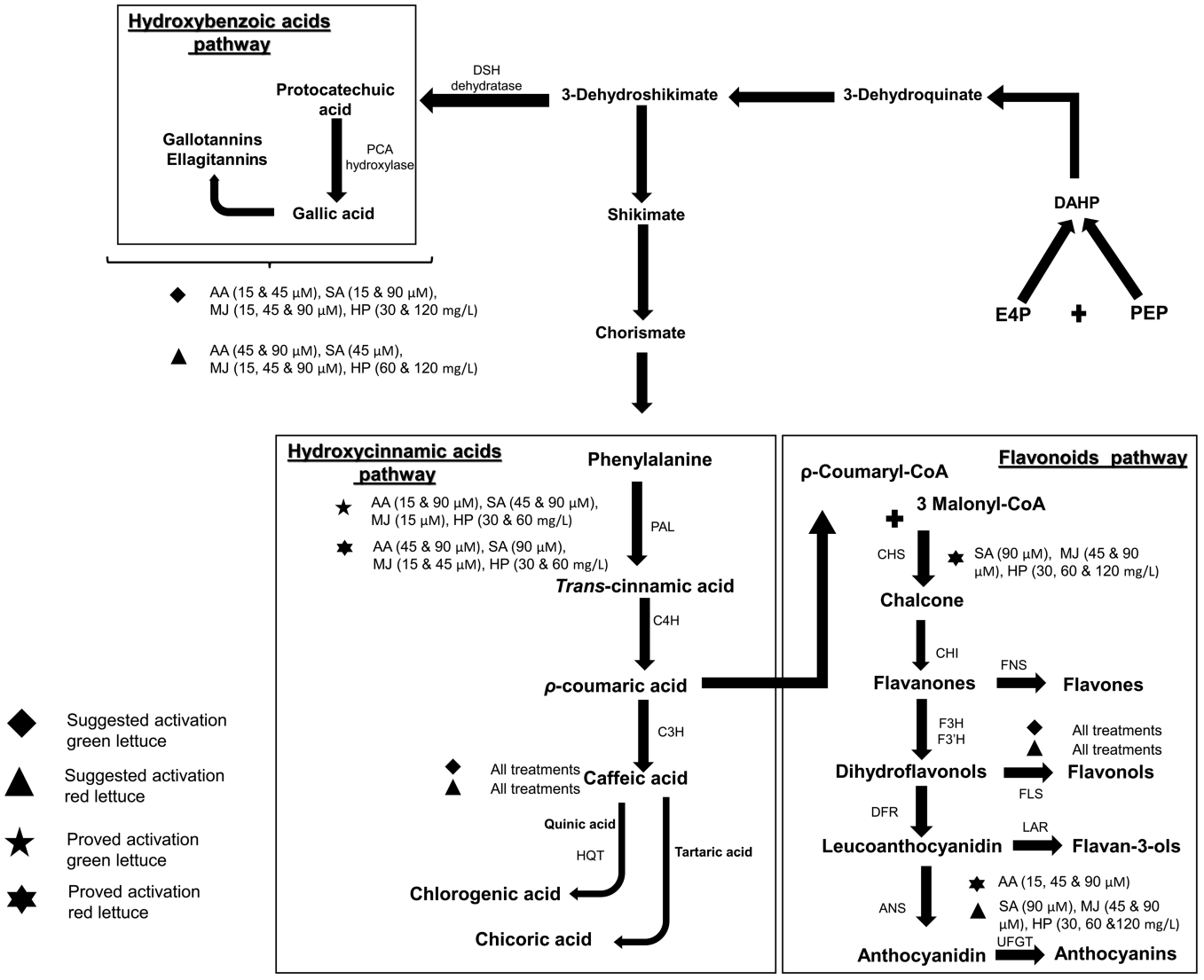

Figure 3. Proposed ways activated by elicitors on the metabolism of phenylpropanoids.

