The effect of protected cultivation on the nutritional quality of lettuce (*Lactuca sativa* var. *capitata* L.)

with a focus on antifogging additives in polyolefin covers

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
CCD	Carotenoid cleavage dioxygenases
CCD4	Carotenoid cleavage dioxygenase 4
CHS	Chalcone synthase
CHYB	β-Hydroxylase
CoA	Coenzyme A
COP1	Constitutive photomorphogenic 1
CRY	Cryptochromes
DAD	Diode-array detection
DLI	Daily light integral
DMAPP	Dimethylallyl diphosphate
ECHA	European Chemicals Agency
EU	European Union
EVA	Ethylene vinyl acetate
FAO	Food and Agricultural Organization of the United Nations
FCM	Food contact materials
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GGPP	Geranylgeranyl diphosphate
(HP)LC	(High performance) liquid chromatography
HY5	Elongated hypocotyl 5
IPP	Isopentenyl diphosphate
IR	Infrared
ISO	International Organization of Standardization
LDPE	Low-density polyethylene
LUE	Light use efficiency
MS	Mass spectrometry
NCED	Nine-*cis*-epoxycarotenoid dioxygenases
NPQ	Non-photochemical quenching
OR/ OR-like	Orange/ Orange-like protein
PAR	Photosynthetic active radiation
PBT	Persistent, bioaccumulative, toxic

PDS	Phytoene desaturase
PE	Polyethylene
PEP	Phosphoenol pyruvate
PHOT	Phototropins
PHY	Phytochromes
PIF1	Phytochrome interacting factor 1
PP	Polypropylene
PPFD	Photosynthetic photon flux density
PSY	Phytoene synthase
PSII	Photosystem II
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
RT-*q*PCR	Reverse transcriptase - quantitative polymerase chain reaction
SDG	Sustainable developmental goals
ToF-MS	Time-of-flight-Mass spectrometry
UN	United Nations
UV	Ultraviolet
UVCB	Unknown or variable composition, complex reaction products or biological materials
UVR8	UV resistance locus 8

INDTRODUCTION

1 Sustainable vegetable production by protected cultivation

Agricultural systems are constantly changing due to new production strategies and technologies, or changing food demands and dietary choices. With the so-called "green agricultural revolution" in the mid-20th century, productivity was more than tripled (from 1960 to 2015) through new techniques and extensive resource use, driving world hunger to historic lows.[1,2] Today's resource-intensive food production systems are no longer future-feasible. The scarcity of resources, especially land and water, is currently slowing the growth of production yields. About 40 % of global land is used for agriculture and it accounts for 70 % of global water withdrawals.[1,3] Moreover, climate change highlights the need to adapt agricultural production systems, as the food and agriculture sector contributes to about 30 % of greenhouse gas emissions.[3] The world's population is estimated to reach 9.7 billion by 2050.[1] Estimates show that by 2050, global agriculture will need to produce 48.6 % more to meet future food demand.[1]

While projections of future food production are subject to some uncertainty, studies show that the planetary health and an adequate food supply are not guaranteed under a business-as-usual scenario.[4] Overcoming them will therefore require multi-sectoral efforts, including policy, research and investment in new and digital agricultural technologies, as well as social and ethical issues. In this context, two major global agendas were agreed in 2015: the Paris Agreement and the UN Sustainable Development Goals (SDG, Agenda 2030). For agricultural food production, the SDG2 “Zero hunger” (defined by end hunger, achieve food security and improved nutrition as well as promote sustainable agriculture) is considered particularly important, among others.[5-7] However, global food consumption is projected to increase by 1.4 % annually over the next decade, which would not be compatible with achieving SDG2.[7]

Since 2015, the number of undernourished people worldwide has been on the rise again, reaching 768 million in 2021.[5] Therefore, in addition to the need for higher production, healthy diets should be promoted to address the "triple burden of malnutrition" of undernourishment, micronutrient deficiencies, as well as overweight and obesity. The EAT-Lancet scientific commission identified targets to achieve future-feasibility of food systems.[3] They developed strategies including a shift to a healthy plant-based diet, a re-orientation from producing high yields to healthy food, a sustainable intensification of agriculture as well as a zero-expansion policy of agricultural land.

Therefore, the challenge for sustainable future food could be summarized as follows: Ensure the planetary health by producing more healthy food by using resources sparingly and responsibly without using additional land.
As diets will change, the demand for plant-based foods will increase by 60 % by 2050, so vegetable production will also need to adapt.[8] One way to meet this challenge and sustainably intensify crop production is through protected cultivation. Protected cultivation is a (semi-) closed cultivation system that allows the influence and control of the environmental conditions. It includes a variety of systems, from low-tech approaches such as row covers or polytunnels to high-tech greenhouses with technological control capabilities.[8] All have their own advantages and disadvantages for sustainable cultivation.
One of the main obstacles to overcome in protected cultivation is energy consumption, especially in heated facilities, which results in high greenhouse gas emissions. Another important issue is managing the plastic waste generated by cover materials. Nevertheless, the benefits outweigh the drawbacks, with higher yields due to the controlled environment, off-season production, and the ability to produce year-round on less agricultural land. For example, greenhouse tomato yields have increased by 118 % from 1980 to 2016.[8] A closed system also offers better opportunities for biological control, improved water use efficiency, reduced pesticide use and the ability to recirculate production.[6,8] They can also be strategically located on land not suitable for open field cultivation, near transport hubs, or on urban sites like rooftop greenhouses.
Taken together, protected cultivation can contribute to the achievement of the SDG and sustainable crop production.[6,9] However, developments and investments towards sustainable and circular production systems are necessary. To achieve this goal, it is important to address the challenge of energy use. Recently discussed options include replacing fossil fuels and maximizing the use of natural sunlight.[8] The latter can be achieved by making strategic choices regarding cover materials. Such selection depends, for example, on region, season, or type of crop being produced. A better understanding of cover materials and their effects on crop quality is seen as an opportunity for high-yielding and nutrient-rich vegetable production[10,11], particularly by targeting the light requirements of crops.

1.1 Agricultural polymer films

The expansion of greenhouse cultivation worldwide is associated to the increase in plastic production that began in the 1950s. The first plastic-covered greenhouses were installed in USA and Japan in 1948.[12] Global plastic production has grown rapidly from 1.5 million tons (1950) to 391 million tons (2021)[13] with 3 to 5 % of global plastic is used in agriculture.[14] To date, 90 % of greenhouses are covered with plastic and 10 % with glass.[8] Global demand for greenhouse, silage and mulch film is projected to increase by 50 % by 2030.[14] Using plastics is advantageous because of the low cost of materials and the variable properties.

Plastics are generally defined as synthetic or semisynthetic organic polymers, which can be homo- or copolymeric. Plastics can be characterized by their basic composition and additives, by their raw materials (fossil or bio-based) or by their biodegradability. Products are made by molding, extruding or pressing.[14]

About 20 different polymers with varying formulations are used in agriculture.[12] The main materials are polyolefin-based, the most common being polyethylene (PE), followed by polypropylene (PP).[12,14] In Europe, the main cover material is PE (as low-density PE; LDPE), while up to 10 % is covered with ethylene vinyl acetate (EVA) or co-extruded triple layer EVA/LDPE films (**Fig. 1**).[12] Cover materials can be single, double or multi-layer films, with the latter having better energy saving properties. However, additional layers reduce light transmission (approximately 10 % reduction per additional layer).[11]

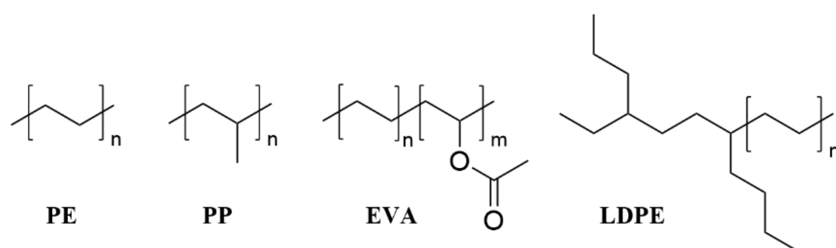

Figure 1: Core structures of polyolefin-based polymers used for agricultural covers. PE, polyethylene; PP, polypropylene; EVA, ethylene vinyl acetate; LDPE, low-density polyethylene (example).

The films have a service life of 3 to 4 years (thickness between 140 to 200 µm), due to weathering the light transmission decreases over time as the material ages.[12] The resultant waste is a source of environmental concern. For example, there are no set targets or regulations for agricultural waste management in the EU.[15] As protected cultivation is expected to make an important contribution to food security and climate change adaptation strategies, recently discussed solutions such as closed-loop systems or product labeling should be pursued.[11,14]

1.2 Additives for improved polymer properties

Plastics are not only made up of the base polymer, but also contain numerous added compounds, so called "additives". Polymer additives can be defined as "any substance intentionally added to plastics to achieve a physical or chemical effect during the processing of a material or to impart functional properties to meet the requirements of the final products".[16] As chemically divers, they are not categorized by chemical identity but by function. There are four main groups of additives: functional additives, colorants, fillers and reinforcements.[17]

Depending on the final product, different types of additives are used. In greenhouse films, for example, the aim is to protect the film from weathering (UV stabilizer) or to improve light transmission (antifogging additives).[12] Additives are often processed as masterbatches, which are highly concentrated systems consisting of a colorant, the additives, and a plastic carrier material such as a resin matrix.[18]

Almost all additives used in plastics are not chemically bound to the polymer matrix.[17,19] This results in migration and leaching from the plastics, which is less for higher molecular weight compounds.[17] Over time, loss of function occurs and leaching of additives poses a potential toxicological and ecotoxicological risk. However, data on environmental fate and possible degradation products are lacking for the majority of additives used.[19] This is despite the fact that the risk of persistent, bioaccumulative or toxic compounds is present, albeit uncertain.

According to the REACH database, 7 % of all registered compounds end up in plastic products. Of these, 40 % have a hazardous potential.[20] A recent study by Wiesinger, Wang [21] identified the use of 10,547 different substances in plastics, 55 % of these were categorized as additives. 25 % of these substances were of unknown or variable composition, complex reaction products or biological materials (UVCBs), i.e. not composed of a single compound, while other substances lack basic information and are registered under trivial names.[21]

Taken together, there are an incalculable number of compounds that can migrate from plastics, some of which are potentially hazardous. However, EU regulations only apply to food contact materials (FCM). Approval is required for the use of compounds and migration limits are set with detailed testing requirements.[22] There is no equivalent regulation for applications such as greenhouse films. A first step forward is the Plastic Additives Initiative of ECHA and industry partners, which has mapped over 400 additives in use.[20] However, this is only a small percentage compared to the compounds identified by Wiesinger, Wang [21].

1.2.1 Antifogging additives as polymer additives

While a wide range of additives are used in plastics, the focus of this thesis is specifically on antifogging additives. They are functional additives used to prevent water droplets from forming on the inside of plastic films.[23] As a component of food packaging, they increase consumer appeal by droplet-free packaging and extend food's shelf life.[18] In greenhouse films, their utilization improves light transmission through the cover material, resulting in higher yields of crops grown underneath. The absence of droplets also prevents microbial contamination of crops and lens effects, both of which reduce yield loss.[23]

The antifog effect is based on their structure. As surfactants, they contain a hydrophobic structural moiety that anchors to the polymer matrix and a hydrophilic moiety that interacts with surface water on plastic films.[23] This interaction causes the water droplets to spread out into a continuous, transparent water layer (**Fig. 2**).

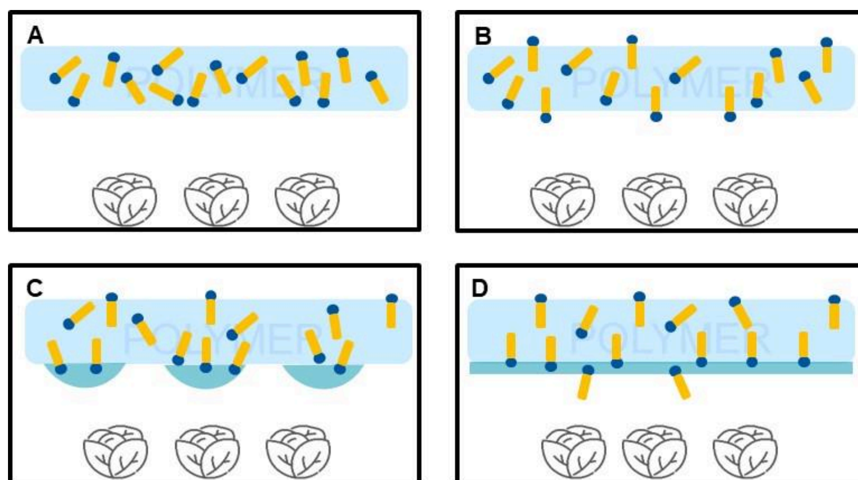

Figure 2: Mechanism of the antifog effect. Antifogging additives equally distributed in polymer matrix (A); Migration to film-air interface (B); Fogging appears, a part of antifogging additives dissolve in surface water (C); Water droplets spread, fogging disappears (D).

The SpecialChem database lists 219 different antifogging additives, whereas only two were reported by the Plastics Additives Initiative's mapping exercise.[20,24] It demonstrates that there has been little focus on this type of additives, although their ability to migrate is of great importance. This is due to the amphiphilic structure, as they are easily leached into surface water due to the hydrophilic moiety. In particular, the use in greenhouse films poses a risk of contamination of the crops grown underneath and of the environment. In addition, these films have a service life of about 18 months due to the loss of antifog properties[25], which is not only an economic but also an environmental issue. The PermAFog project (grant no. 854 798 by Landwirtschaftliche Rentenbank) aims to achieve permanent antifog solutions, with an extended film service life of up to 36 months.

Given the potential issues with antifogging additives, analytical methods are needed for their characterization and determination, especially after leaching from films. To date, they have only been determined in the polymer matrix using Fourier Transform Infrared Spectroscopy (FTIR).[26] Studies on leached additives are lacking and their identity presents an analytical challenge. First, structurally different compounds are used as antifogs, including sorbitan-, glycerin-, polyglycerol- or amine-esters, and alkylphenol polyoxyethylene ethers (**Fig. 3**).[23]

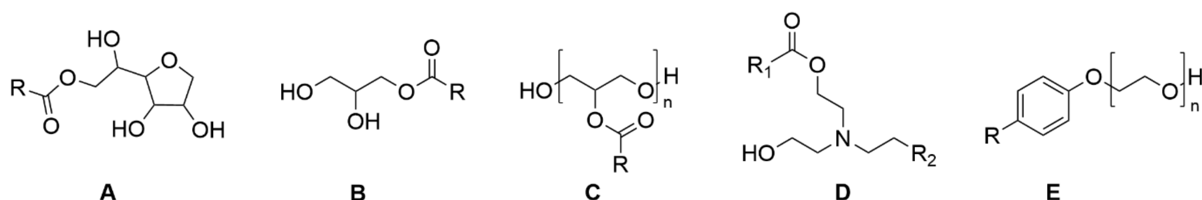

Figure 3: Example structures of compounds used as antifogging additives. A, sorbitan-; B, glycerin-; C, polyglycerol-; D, amine-ester; E, alkylphenol polyoxyethylene ether.

Second, antifogging additives are UVCBs. Consequently, one additive can contain a range of compounds depending on processing conditions and raw materials. However, almost all have a fatty acid moiety. For this reason, a method for the determination of antifog fatty acids by gas chromatography-mass spectrometry (GC-MS) was developed to study their fate within this thesis.

1.3 Impact of cover material on vegetable production

Cover materials utilized in protected cultivation have different radiometric and physical properties. These include heat transfer and retention, ultraviolet and infrared (UV and IR) reflectivity, and light transmission across the spectrum.[11] Consequently, the selected cover material affects the prevailing cultivation conditions. For example, for winter crops, high solar transmitting materials are beneficial to ensure effective photosynthesis, while for summer crops, lower transmittance is preferred due to the risk of overheating.[11]

In protected cultivation, light and temperature are key factors modified by using covers. Solar radiation and IR absorption by the cover material affect the temperature in the cultivation system,[10] known as the greenhouse effect.[27] Light intensity is reduced by cover materials and also spectral quality may vary. Conventional PE greenhouse films transmit about 80 % of visible light,[27] whereas, UV blocking materials are used to prevent material's degradation and modern materials can be designed to transmit specific wavelengths.[11] In addition, covers affect direct and diffuse light.[10,11]

As a result, the cover material influences the microclimate underneath, which can affect the crops. Higher temperatures increase photosynthesis to a certain threshold, leading to increased biomass accumulation.[28] Light also affects crop yield, for example reduced transmittance of photosynthetic active radiation (PAR) by covers is associated with reduced yields.[29] The PAR proportion of the light spectrum is most important for photosynthesis. Plants receive light through photoreceptors detecting red (660-730 nm; phytochromes, PHY), blue (320-500 nm; cryptochromes, CRY; phototropins, PHOT) and UVB light (280-315 nm; UV resistance locus 8, UVR8), and they can respond and adapt to adverse conditions by altering metabolite profiles.[30]

Early studies examining the effects of cover materials focused on crop yield.[31-33] In recent years, however, there has been increasing interest in researching the effects of cover materials on the nutritional quality of crops. These studies have compared different materials used for polytunnels or greenhouses or with open field. Cover materials have been shown to affect the micro- and macro-nutrients such as minerals, proteins, and sugars in lettuce and tomatoes.[34-36] Additionally, effects on plant secondary metabolites and antioxidant activity were observed in a variety of vegetables such as lettuce, tomato or cucumber.[34-40] Here, most studies focus on covers with different UV transmittance and phenolic compounds.[34,38-40] Higher UV transmittance was positively associated with flavonoids and anthocyanins and negatively associated with yield. Research on other phytochemicals, such as carotenoids, has rarely been conducted especially for leafy vegetables. Although the studies found an effect of cover material on yield and nutrients, it depends on the species, cultivar and the time of harvest.[35-38] Taken together, the choice of cover material in protected cultivation offers the opportunity to produce high-yielding and nutrient-rich crops, which is of importance in regard to sustainable future food production. However, He, Maier [10] noted that there is a lack of fundamental systematic research on the influence of cover materials. This is especially required due to the complexity of materials (polymer + incorporated additives). In particular, understanding the response of plants to the conditions created is an important consideration.

2 Lettuce (*Lactuca sativa* L.) a source of micronutrients?

Lettuce (*Lactuca sativa* L.) originated in the Mediterranean, possibly from the wild form *Lactuca serriola* L.[41] Today it is a vegetable consumed worldwide. In 2021, about 27 million tons of lettuce and chicory were produced globally on an area of 1.2 million ha.[42] In Germany, lettuce is grown in the open field from April to September and under protective cover almost in March/April and October/December.[41] Here, about 42,400 tons of lettuce were produced in the open field on 1,317 ha, while 2,334 tons were produced in protected cultivation on 60.78 ha in 2021.[43]

Lettuce belongs to the Asteraceae family, which includes crops such as chicory (*Cichorium intybus*) or endive (*Cichorium endivia*). In Europe, the most common lettuce cultivars are romaine (*Lactuca sativa* var. *longifolia* Lam.), leaf (*Lactuca sativa* var. *crispa* L.) and head lettuce (*Lactuca sativa* var. *capitata* L.). The ladder can be subdivided into crisphead (iceberg) and butterhead lettuce.[41] In this thesis, the red and green lettuce cultivars 'Merveille de 4 Saison', 'Attractie' and 'Veronique' were investigated (**Fig. 4**). According to the supplier, ‘Veronique’ is particularly tolerant to heat.

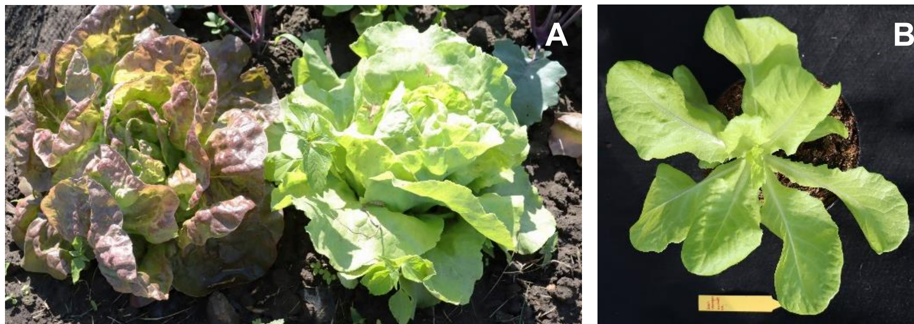

Figure 4: Butterhead lettuce cultivars ‘Merveille de 4 Saison’ (left, A), ‘Attractie’ (right, A) both fully developed and ‘Veronique’ (B) in 8 leaf-stage.

With a per capita consumption of 5.7 kg, lettuce is one of the most consumed vegetables in Germany (2021).[43] It is consumed in the vegetative stage (heads), after which the shoot elongation (generative stage) begins. The consumption of the latter is inappropriate due to the accumulation of bitter sesquiterpene lactones.[44]

Lettuce is often considered a nutrient-poor vegetable. This is probably due to its high water content of 94 to 95 %.[45,46] However, it is a source of vitamins such as tocopherol, phylloquinone, folic acid, riboflavin, and ascorbic acid; minerals such as potassium, magnesium, calcium, iron, and zinc; and dietary fiber (**Tab. 1**).[45,46] Compared to other leafy vegetables such as spinach, endive or lamb's lettuce, these are present in moderate but also

comparable amounts.[46] Nutrient content varies by cultivar.[45,47,48] Crisphead has the lowest nutrient content compared to romaine, leaf and butterhead lettuce. These latter cultivars, however, have nutritional content partially comparable to spinach.[45] In addition, the mineral absorption inhibiting oxalate present in spinach was not found in lettuce.[45,46] Furthermore, lettuce is a good source of plant secondary metabolites like carotenoids, phenolic acids and flavonoids.[45,47,48] Again, crisphead has the lowest levels.[45] Levels of phenolic compounds tend to be higher in red than in green cultivars.[47] The carotenoid content of butterhead, romaine, and leaf lettuce is comparable to that of spinach.[45] Because both, carotenoids and phenolic compounds such as flavonoids are associated with a number of health benefits, they were focused in this thesis.