as observed by the gene expression increment of this enzyme by all elicitor treatments in green lettuce. In the case of CHS, the effect of elicitors on this gene transcript was smaller than that observed for PAL although the effect on total flavonoid concentration had a notorious change due to elicitation. There is a hypothesis that increases in flavonoids such as naringenin and luteolin influence the inhibition of CHS to avoid toxic levels that could affect the plant, this being a possible reason for why the flavonoid content increases but the CHS transcript was not highly modified.[41]

AA (90 μM) increased PAL gene expression, in agreement with the observed increase on TFC in red lettuce. Other elicitors also increased PAL gene expression even though in a lesser degree. Similar results were observed in green lettuce, suggesting that in both lettuces varieties, elicitors activate the enzyme transcript, which may synthesize more phenolic compounds. Flavonol synthesis was also activated by SA and MJ treatments in red lettuce, suggesting that these elicitors could be activating the biosynthesis of these polyphenol families. Similar to green lettuces, the content of hydroxycinnamic acids, caffeic acid derivatives in red lettuces, increased with all treatments to a lesser degree than in green samples.

LBCY transcript increased after elicitation with almost all treatments in green lettuce. AA and HP protein were the elicitors with the highest effect on the expression of these gene transcripts. Total CAR content was also higher with these two elicitors, suggesting that both elicitors activated the CAR synthesis through the activation of the LBC enzyme. In red samples, LBCY gene was only activated by AA and MJ. However, total CAR content increased in almost all treatments, suggesting that even though only slight increases in LBCY gene expression were observed, this enhancement was enough to have an effect on the total carotenoid content. These results set the basis to generate information that could lead to clear the mechanism of elicitors. However, it is still needed to perform more experiments to confirm the pathways activated by elicitors applied to lettuce samples.

3. CONCLUSIONS

The use of elicitors can be a useful tool to increase bioactive molecules in lettuce. MJ (90 μM) was the treatment with the highest increment of phytochemicals in green and red lettuce. MJ enhanced polyphenol content in both varieties, while AA and HP had the main effect on carotenoids in both varieties. This increase in phytochemicals could be linked by the

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increment of gene transcripts, which leads to an enhanced phytochemical biosynthesis. Further investigations are needed to better understand the pathways activated by elicitations that result in a phytochemical increment in fruits and vegetables.

4. MATERIALS AND METHODS

4.1. Plant Materials, Growing Conditions, and Elicitor Administration. Butterhead lettuce plants (*Lactuca sativa* L. var. *capitata*) of green (FVM02 seed) and red (FRM02 seed) varieties were hydroponically grown (April−May 2017) in a recirculating system in a greenhouse with an average photoperiod of 10 h/day, at 25−28 °C, 40−60% relative humidity, and 35% of sunlight blocking at InnoBio Hidroponia, Inc. A hydroponic technique was used due to its capacity to obtain a higher yield compared with other agricultural methods.[42] Mineral nutrients consisted of N (16%), P (4%), K (17%), and a stage II micronutrient solution mix (InnoBio Hidroponia, Inc.; pH 6.8, EC = 1800 mS). Elicitors used were arachidonic acid (AA), salicylic acid (SA), and methyl jasmonate (MJ) at 15, 45, and 90 μM, as well as Harpin protein (HP) at 30, 60, and 120 mg/L dissolved in deionized water (elicitors were previously dissolved in 1 mL of ethanol).[9,14] A group of samples and water with only 1 mL of ethanol were added. Control samples with no treatment were added. The concentrations for each elicitor were selected based on those concentrations previously reported at which no toxic effect was observed.[2,9,43,44] Elicitor treatments were applied on the 7th preharvest day on green lettuces and the 15th preharvest day on red lettuces, according to our previous report.[2] Each experimental unit consisted of five lettuces randomly selected and assigned to one treatment, with a total of 70 lettuces per variety (70 green and 70 red lettuce). Each sample was treated by foliar aspersion, with 3 sprays of each elicitor (approximately 1.70 mL). Lettuce samples were harvested 60 days after being transplanted, transported to the laboratory, freeze-dried (Labconco 6 Freezone, Labconco Corporation, Kansas City, MO, USA), homogenized, sieved through a mesh (0.42 mm), and vacuum stored at −80 °C until analysis.