Table 1: Nutrient content (fibers, minerals, vitamins and plant secondary metabolites) in selected lettuce types and leafy green vegetables, in 100 g fresh weight (rounded values). Data from figures were extracted using WebPlotDigitizer (Ankit Rohatgi, Version 4.3.). Car, carotenoids; Flav, flavonoids; ch, crisphead; bh, butterhead; rm, romaine; lf, leaf; gr, green, n/a, data not available.

Vegetable	Dietary fibers	Minerals					Vitamins					Plant secondary metabolites		References
		K	Mg	Ca	Fe	Zn	K	E	C	B_2	B_9	Car	Flav	
	[g]	[mg]	[mg]	[mg]	[µg]	[µg]	[µg]	[µg]	[mg]	[µg]	[µg]	[mg]	[mg]	
Lettuce, head[#]	1.4	177	9	21	314	372	109	907	13	78	59	1	n/a	46
Lettuce, ch	1.2	168	10	27	640	160	75	200	6	53	34	3	n/a	49
Lettuce, ch	n/a	n/a	n/a	n/a	416	n/a	n/a	180-220	3- 4	n/a	30- 40	1	1	45
Lettuce, bh	n/a	n/a	n/a	n/a	954	n/a	n/a	180-750	4- 7	n/a	70	4	6-20	45
Lettuce, rm	1.3	194	13	36	860	180	126	220	9	80	38	6	n/a	49
Lettuce, rm	n/a	n/a	n/a	n/a	563	n/a	n/a	130-550	3-11	n/a	30-220	6	2-17	45
Lettuce, lf (gr)	n/a	277	13	40	320	310	n/a	n/a	15	n/a	n/a	n/a	n/a	49
Lettuce, lf (gr)	n/a	n/a	n/a	n/a	858	n/a	n/a	220-740	7-30	n/a	40- 70	7	2	45
Lettuce, lf (red)	n/a	321	12	43	400	300	n/a	n/a	9	n/a	n/a	n/a	n/a	49
Lettuce, lf (red)	n/a	n/a	n/a	n/a	1200	n/a	n/a	150-240	4-15	n/a	40-130	5	4-28	45
Endive	1.2	330	10	54	1400	356	n/a	n/a	9	120	109	2	n/a	46
Kale	4.2	451	31	212	1900	330	817	1700	105	250	187	5	21	46
Kale	4.1	348	33	254	1600	390	390	660	93	347	62	9	n/a	49
Lamb's lettuce	1.5	421	13	35	2000	430	n/a	600	35	80	145	4	n/a	46
Spinach	2.6	554	62	117	3400	617	305	2300	51	202	145	5	n/a	46
Spinach	1.6	460	93	67	1050	420	n/a	n/a	30	192	113	12	n/a	49

[#]head lettuce type not specified

3 The function of carotenoids in nature

Carotenoids belong to the plant secondary metabolites. These are ubiquitous in the plant kingdom, and several of them are associated with health-promoting effects when consumed in the human diet. They are synthesized in a secondary metabolism, however, the distinction between primary and secondary metabolites is not always clear-cut.[50]

Carotenoids are isoprenoid pigments, with about 700 different compounds known naturally. They are synthesized not only by plants, but also by other organisms such as algae, bacteria and fungi.[51] Based on their structure, carotenoids can be divided into carotenes (polyene chain) and xanthophylls (oxygen function).[51] Due to their conjugated double bonds, carotenoids appear in a range of colors from red to orange to yellow. They accumulate in various plant organs such as leaves, flowers or fruits. Here, the colored flowers and fruits, as well as carotenoid-derived volatile compounds, attract insects and birds that pollinate the plants and disperse their seeds.[51]

In green leaf tissue, carotenoids function alongside chlorophylls as photosynthetic pigments. In the photosynthetic antenna, carotenoids are involved in light harvesting. In addition, they serve as photoprotectors due to their antioxidant properties and their involvement in non-photochemical quenching (NPQ).[51] Furthermore, carotenoids are precursors of signal molecules, such as the phytohormones abscisic acid (ABA) and strigolactones.[51]

Besides their multiple functions in plants, carotenoids may benefit to human health. For example, the carotenoids α- and β-carotene, as well as β-crypthoxanthin have pro-vitamin A activity in humans.[52] In addition, epidemiological studies suggest a positive effect on non-transmissible diseases, such as cardiovascular diseases and certain types of cancer.[53] While, lutein and zeaxanthin have been shown to affect the development and progression of age-related macular degeneration.[54]

Because carotenoid contents vary with cultivation conditions among other factors, it is important to shed light on the regulation of biosynthesis, storage and degradation.

3.1 The carotenoid biosynthetic pathway

Plants synthesize and store carotenoids in organelles called plastids. In green leaf tissue, these are chloroplasts. Here, carotenoids are located at the thylakoid and envelope membrane as well as in plastogloboli.[55] Carotenoid levels are not static, but defined by steady state. This steady state describes the balance between carotenoid biosynthesis, accumulation, and degradation.

The biosynthesis of carotenoids starts with phytoene synthesis using substrates from the methylerythritol 4-phosphate pathway (**Fig. 5**). This reaction is catalyzed by the enzyme

phytoene synthase (PSY), which has been described as a rate-limiting and important regulatory step.[51] Lycopene is then formed by multiple enzymatic reactions, followed by branching of the pathway to synthesis of α- and β-carotene. This is followed by their hydrolysis and further steps to a number of xanthophylls, including lutein (α-branch), violaxanthin and neoxanthin (β-branch).[51,56] However, there is a third branch in lettuce that produces ε-carotene, which in turn is used to synthesize lactucaxanthin. Together with lutein, this carotenoid is present in the antenna of photosystem II (PSII) in some species of Asteraceae.[57,58]

The carotenoid storage capacity of plastids depends on the size of the compartment and their type.[51] Besides oxidative degradation, two groups of carotenoid cleavage enzymes degrade carotenoids to apocarotenoids. The nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) are involved in the production of ABA, while the carotenoid cleavage dioxygenases (CCDs) synthesize a number of apocarotenoids, including volatiles, aroma and color compounds, and stricolactones.[51]

3.2 Regulation of carotenoid pathway by light and temperature

The carotenoid pathway is highly conserved in plants, including vegetables. However, the regulation of carotenoid steady state is less well understood. Carotenoid levels in vegetables depends on genetic, environmental as well as agronomic factors.[59] Furthermore, their regulation appears to be different in photosynthetic and non-photosynthetic tissue.[60] As photosynthetic pigments, carotenoids are crucial for photosynthesis. Both light intensity and quality can affect photosystems and photosynthetic capacity, resulting in carotenoid adaptation.[61] For example, different levels of carotenoids were accumulated in kale and spinach, grown at light intensities between 125 to 620 µmol m^{-2} s^{-1}.[62] Furthermore, carotenoids differ in lettuce, spinach and komatsuna grown under white, blue and red fluorescent lamps.[63] Temperature can also induce photosystem adaptations. Thus, carotenoids are important in the response to higher temperatures by scavenging reactive oxygen species (ROS) generated by PSII and stabilizing the thylakoid membrane.[64] Carotenoids tend to increase in spinach and kale grown at elevated temperatures (10 to 30 °C).[65]

The accumulation of carotenoids appears to be regulated at multiple levels: transcriptional, post-transcriptional, post-translational, storage and degradation as well as feedback regulation by endproducts.[64] Plants sense light through photoreceptors for blue (CRY, PHOT), red (PHY), and UV light (UVR8).[30] In addition, there are light modulated processes such as shade avoidance.[66]

The *PSY* gene is known to respond transcriptionally to environmental factors such as light and temperature. For example, increased *PSY* transcription is associated with carotenoid accumulation induced by specific light regimes in *Arabidopsis thaliana*.[67]

Several transcription factors have been identified that interact with *PSY*. Elongated hypocotyl 5 (HY5) and phytochrome interacting factor 1 (PIF1) are both light- and temperature-responsive, modulating *PSY* transcripts (**Fig. 5**). HY5 is an integration point downstream of all photoreceptors and functions as a transcriptional activator of *PSY* in light and lower temperatures.[64,68] PIF1 is a HY5 antagonist that represses *PSY* transcription in the dark, in shade, and at higher temperatures.[64,68]

There is increasing research into the influence and regulation of individual factors such as light and temperature on carotenoid accumulation. In protected cultivation, these factors are closely correlated and carotenoid steady state depends on their interaction.

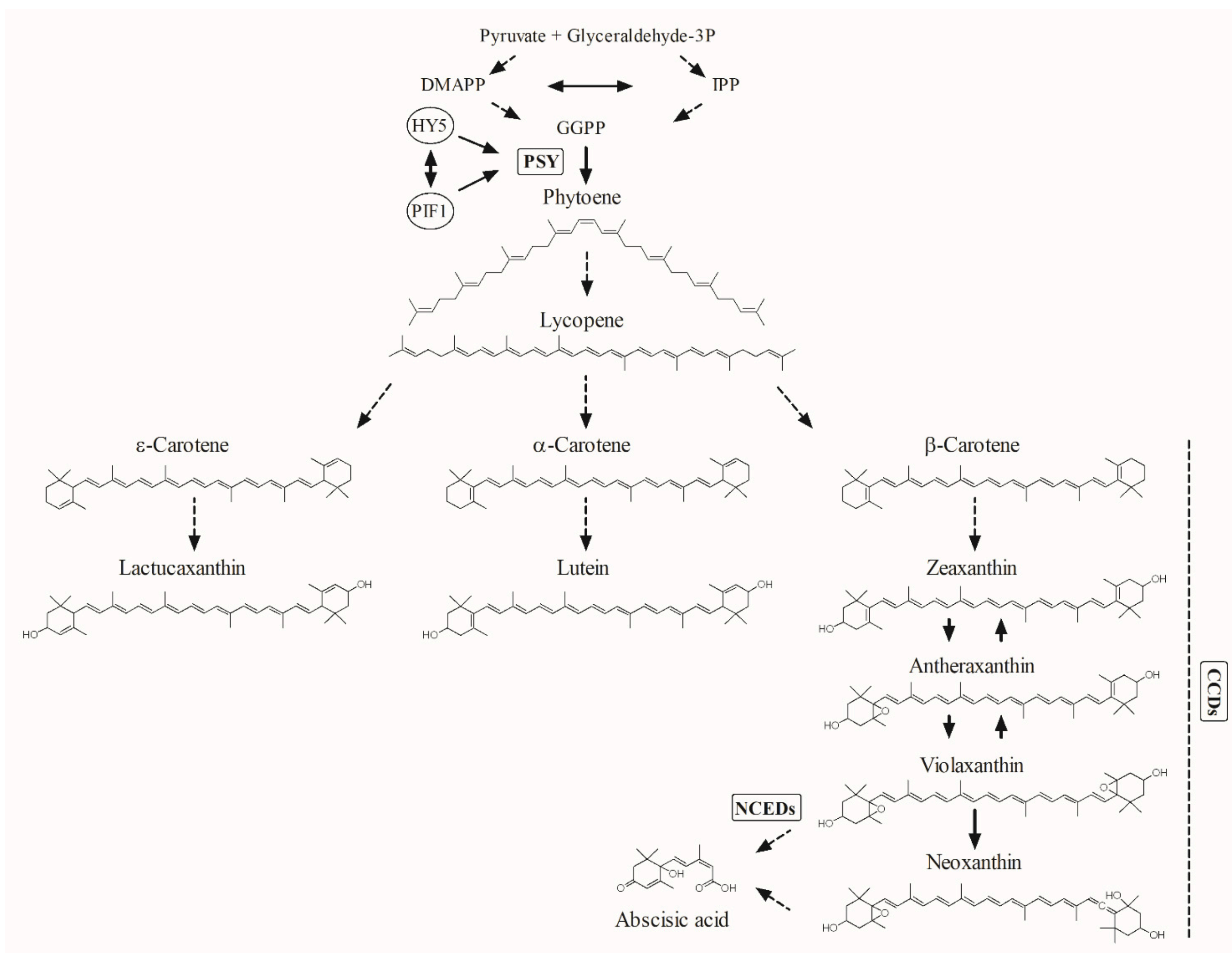

Figure 5: Carotenoid biosynthetic pathway in lettuce. The rate-limiting enzyme PSY, transcription factors (HY5 and PIF1) analyzed in this thesis and degradation enzymes are shown. Dashed lines indicate multiple reaction steps, and continuous line indicate one reaction step. DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; HY5, elongated hypocotyl 5; PIF1, phytochrome interacting factor 1; PSY, phytoene synthase; CCDs, carotenoid cleavage dioxygenases; NCEDs, nine-*cis*-epoxycarotenoid dioxygenases.

4 The function of flavonoids in nature

Polyphenols are the largest group of plant secondary metabolites. Over 8,000 different polyphenolic structures have been identified in the plant kingdom.[69] Phenolic acids and flavonoids are the polyphenols identified in lettuce.[70]

Phenolic acids can be divided into hydroxybenzoic acid and hydroxycinnamic acid derivatives.[69] The latter group includes lettuce caffeic acid derivatives.[70]

Flavonoids are structural derivatives of chroman with a C_6-C_3-C_6 backbone. The group of flavonoids can be further divided into six subgroups: flavonols; flavones; *iso*-flavones; flavonones; flavanols and anthocyanins.[69] These represent the aglycones. However, in plants,

most polyphenols are glycosylated with various sugar moieties, which in turn can be acylated. In lettuce, flavonols (quercetin) and flavones (luteolin) were detected.[70]

Various plant organs such as flowers, fruits and leaves contain flavonoids, where they perform different biological functions. They act as defenses against plant pathogens and herbivores, and are involved in controlling phytohormone transport (auxin). In legumes, flavonoids are involved in nodulating.[71] In addition, they have a key function in the protection of plant photosynthesis, e.g. from excessive light and UV radiation.[71,72] This is because flavonoids have UV light screening properties and, as antioxidants, are able to scavenge ROS generated during the photosynthesis.[72]

Like carotenoids, flavonoids are not only beneficial to plants, but are also associated with health-promoting properties in humans. In cell and animal studies, a number of flavonoids have shown inhibitory effects on several types of cancer cells, including lung, breast and colon cancer.[73] In addition, epidemiologic studies suggest that flavonoid intake is associated with reduced risk of cardiovascular disease, breast cancer, and type 2 diabetes.[74-76] Controlled clinical studies have also demonstrated the potential of flavonoids for cardiovascular disease, as well as indications of neuroprotective properties.[77] Besides flavonoids, phenolic acids are also associated with a number of health-promoting effects, including antiinflammatory, anticancer, and neuroprotective properties.[69]

4.1 The flavonoid biosynthetic pathway

Plants accumulate flavonoids in a number of different cellular and subcellular compartments. They are found in cell walls, vacuoles, and trichomes. In addition, flavonoids also accumulate at the envelope membrane of the chloroplasts.[72] However, they are synthesized in the endoplasmic reticulum with a multi-enzyme complex.[71] Therefore, they have to be transported intra- and extracellularly to the compartments.[72] Furthermore, the chloroplastic biosynthesis of flavonoids is discussed.[72]

Substrates of the shikimate and phenylpropanoid pathways are required to synthesize flavonoids. The synthesis of naringenin chalcone is the first step in flavonoid biosynthesis (**Fig. 6**). This reaction is catalyzed by chalcone synthase (CHS), a key pathway enzyme.[71] In a next step the flavanone naringenin is synthesized. Here the flavonoid pathway branches depending on the flavonoids to be synthesized. Naringenin can be oxygenated to dehydrokaempferol, which is further hydroxylated and converted to quercetin. Additionally, naringenin can be converted to the flavone apigenin and further hydroxylated to luteolin. These

aglycones are subsequently glycosylated.[56] Besides, lettuce caffeic acid derivatives can be synthesized in several steps from coumaroyl-Coenzyme A (CoA).[56]

4.2 Regulation of flavonoid pathway by light and temperature

Flavonoid biosynthesis is highly conserved in plants. However, flavonoid levels are dependent on genetic, environmental and agronomic factors.[59] Some key factors for flavonoid regulation under different environmental conditions have been identified, however, research is ongoing.
In lettuce, flavonoids accumulated at higher light intensities (410 to 225 µmol m^{-2} s^{-1}), as well as at UVB light supplementation (0.5 kJ m^{-2} d^{-1} for 1 h).[78,79] Moreover, lower temperatures were found to be associated with higher flavonoid content in lettuce (13/10 °C and 25/20 °C, day/night temperatures) and *Arabidopsis thaliana* (10 °C and 22 °C).[80,81]
Like carotenoids, flavonoids are regulated at multiple levels, including transcriptional, post-transcriptional and phytohormonal. The transcription of the key enzyme *CHS* in *Arabidopsis thaliana* is known to be induced by high light and UV light.[72,82] *CHS* is described as having light regulatory units associated with photoreceptor signaling. Furthermore, low temperature also induce *CHS* transcription in *Arabidopsis thaliana*.[80] In UV light regulation of flavonoid biosynthesis, UVR8, COP1, and HY5 have been identified as key transcription factors (**Fig. 6**). Constitutive photomorphogenic 1 (COP1) is an E3 ubiquitin ligase that continuously degrades HY5. The interaction of UVR8 with COP1 in the nucleus prevents degradation and stabilizes HY5.[83] HY5 has already been mentioned as a transcriptional activator downstream of several photoreceptors. It can also activate genes of the flavonoid biosynthesis such as *CHS*.[84] As described above, HY5 is also stabilized at lower temperatures. In *Arabidopsis thaliana*, HY5 appeared to be a central activator of genes for flavonoid biosynthesis at low temperatures, in a light-dependent manner.[80]
Protected cultivation affects light and temperature. These factors can be modified by the choice of cover material. Since most cover materials are UV blocking due to their service life (see **1.3**), this is important for the accumulation of flavonoids in vegetables.

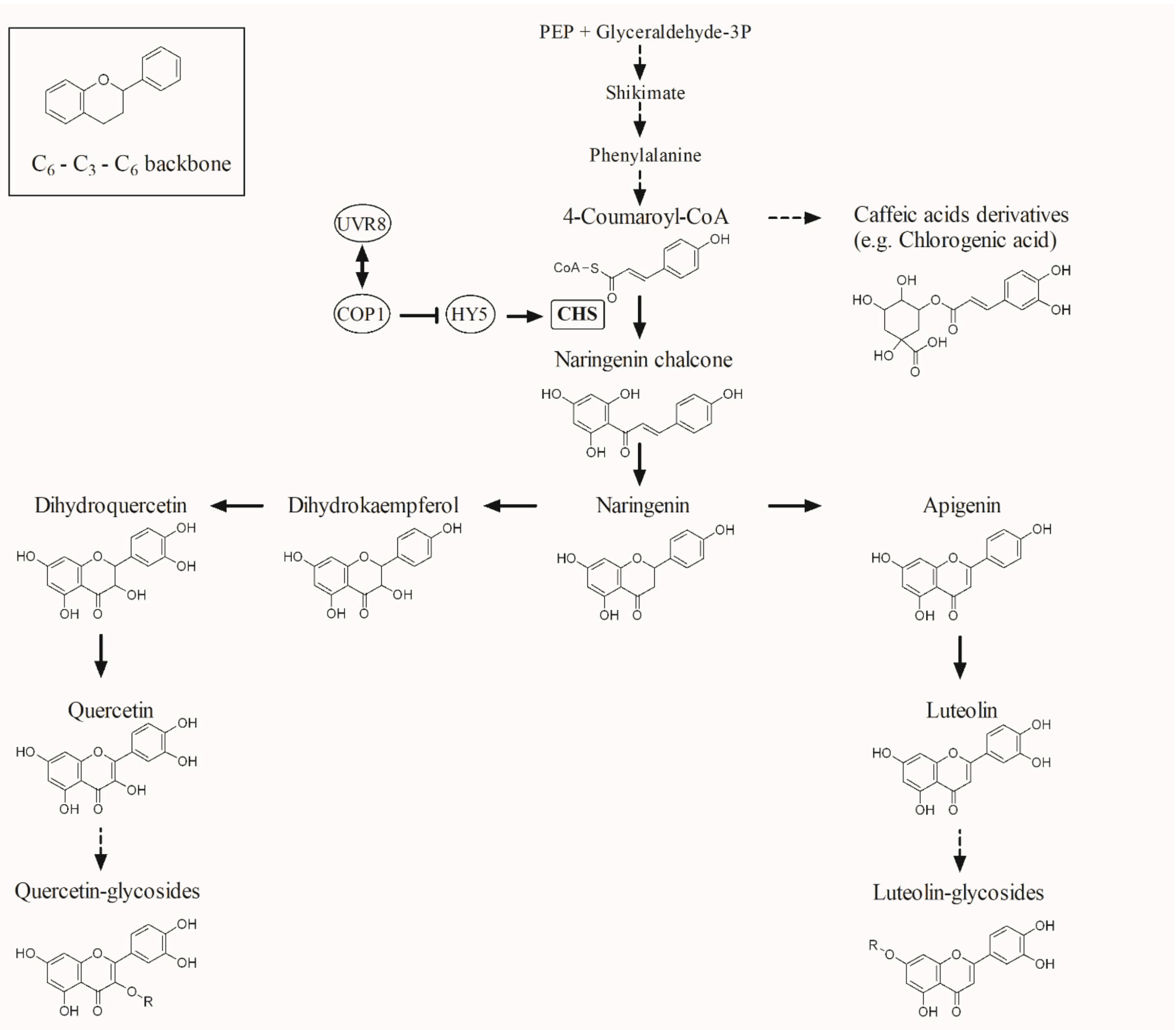

Figure 6: Flavonoid biosynthetic pathway in lettuce. The rate-limiting enzyme CHS and key regulators analyzed in this thesis (HY5, UVR8) are shown. Dashed lines indicate multiple reaction steps, and continuous line indicate one reaction step. Displayed are quercetin-3-glycosides and luteolin-7-glycosides found in lettuce. UVR8, UV resistance locus 8; COP1, constitutive photomorphogenic 1; HY5, elongated hypocotyl 5; CHS, chalcone synthase; CoA, coenzyme A; PEP, phosphoenol-pyruvate.