4.2. Extraction and Quantification of Polyphenols. Polyphenols were extracted with 80% (v/v) methanol in water, according to Moreno-Escamilla et al.[2] Total phenolic content (TPC) was determined by the Folin−Ciocalteu method as previously reported.[45] Results were expressed as mg of gallic acid equivalents (GAE)/g of dry sample.

Total flavonoids (TF) were determined by the aluminum chloride method with slight modifications.[46] Catechin was used as a standard, and results were expressed as milligrams of catechin equivalents (CE) per gram of dry sample.

4.3. Extraction and Quantification of Anthocyanins. Total anthocyanin (TAN) content was extracted and quantified only in red lettuce using the method developed by Lee et al.,[47] modified to apply it in lettuce by Moreno-Escamilla et al.[2] Absorbance was measured at 520 and 700 nm on a microplate after 30 min of incubation at room temperature. Absorbance (A) was calculated using eq 1.

$$A = (A_{520\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5} \quad (1)$$

Total anthocyanin content was calculated using the eq 2 (mg of cyanidin 3-rutinoside/g). Results were expressed as mg of cyanidin 3-rutinoside/g of dry sample.

$$\text{total anthocyanins} = (A \times 449.2 \times 25 \times 1000)/(26900 \times 1) \quad (2)$$

4.4. Extraction and Quantification of Carotenoids. Total carotenoids (CAR) were quantified in green and red lettuce after extracting samples with acetone.[48] Fifty milligrams of the freeze-dried sample was mixed with 10 mL of acetone, sonicated for 20 min (40 kHz), and centrifuged at 2000×g for 10 min (4 °C). The supernatant was collected, and the residue was re-extracted two more times under the same conditions. Supernatants were combined, and a total of 250 μL were taken and placed on a microplate to be read at 474 nm. Total carotenoids were determined using eq 3

$$\text{mg } \beta - \text{carotene/mg sample} = (A \times V \times \text{DF} \times 10)/(g \times \text{E1\%cm}) \quad (3)$$

where A = absorbance, V = volume (30 mL), DF = dilution factor, g = grams of sample, and E1%cm = the specific extinction coefficient of β-carotene, which is 2500 (Jeffrey, 1997). Results were expressed in mg β-carotene/g of dry sample.

4.5. Identification of Phenolic Compounds by HPLC-ESI-QTOF/MS−MS. Freeze-dried butterhead lettuce samples were weighed (50 mg) and ultrasonically extracted using 10 mL of methanol (80% v/v) for 30 min, repeating this process for recovering and mixing supernatants. The supernatant was filtered through a 0.45 μm membrane filter and transferred to an autosampler vial for HPLC-ESI-QTOF/MS−MS analysis.[2] An HPLC 1200 Series system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, an autosampler, and a binary pump was used to identify polyphenols. The separation was performed at 25 °C using a rapid resolution high-definition (RRHD) reverse-phase C-18 analytical column (2.1 × 50 mm, 1.8 μm particle size; ZORBAX Eclipse Plus) protected with a guard cartridge of the same packing. The mobile phase consisted of formic acid (0.1%) in Milli-Q deionized water (A) and acetonitrile (B). A volume of 1 μL (methanolic extract) was injected at a flow of 0.4 mL/min. The gradient used was 0−4 min, 90% A; 4−6 min, 70% A; 6−8 min, 62% A; 8−8.5 min, 40% A; and 8.5−9.5 min, 90% A. The column was re-equilibrated for 2 min before each injection. MS was obtained using a quadrupole time-of-flight (QTOF) mass spectrometer (G6530BA, Agilent Technologies, Palo Alto, CA, USA). ESI-MS measurements were done by negative ionization with the gas temperature set at 340 °C, drying gas flow of 13 L/min, nebulizer of 30 psi. The MS was recorded in the range of 100 to 2000 m/z. Phenolic compound identification was carried out using the software MassHunter Qualitative Analysis B.07.00 (Agilent Technologies Inc., Santa Clara, CA, USA). A semiqualitative analysis was performed, identifying phenolic compounds according to their exact mass and isotopic profile and classifiying them in different groups according to their polyphenol family.