AIM OF THE DISSERTATION

Major challenges have been identified with respect to the future of food production, including the efficient use of resources, particularly land and water, and the inclusion of healthy plant-based diets. Protected cultivation can contribute to sustainable agriculture, but there are obstacles to overcome. An important issue is the cover material used to protect crops in greenhouses or polytunnels. As a low-cost material with modifiable properties, plastic covers are of particular interest. There are several factors to consider when using such materials, including service life and waste, plastic additives that can migrate from the films, and the impact on crop yield and nutritional value. The influence of the materials used for covers with the various incorporated additives is complex, and systematic research is lacking. This thesis addresses this problem and focuses on covers with one specific type of plastic additives: antifogging additives. These additives may affect vegetables grown under antifog covers directly by migration and leaching or indirectly by modifying the climatic conditions. Changes in these conditions, which can also be caused by plastic covers in general, can affect nutritionally valuable compounds in vegetables.

Hence, the first part of this thesis examines the direct effects of antifogging additives through leaching simulation experiments.

i. A GC-MS method was developed to characterize three structurally different antifogging additives based on their fatty acid moieties, which is summarized in the first publication. This method was applied to investigate the fate of additives by simulated leaching. For this purpose, lettuce leaves were treated with additives and their removability was tested. Furthermore, treatment effects on plant physiological parameters including gas exchange measurements as well as carotenoids and chlorophylls by HPLC-DAD-ToF-MS were analyzed. All antifogging additives have been shown to exceed the manufacturer's specified fatty acids. Based on this approach, it was demonstrated that antifogging additives adhered to the lettuce leaves and could not be removed by either water or hexane. However, the physiological parameters and metabolites were not affected.

A healthy nutrition is crucial for a future-feasible food system. Thus, the second part examines the indirect influence of antifogging additives utilized in protected cultivation on carotenoids

and flavonoids in lettuce (*Lactuca sativa* var. *capitata* L.). Both metabolites are associated with several health-promoting properties.

ii. The second publication determines the effects of an LDPE/EVA cover material with and without antifogging additives on microclimate and lettuce quality. In order to evaluate the general effects of protected cultivation, experiments with both, polytunnels covered with such films and without polytunnels were performed. Climatic conditions were monitored and carotenoids and chlorophylls (HPLC-DAD-ToF-MS), flavonoids and caffeic acid derivatives (HPLC-DAD-MS/MS), and fatty acids (GC-MS) were analyzed to provide insight into plant metabolism. The incorporation of antifogging additives in polytunnel covers negatively affected carotenoids, but not other metabolites studied. This has been suggested to be related to light transmission and carotenoid involvement in photosynthesis. On the other hand, protected cultivation showed an effect on all the metabolites analyzed, probably associated to the different light and temperature regimes. In further experiments, the influences of novel "permanent" antifog covers and antifogging additives in a PP food packaging material on valuable metabolites in lettuce were evaluated.

iii. Since the accumulation of carotenoids and flavonoids was reversed in lettuce grown under and without polytunnels, a linkage of both pathways was hypothesized. To shed light into the underlying mechanisms in lettuce under such protected cultivation, the third publication covers the analysis of transcripts of genes for key metabolic enzymes (*PSY* and *CHS*) and light and temperature related transcription factors by RT-*q*PCR as well as phytohormone ABA involvement by HPLC-MS/MS. In addition, the carotenoid metabolic flux was analyzed using phytoene accumulation (by HPLC-QToF-MS) due to a norflurazon treatment. The use of antifogging additives showed no effect in this study. Nevertheless, lettuce flavonoids were reduced under polytunnels. This was shown to be related to the UV transmissivity and regulated at the transcript level (*CHS*, *UVR8*). In contrast, higher carotenoids in lettuce under polytunnels were not related to transcripts of the key metabolic enzymes, but higher metabolic flux suggested post-transcriptional mechanisms.

OVERVIEW OF THE MANUSCRIPTS

Publication 1

Title	Comprehensive profiling and quantification of antifogging additives based on fatty acid composition by GC-MS and its application in different matrices
Authors	V. Harbart, B. Hönig, S. Baldermann
Published in	*Microchemical Journal*, 2023, 193, 109133.
DOI	10.1016/j.microc.2023.109133
Impact factor	5.304 (2023)

Publication 2

Title	Antifogging additives for greenhouse covers – effects on phytochemicals and nutritional quality of lettuce
Authors	V. Harbart, H.-P. Kläring, S. Baldermann
Published in	*Journal of Applied Botany and Food Quality*, 2022, 95: 76 – 84.
DOI	10.5073/JABFQ.2022.095.010
Impact factor	1.481 (2021)

Publication 3

Title	Regulation of carotenoid and flavonoid biosynthetic pathways in *Lactuca sativa var capitate* L. in protected cultivation
Authors	V. Harbart, K. Frede, M. Fitzner, S. Baldermann
Published in	*Frontiers in Plant Science*, 2023, 14, 1124750.
DOI	10.3389/fpls.2023.1124750
Impact factor	6.627 (2021)

Statement of contribution

Publication 1	
Own contribution	Organization and conduction of experiments, method development, data analysis, statistics, visualization, writing of original draft including tables and figures, corresponding author
Publication 2	
Own contribution	Organization and conduction of experiments, data analysis, statistics, visualization, writing of original draft including tables and figures, corresponding author
Publication 3	
Own contribution	Organization and conduction of experiments, data analysis, statistics, visualization, writing of original draft including tables and figures, corresponding author

Publication 1

Comprehensive profiling and quantification of antifogging additives based on fatty acid composition by GC-MS and its application in different matrices

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Comprehensive profiling and quantification of antifogging additives based on fatty acid composition by GC–MS and its application in different matrices

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A R T I C L E I N F O

Keywords:
Plastic additives
Fatty acid methyl esters (FAME)
GC–MS
Mass spectrometry
Metabolites
Carotenoids and chlorophyll

A B S T R A C T

As polymer additives, antifogging additives are used for, among other things, food packaging or in agricultural films. Here, they prevent the formation of water droplets on the inside of the film. Since they contain a hydrophilic structural moiety, they can be leached out of the polymer by interacting with water. Therefore, methods are required to study the fate of the (leached) additives. Since additives on the market comprise diverse structures, and also contain a range of compounds, a method was developed to determine the hydrophobic fatty acid moiety of antifogging additives which is based on gas chromatography coupled with mass spectrometry (GC–MS). Different extraction and derivatization protocols were tested to optimize the method and determine the best recoveries (between 106 and 117 %), resulting in rapid and low effort sample preparation. Limits of detection varied between 0.01 pg and 12.15 ng on column. The method presented can not only be used to detect the additive fatty acids, but is also suitable for detecting leached antifogging additives in matrices such as plant leaves and soil.

In summary, three different commercially available antifogging additives were analyzed with the method. All showed a structural diversity beyond the fatty acids specified by the manufacturer. Using this fatty acid approach, it was observed that all three additives adhered to leaves when foliarly applied. It was also shown that significantly increased amounts of fatty acids were detectable even after washing the leaves with hexane or water, indicating a fatty acid residue on the treated leaves. However, a negligible effect of the adherent antifogging additives on plant physiological parameters as well as on selected metabolites was observed within the short experimental period.

1. Introduction

Plastic additives help to improve the daily lives of consumers and producers. Used in agricultural films, UV blockers can, for example, protect crops from damage related to insect populations and fungal diseases [1]. Antifogging additives among other plastic additives are incorporated into polymers to improve the desired properties of plastic films. As surfactants, antifogging additives reduce the surface tension and the contact angle of water droplets, which then merge into a continuous water layer on the film [2]. The antifogging hydrophilic moiety can thus interact with the adhering water while the hydrophobic moiety anchors in the polymer. They are used in agricultural films or

Abbreviations: CLP, Regulation on Classification, Labelling and Packaging of Substances and Mixtures; DW, dry weight; ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; EI, electron impact ionization; FAME, fatty acid methyl ester; FID, flame ionization detector; FT-IR, Fourier-transform infrared spectroscopy; GC–MS, gas chromatography - mass spectrometry; HPLC-DAD-ToF-MS, high performance liquid chromatography - diode array detection - time of flight - mass spectrometry; MeOH, methanol; NMR, nuclear magnetic resonance spectroscopy; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; TAG, triacylglycerides; THF, tetrahydrofuran; TIC, total ion current; TLC, thin layer chromatography; SFC, supercritical fluid chromatography; UVCB, unknown or variable composition, complex reaction products or biological materials.

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food packaging to prevent the formation of water droplets on the inner side. In agricultural films this increases crop yield and productivity, since condensed water droplets both reduce light transmission and favor microbiological contamination [2]. The same applies to food packaging, since it can reduce food spoilage caused by water droplets. In addition, droplet-free films are preferred by consumers [3].

However, the additives are not permanently bound and can migrate in and out of the polymer matrix [4]. This could be a problem not only for economic efficiency and sustainable crop production (due to the short service life of antifogging films of about 18 months [5]), but also for food security. As a known problem, cost-effective solutions for permanent antifogging additives have been developed [5–7]. However, these currently available additives described as "permanent", are characterized by a higher molecular weight and migrate more slowly to the surface.

Plastic additives in general have been determined using several techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC), mass spectrometry (MS) and Fourier-transform infrared spectroscopy (FT-IR), often as extractables and leachables from plastics [8,9]. The antistatic additive glycerol monostearate, which is also used as an antifogging additive, was analyzed by GC–MS [10]. To the best of our knowledge, antifogging additives have only been characterized in a polymer matrix using FT-IR [9] and those additives outside the polymer matrix have not been the focus of any studies. Therefore, instrumental analytical methods are lacking. In addition, effects on plant metabolism due to leached additives have rarely been investigated.

Two main analytical challenges were identified for the analysis of antifogging additives. Firstly, there are a variety of different compounds on the market, for example sorbitan- or glycerol- fatty acid esters as well as fatty amine polyoxyethylene esters [2]. Secondly, these compounds are classified by ECHA (European Chemicals Agency) as UVCB (unknown or variable composition, complex reaction products or biological materials) [11–13]. Specifically, antifogging additives not only consist of one chemical compound, usually specified by the manufacturer, but there are varieties of possible structures. This depends on the synthesis

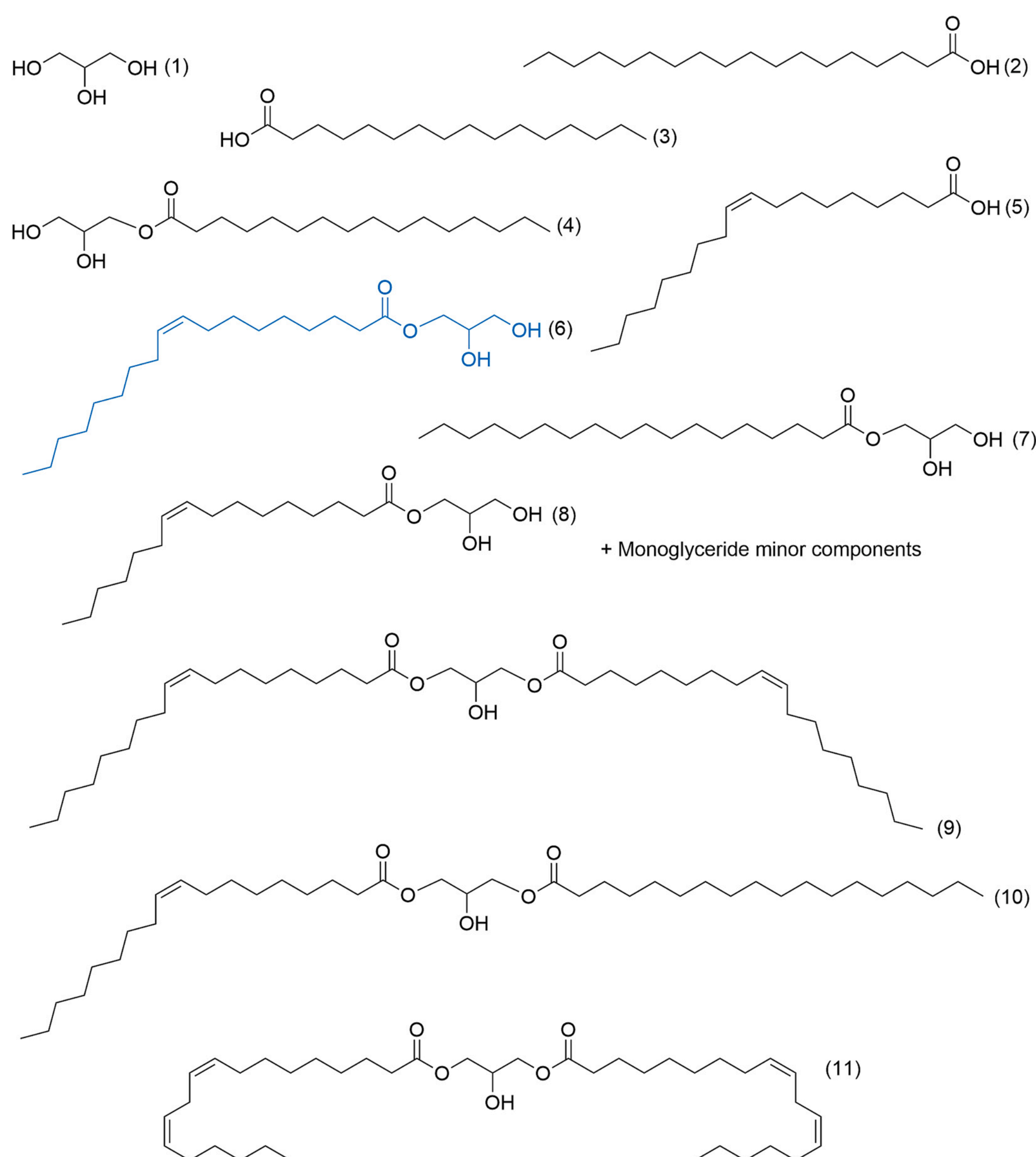

Fig. 1. Predicted exemplary structural diversity of the antifogging additive Atmer 1440 by Croda, specified as glycerol monooleate. The glycerol monooleate structure is highlighted in blue (6). Structures are: (1) glycerine (unreacted monomer), (2) stearic acid, (3) palmitic acid, (4) glycerol mono-palmitate, (5) oleic acid, (6) glycerol monooleate, (7) glycersol mono-stearate, (8) glycerol mono-palmitoleate, (9) glycerol di-oleate, (10) glycerol oleate stearate di-ester, and (11) glycerol di-linoleate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions and the raw materials. For example, the additive Atmer 1440 from Croda (Croda International Plc, UK) is listed as glycerol monooleate [14]. Depending on the raw material indicated as "vegetable derived" and the processing conditions, different fatty acids may be esterified or different types of esters (di-, triesters) or unreacted monomers may be present in a single additive (Fig. 1).

To help address this problem, the present paper describes a rapid and simple method for characterizing such additives by determining the fatty acid moieties, which is applicable to additives of different structures. The analysis of fatty acids as methyl esters (FAMEs) is an affordable, robust and a long proven analytical approach, used for several different matrices [15–22]. Derivatization to FAMEs is also suitable for complex matrices with a potentially high diversity of different compounds such as plant tissues. Here, three different antifogging additives were analyzed using this method. In addition, simulation experiments were conducted to examine the fate of leached antifogging additives on plant leaves. We analyzed not only the additives themselves, but also the effects on plant metabolites and physiological parameters. As a low fatty acid-content vegetable, lettuce was used for these experiments. Additionally, the method was applied to soil and soil-added additives.

Our method was able to detect and quantify fatty acids of foliar applied antifogging additives. Although the applied additives had negligible effects on plant metabolite levels and physiological parameters, the problem of non-removability from the leaves raises new concerns that have not been considered so far.

2. Material and methods

2.1. Chemicals and standards

Boron-trifluoride-methanol solution (~10%, for GC derivatization, LiChropur™), tetrahydrofuran (THF, ≥99.9 %, LiChroSolv®), *n*-hexane (SupraSolv® for GC–MS), dichloromethane (<99.8%, SupraSolv®), chlorophyll *a* and b (analytical standards), 2,2-dimethoxypropane (98 %), FAME standard mix (C8 - C24, certified reference material) as well as heptadecanoic acid (≥98 %) and heptadecanoic acid methyl ester (≥99 %) were purchased from Merck KGaA (Darmstadt, Germany). Hydrochloric acid (37 %), 2-propanol (≥99.9 %, ROTISOLV®), potassium hydroxide solution (50 %) and sodium chloride (>99.8 %) were obtained from Carl Roth GmbH (Karlsruhe, Germany). Sodium sulphate (anhydrous, >99.4 %) was from VWR International GmbH (Vienna, Austria). Methanol (Chemsolute®) was purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany) and carotenoid standards were from CaroteNature GmbH (Münsingen, Switzerland). The antifogging additives (Sabofog MS P and Sabostat A300 from SABO S.p.A., Italy and Atmer 1440 from Croda International Plc, UK) were kindly provided by CONSTAB polyolefin additives GmbH (Rüthen, Germany). All solvents were of LC-MS or GC–MS quality, and the water was of ultra-pure quality.

2.2. Fatty acid extraction and derivatization

2.2.1. Acid hydrolysis with methanolic hydrochloric acid

The fatty acids were simultaneously saponified and derivatized to methyl esters as described previously [23]. Briefly, 15 mg of lettuce leaves or 5 mg soil, both lyophilized and powdered, or 100 μL of an antifogging additive aliquot (final concentration of 2 mg 100 μL^{-1}) diluted in THF/*iso*-propanol (1:1, v/v) were used for the analysis. 1000 μL methanolic-hydrochloric acid reagent (3 M HCl/methanol 1:2 v/v, with 5 % 2,2-dimethoxypropane) was added as well as 500 μL heptadecanoic acid (0.2 mg mL^{-1}) as internal standard. This mixture was shaken continuously for 60 min at 80 °C under nitrogen atmosphere to protect unsaturated fatty acids from oxidation. Next, 750 μL hexane and 1000 μL saturated sodium chloride solution were added to extract the FAMEs into the upper hexane phase. After centrifugation and filtration (over anhydrous sodium sulphate) of the upper hexane phase, FAMEs were determined by GC–MS.

2.2.2. Transesterification with boron-trifluoride-methanol complex

The transesterification protocol with prior saponification was modified according to the methods previously discussed by Cavonius, Carlsson and Undeland [24]. Using this protocol, the esterified fatty acids in antifogging additives were first saponified and then derivatized. 100 μL of the antifogging additive aliquots described above were used. 1000 μL of methanolic potassium hydroxide solution (0.5 M) was added to the aliquots for saponification. In addition, 500 μL of the internal standard (**2.2.1**) was added and this was shaken continuously at 80 °C for 10 min. Afterwards 1000 μL of boron-trifluoride-methanol-complex solution was added for derivatization and the mixture was again shaken continuously at 80 °C for 10 min. The extraction of the FAMEs into the hexane phase was proceeded similarly to that described under **2.2.1** following centrifugation, filtration and GC–MS analysis.

2.3. Gas chromatography coupled to mass spectrometry (GC–MS)

Analysis was performed using an Agilent 6890 GC equipped with a J&W DB-23 GC column (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany, 30 m × 0.25 mm × 0.25 μm). The injector temperature was set to 230 °C with splitless injection of 1 μL. Helium was used as carrier gas with a constant flow of 1.2 mL min^{-1}. The oven temperature program was as follows: 80 °C for 2 min, 80 °C to 120 °C with 5 °C min^{-1}, 120 °C to 220 °C with 2 °C min^{-1}, held at 220 °C for 5 min. FAMEs were detected with an Agilent 5973 mass selective detector with a source temperature of 230 °C and a quadrupole temperature of 150 °C. The transfer line temperature was set to 230 °C and the voltage was set to 953 V. Scan mode was used for analysis (mass range between m/z 90 to 400). Fatty acids were identified as their methyl esters by comparing retention time and mass spectra with authentic standards. The quantification was performed with the internal standard together with experimentally determined response factors. Data acquisition and processing were conducted by GC/MSD ChemStation Software and Agilent MassHunter Workstation Software.

2.4. Lettuce cultivation and foliar application of antifogging additives

Lettuce seeds (*Lactuca sativa* L. cultivar 'Attractie') were germinated in a climate cabinet (Vötsch Industrietechnik GmbH, Balingen-Frommern, Germany) under the following conditions: 10 °C for 48 h followed by 12 °C until transplanting, 75 % relative humidity, 350 μmol s^{-1} m^{-2} light intensity with a day/night rhythm of 12/12 h. When the seedlings had entered the two-leaf stage, they were transplanted into 13 cm pots filled with soil (Einheitserde classic, Einheitserde Werkverband e.V., Sinntal-Altengronau, Germany) and transferred to a climate chamber. The climate chamber was set to 22 °C during the day and 18 °C at night with a relative humidity of 70 %. The light intensity was set to 350 μmol s^{-1} m^{-2} with a day/night rhythm of 14/10 h. When the lettuces reached the 8-leaf stage (approximately after 30 days), the antifogging additive treatment was applied.

Two experimental repetitions were performed. In a first experiment, the fatty acid content of lettuce leaves was analyzed. In a subsequent experiment, the fatty acid analysis was repeated and additionally plant physiological characteristics were determined (experimental procedure is shown in Supplemental Fig. S1).

Solutions of antifogging additives (Sabofog MS P, Sabostat A300 and Atmer 1440, 10 mg mL^{-1} in THF/*iso*-propanol 1:1, v/v) were prepared. Approximately 1 mL of each solution or pure solvent (THF/*iso*-propanol 1:1, v/v) or water, as controls, were nebulized onto lettuce leaves with a solvent-proof spray bottle, resulting in approximately 10 mg of additive on all lettuce leaves per plant. Lettuces were harvested 24 h after the additive treatment. Half of the plants were immediately frozen in liquid nitrogen, the other half were first washed with hexane (two times 60 sec in a hexane filled beaker). Six replicates were performed per treatment.

A subsequent set of lettuce was treated in the same way (each with four identical replicates). After harvest one third of the plants were directly frozen in liquid nitrogen, one third was washed with hexane as described above, and the other third was washed with water (two times 60 sec under tap water; fatty acid results of this subsequent experiment are shown in Supplemental Fig. S2).

Within this subsequent experiment, fresh weight assessment, plant physiological parameters and metabolite analyses were carried out. All samples were lyophilized and stored vacuum-packed at room temperature in the dark until further analysis. Before analysis, samples were ground to a fine powder with a mill (Retsch® MM 400, 45 sec, 2 repetitions at 25 s^{-1}).