4.6. Quantification of Enzyme Gene Transcripts. The extraction of total mRNA was carried out using the QIAGEN RNeasy Plant Mini Kit (Cat. 74904), following the manufacturer's specifications. The concentration and quality of RNA were evaluated by spectrometry using A_{260}/A_{280} ratios. A retro transcription was performed using the QIAGEN

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QuantiTect Reverse Transcription Kit (Cat. 205311), according to the manufacturer's instructions.

The real-time qRT-PCR analysis was performed using the iQ5 Real-Time PCR detection system (Bio-Rad). The QuantiTect SYBR Green PCR Kit (QIAGEN; Cat. 204143) was used following the manufacturer's instructions with some modifications. Primers of enzymes related with phytochemical biosynthesis phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT), lycopene beta cyclase (LBC), and serine/threonine-protein phosphatase PP2A-1 catalytic subunit (PP2A-1) housekeeping were evaluated (Table 1s, Supporting Information). Each reaction contained 12.5 μL of 2x QuantiTect SYBR Green PCR Master Mix, 2.0 μL of cDNA, and direct and reverse primers at the appropriate concentration (5 μM) in a total volume of 25 μL supplemented with RNase-free sterile distilled water. The amplification conditions were the following: 95 °C for 15 min followed by 40 cycles of 15 s at 95 °C, 30 s at the specific primer temperature, and final elongation at 72 °C for 2 min. The melting curve was performed by melting the amplified mold from 65 to 95 °C, rising the temperature by 0.5 °C/cycle. Negative controls were included. Three technical repetitions were used for each sample. The relative concentration of genes of enzymes related to secondary metabolites was assessed by using the housekeeping gene PP2A-1.

4.7. Statistical Analysis. A pool of all individuals for each treatment (five lettuces) was made to minimize the variability among individual samples. All analyses were carried out in triplicate. Values are expressed as the mean ± standard deviation (SD). For phytochemical quantification and gene concentration, a one-way ANOVA and Tukey analyses were performed to determine statistical differences ($p < 0.05$) between elicitors and their concentrations in the varieties separately. For HPLC analysis, a principal component analysis (PCA) was carried out to elucidate the effect of elicitors over the different polyphenol groups. Data values were analyzed using XLSTAT (Addinsoft, New York, NY, USA). Relative gene expression results were analyzed by Livak and Schmittgen's method[49] and were carried out with the Excel program (Microsoft, Redmond, WA, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00680.

PCA analysis of the effect of elicitors in polyphenol profile in green and red butterhead lettuce (AA, arachidonic acid; SA, salicylic acid; MJ, methyl jasmonate; HP, Harpin protein) (PDF)

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Notes

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■ ABBREVIATIONS

AA, arachidonic acid; ANS, anthocyanidin synthase; C4H, cinnamate-4-hydroxylase; C3H, coumaryl 3-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; COMT, caffeic acid o-methyltransferase; DAHP, 3-deoxy-D-arabinoheptulosonate-7-phosphate; DFR, dihydroflavonol 4-reductase; DHS, 3,5-didehydroshikimate; DQH, dehydroquinate; E4P, eristrose-4-phosphate; F3H, flavonone 3-hydroxylase; F3'H, flavonoid 3′-hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; F5H, ferulic 5-hydroxylase; HQT, hydroxycinnamoyl-coenzyme A quinate transferase; HP, Harpin protein; MJ, methyl jasmonate; LAR, leucoanthocyanidin reductase; PEP, phosphoenolpyruvate; PCA, protocatechuic acid; PAL, phenyl-

alanine ammonia-lyase; SA, salicylic acid; UFGT, UDP-glucose flavonoid 3-O-glucosyltransferase

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