2.5. *Physiological measurements and metabolite analysis*

The plant physiological parameters (assimilation rate, transpiration rate, stomatal conductance and leaf temperature) were measured using a LI-6800 gas exchange system (LI-COR Biosciences GmbH, Germany). These were determined for lettuce in the afternoon, immediately before treatment with the antifogging additives and 20 h after treatment. Four plants were measured for each treatment. The conditions were PAR 229 $\mu mol\ m^{-2}\ s^{-1}$, temperature at 22 °C and 70 % relative humidity. The carbon dioxide concentration in the chamber was set to 400 $\mu mol\ mol^{-1}$ with a flow rate of 500 $\mu mol\ s^{-1}$ and fan speed of 8000 rpm. Carotenoids and chlorophylls were extracted with tetrahydrofuran/methanol (1:1, v/v) and analyzed by high performance liquid chromatography-diode array detection-time of flight- mass spectrometry (HPLC-DAD-ToF-MS) as previously described [25]. Identification was based on comparing retention time, absorption maxima and mass spectra with standards or with the literature. External calibration with authentic standards was used for quantification at 450 nm.

2.6. *Soil application of antifogging additives*

Soil (20 g; 66.25 % water content) was homogenized with a solution of the antifogging additive Sabofog MS P in THF/*iso*-propanol (1:1, v/v), with an end concentration of 0.25 mg g^{-1} additive in standardized soil (Einheitserde classic). The same amount of pure solvent was also homogenized with soil as a control. These mixtures served as samples for fatty acid analysis (both in triplicate) and were frozen (-60 °C), lyophilized and stored in darkness at room temperature until further analysis.

2.7. *Statistical analysis*

For the statistical analysis, SigmaPlot 14 (Systat Software GmbH, Erkrath, 200 Germany) was used. Differences between treatments were analyzed using a *t*-test (for comparison of two groups) or one-way ANOVA followed by Tukey's HSD post hoc (for comparison of more than two groups). If the assumption of normal distribution was violated, the data were analyzed with the Kruskal-Wallis test. The data are presented as mean ± standard error.

3. Results and discussion

3.1. *Characterization of antifogging additives based on the fatty acid profiles via GC–MS*

Three commercially available antifogging additives with different structural properties were selected for the present study. The trade names of these additives are Sabofog MS P, Atmer 1440, and Sabostat A300. According to the SpecialChem database, these antifogging additives consist of the following specified compounds: sorbitan stearate (Sabofog MS P), glycerol monooleate (Atmer 1440) and stearyl diethanolamine stearate (Sabostat A300) [26–28].

The fatty acids of the antifogging additives were extracted after saponification to remove the hydrophilic structural moiety, and derivatized to methyl esters followed by GC–MS analysis. Two different extraction protocols were tested: transesterification with boron-trifluorid-methanol-complex and acid hydrolysis with methanol-hydrochloric acid. Since the latter resulted in better recoveries and required a less toxic reagent, this protocol was used for further analyses (Supplemental Table S1). It has been described before that the highest recoveries were achieved with a one-step extraction and methylation process as opposed to multi-step methods [29]. As antifogging additives may contain different saturated and unsaturated fatty acids and their isomers, a polar column (cyanopropyl base) was used as it is capable for such separations [17]. Four fatty acids were identified in Sabofog MS P, three in Sabostat A300, and nine different fatty acids in Atmer 1440. Thus, all antifogging additives contain more than the specified fatty acids (Fig. 2, Table 1).

To capture experimental fluctuations an internal standard was used to quantify the fatty acids as their corresponding methyl esters in relation to experimentally determined response factors of the fatty acids of interest. Heptadecanoic acid was used as an internal standard, since it is not present in antifogging additives, nor in a plant and soil matrix. Qualifiers and quantifier for each fatty acid were selected using the MassHunter quantitative data analysis software package (Table 2).

Only Sabostat A300 primarily contained the specified fatty acid stearic acid in major quantities (93.63%). In addition to the main fatty acid indicated, Atmer 1440 contained many fatty acids in minor quantities such as linoleic acid (9.86 %) and palmitic acid (8.01 %), so that the proportion of the most abundant fatty acid, oleic acid, was about 72.33 %. In contrast, Sabofog MS P contained 53.88 % palmitic acid and only 45.48 % of the specified main fatty acid stearic acid. This shows that the method presented can be used to characterize additives on the basis of their fatty acid composition, despite their structural diversity and variability.

The simplification of sample complexity by the FAME approach is also known from the analysis and quantification of lipids such as triacylglycerides (TAGs) [30]. Similar to antifogging additives (e.g. "glycerol monooleate"), TAGs consist of a glycerol core structure to which several possible fatty acids are esterified. However, it is necessary to determine intact molecules in order to answer certain questions about the stability of migrated additives on or in plants, or the uptake of intact molecules. For the analysis of intact lipids, various analytical methods were used that could also be of interest for considering antifogging additives, such as thin layer (TLC), or supercritical fluid chromatography (SFC), HPLC, nuclear magnetic resonance spectroscopy (NMR) and MS-based methods [30–32]. Each analytical approach offers its advantages.

MS-based methods are the best choice in terms of high sensitivity, specificity and provide structural information that is not available from the flame ionization detector (FID) systems often-used in FAME analysis. In contrast FID is advantageous due to a wide linear response range [16,17]. However, quantification could be somewhat challenging due to the ionization yield and response of the MS analyzer [16]. The electron impact ionization (EI) used in this study has long been proven to be suitable for small molecules such as FAMEs, but is limited for higher mass molecules such as intact lipids [33]. Depending on the research questions, a combination of complementary analytical methods may provide the greatest benefit, as in the case of lipid analysis. However, for the determination of several structurally different antifogging additives, the FAME analysis presented here provides a promising approach.

3.2. *Method validation*

The limit of detection (LOD) was determined based on a signal to noise ratio of three. Likewise, the limit of quantification (LOQ) was determined using a signal to noise ratio of ten. Limits of detection varied

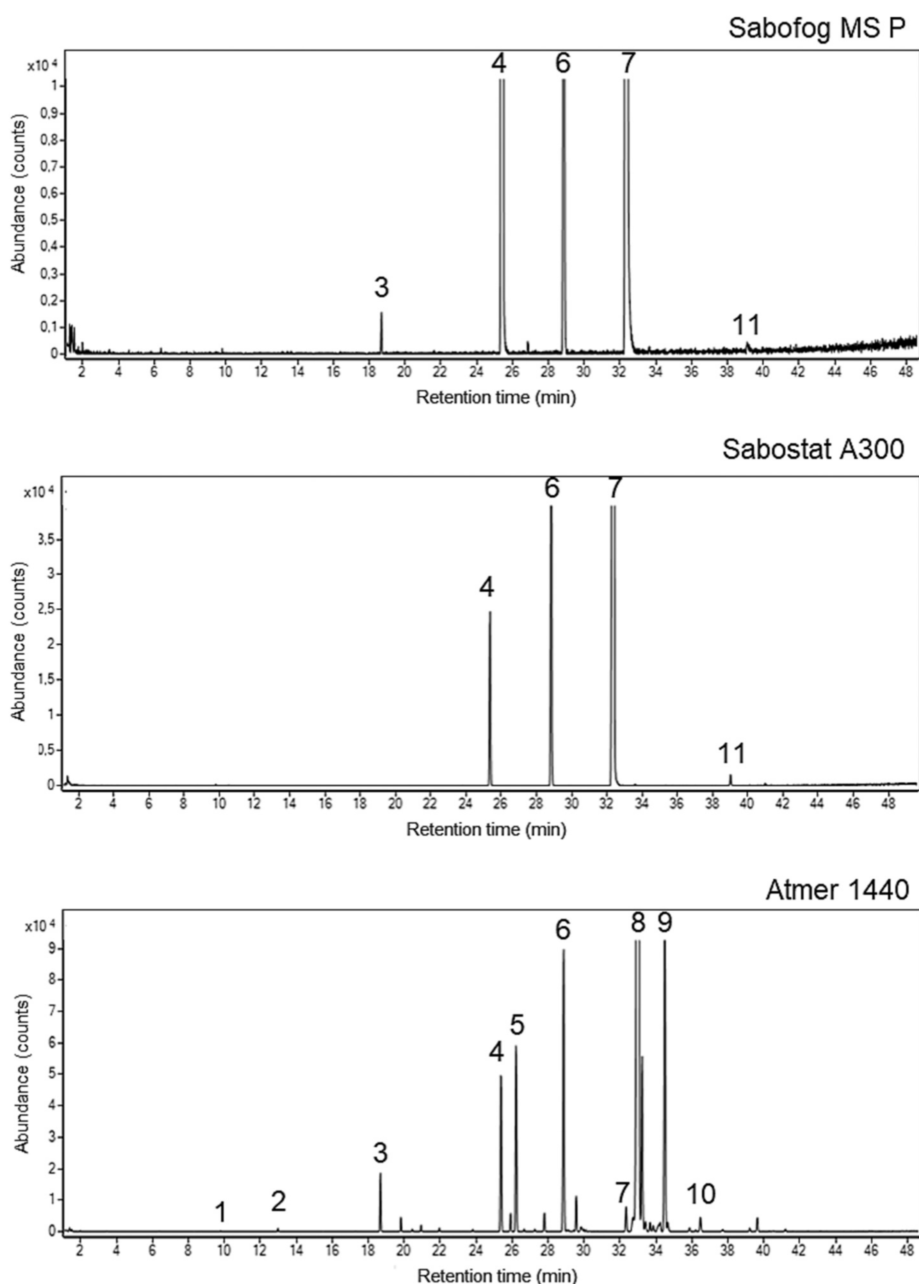

Fig. 2. Chromatogram (total ion current, TIC) of FAMEs of selected antifogging additives: Sabofog MS P (sorbitan stearate), Sabostat A300 (stearyldiethanolamin stearate) and Atmer 1440 (glycerol monooleate). The chromatograms show zoomed regions that allow the recognition of minor fatty acids. The following fatty acids were detected: (1) capric acid, (2) lauric acid, (3) myristic acid, (4) palmitic acid, (5) palmitoleic acid, (6) magaric acid – internal standard, (7) stearic acid, (8) oleic acid, (9) linoleic acid, (10) linolenic acid, and (11) arachidic acid. Peak assignment follows the numbering in Table 2.

between 0.01 pg and 12.15 ng on column, whereas limits of quantification varied between 0.04 pg and 40.51 ng on column (Table 2). Both LOD and LOQ tendentially increased with both increasing fatty acid chain length and degree of saturation, with the exception of oleic acid which shows the highest limits.

Additionally, the reproducibility of the method was verified by determining the inter- and intra- day variability (Table 2). The analysis revealed coefficients of variation overall under 10 %. Recoveries were performed for both extraction protocols with selected fatty acids (Supplemental Table S1). Here, soil samples were spiked with pamitic and stearic acid as the main fatty acids of Sabofog MS P. However, the recovery may vary between different fatty acids, and the method needs to be revalidated for the analysis of antifogging additives composed of different major fatty acids. The determined recovery for palmitic acid was 106.1 % and for stearic acid 116.7 %. Since the acid hydrolysis revealed the best recoveries, this protocol was selected for further analyses.

3.3. Determination of fatty acids in different matrices

The fatty acid approach can be used not only to determine the composition of additives, but also to characterize the matrices with which the additive is likely to come into contact. In preparation for further simulation experiments, the fatty acid analysis was tested for plant leaves and soil.

3.3.1. Lettuce leaves

Lyophilized lettuce leaves were used for the analysis and nine different fatty acids were identified in lettuce (Fig. 3). Four saturated fatty acids were detected (lauric, myristic, palmitic and stearic acid). In addition, four unsaturated fatty acids (palmitoleic, oleic, linoleic and linolenic acid), were all detected in quantifiable amounts. Palmitic acid as well as linoleic and linolenic acid were the most abundant fatty acids (Table 1). The fatty acids detected in lettuce are comparable to the data from food composition and nutrition tables by Souci, Fachmann, Kraut

Table 1

Fatty acids determined as their methyl ester (FAME) of the selected antifogging additives Sabofog MS P (sorbitan stearate), Sabostat A300 (stearyldiethanolamin stearate) and Atmer 1440 (glycerol monooleate, mg g^{-1}), lettuce leaves, soil, as well as soil spiked with Sabofog MS P (0.25 mg g^{-1} in soil FW, corresponding to 15.5 mg g^{-1} in soil DW, proportion of palmitic and stearic acid in Sabofog MS P can be seen below; mg g^{-1} DW) were analyzed. Values represent mean ± SD of three independent measurements. Abbreviations: ND - not detected, DW - dry weight, FW - fresh weight.

Fatty acid		**Antifogging additive fatty acids (mg g^{-1})**			**Matrix fatty acids (mg g^{-1} DW)**		
Name	Peak	Sabofog MS P	Sabostat A300	Atmer 1440	Lettuce leaves	Soil	Soil added AF
Saturated							
Capric acid	1	ND	ND	0.75 ± 0.24	ND	ND	ND
Lauric acid	2	0.22 ± 0.21	ND	2.26 ± 0.91	1.27 ± 0.78	0.01 ± 0.01	0.02 ± 0.01
Myristic acid	3	2.97 ± 0.89	ND	19.76 ± 1.34	0.21 ± 0.10	0.02 ± 0.01	0.12 ± 0.01
Palmitic acid	4	446.15 ± 18.50	31.08 ± 0.80	58.87 ± 1.70	6.51 ± 0.84	0.11 ± 0.01	7.04 ± 0.47
Stearic acid	7	376.56 ± 9.66	496.23 ± 39.36	10.35 ± 0.19	0.51 ± 0.09	0.04 ± 0.01	7.17 ± 0.60
Arachidic acid	11	2.13 ± 0.11	2.72 ± 0.18	ND	0.03 ± 0.03	0.02 ± 0.01	0.06 ± 0.01
Unsaturated							
Palmitoleic acid	5	ND	ND	34.18 ± 0.50	0.35 ± 0.12	ND	ND
Oleic acid	8	ND	ND	531.59 ± 27.45	0.13 ± 0.09	< LOQ	< LOQ
Linoleic acid	9	ND	ND	72.44 ± 3.58	4.80 ± 1.02	< LOQ	< LOQ
Linolenic acid	10	ND	ND	4.56 ± 0.21	6.97 ± 2.13	< LOQ	< LOQ

Table 2

Method parameters. Fatty acids, retention times (RT), ions used for qualification and quantification as well as nominal masses of the fatty acid methyl esters (FAMEs). Determined limits of detection and quantification (LOD, LOQ) and intra- and inter-day variability of each fatty acid are represented. The peak numbers correspond to the assignment in the chromatograms of Figs. 2 and 3. Rel. resp. – relative response (ratio of Qualifier to Quantifier).

Fatty acid			**RT**	***m/z***		**Mass**	**LOD**	**LOQ**	**Intra-day variability**	**Inter-day variability**
Saturated fatty acids										
Peak no	Name	Compound	min	Quantifier	Qualifier (rel. resp.)	(FAME)	pg on column	pg on column	CV %	CV %
1	Capric acid	C10:0	8.71	143	155 (55.3); 101 (31.5)	186	0.01	0.04	2.02	9.92
2	Lauric acid	C12:0	12.98	183	171 (136.2); 143 (155.2)	200	0.03	0.10	1.91	8.03
3	Myristic acid	C14:0	18.69	143	199 (86.3); 211 (43.3)	242	0.02	0.06	2.07	7.99
4	Palmitic acid	C16:0	25.41	236	129 (37.1); 199 (28.6)	270	0.02	0.08	2.97	9.04
6	Magaric acid	C17:0	28.89	284	241 (120.4); 143 (141.3)	284	0.04	0.12		
7	Stearic acid	C18:0	32.35	255	298 (94.0); 143 (118.5)	298	0.03	0.09	4.50	5.63
11	Arachidic acid	C20:0	39.07	326	283 (77.4); 143 (96.4)	326	0.02	0.07	4.93	4.59
Unsaturated fatty acids										
Peak no	Name	Compound	min	Quantifier	Qualifier (rel. resp.)	(FAME)	ng on column	ng on column	CV %	CV %
5	Palmitoleic acid	C16:1	26.22	236	237 (86.1); 96 (152.0)	268	1.39	4.63	1.33	6.24
8	Oleic acid	C18:1	32.93	265	264 (134.7); 222 (77.1)	296	12.15	40.51	4.76	4.12
9	Linoleic acid	C18:2	34.48	95	294 (26.5); 263 (22.8)	294	0.46	1.53	4.17	7.04
10	Linolenic acid	C18:3	36.47	95	93 (91.4); 108 (67.6)	292	0.45	1.51	3.70	7.62

Note: kindly check Table 2 alignment.

[34] as well as Kim et al. [35]. Thus, the method can be used to examine the fate of additives in simulation experiments.

3.3.2. *Soil and soil-added additives*

Similarly, to the lettuce leaves, the fatty acids were analyzed in lyophilized soil. Here, eight fatty acids were identified (Fig. 3). The saturated fatty acids lauric, myristic, palmitic, stearic acid as well as arachidic acid were detected. Also, the unsaturated fatty acids oleic, linoleic and linolenic acid were identified. Except for the saturated fatty acids, which were present in quantifiable amounts, the unsaturated fatty acids were below the limit of quantification (Table 1). A mixture of soil and Sabofog MS P was also prepared. Here, 89.16 ± 3.95 % palmitic acid and 108.24 ± 5.75 % stearic acid were recovered from Sabofog MS P in the soil (calculated based on data in Table 1). With an overall additive recovery of 97.59 ± 4.96 %, the fatty acid approach is suitable for determining of intentionally added additives in soil.

3.3.3. *Foliar application of antifogging additives*

Due to their structural properties, antifogging additives migrate to the polymer surface and might get washed out. In a simulation experiment, we analyzed the impact of antifogging additives on lettuce leaves.

The three antifogging additives Sabofog MS P, Sabostat A300 and Atmer 1440 were dissolved in THF/*iso*-Propanol (10 mg mL^{-1}, 1:1, v/v) and 1 mL was sprayed onto the leaves. Treatment with water was used as

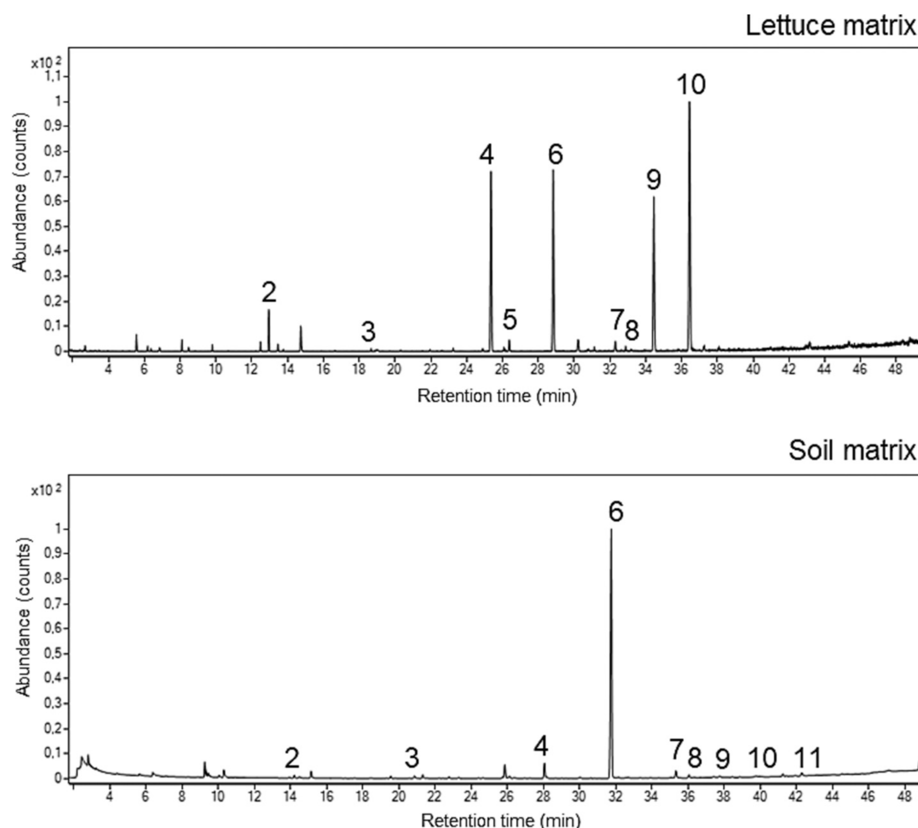

Fig. 3. Chromatogram (total ion current, TIC) of FAMEs in lettuce and soil matrix. The following fatty acids were detected (2; l,s) lauric acid, (3; l,s) myristic acid, (4; l,s) palmitic acid, (5; l) palmitoleic acid, (6; l,s) magaric acid – internal standard, (7; l,s) stearic acid, (8; l,s) oleic acid, (9; l,s) linoleic acid and (10; l,s) linolenic acid, and (11; s) arachidic acid in lettuce (l) and soil (s). Peak assignment follows the numbering in Table 2.

a first control and with pure solvent (THF/*iso*-Propanol 1:1, v/v) as a second control to ensure that the effects were not due to the solvent but to the additives. The treated leaves were harvested after 24 h. They were (A) analyzed directly; (B) first washed with hexane to assess the residual additives after removal of the top layers of waxy cuticle, or (C) simply washed with water (Fig. 4).

For lettuce treated with Sabofog MS P, significantly 6.9-fold higher stearic acid and 1.4-fold higher palmitic acid contents were determined compared to the two controls. Both were identified as the main fatty acids of this additive. Lettuce naturally contains fatty acids which are not added by antifogging additive treatment (Fig. 3). These naturally occurring and non-antifog fatty acids did not increase (Fig. 4). The additive-treated hexane-washed lettuce also revealed a significantly higher 2.2-fold stearic acid content, but this was not observed for palmitic acid. The main fatty acid in Sabostat A300, stearic acid, also showed significantly 9.7-fold higher amounts in the additive-treated lettuce than in the two controls. This significant difference was also present, although reduced, for the hexane-washed lettuce (1.6-fold). Again, the other fatty acids were not significantly affected by the treatments. The main fatty acid identified in Atmer 1440 was oleic acid, which occurs less abundantly in lettuce (Fig. 3). There were significant differences between controls and additive-treated lettuce for both

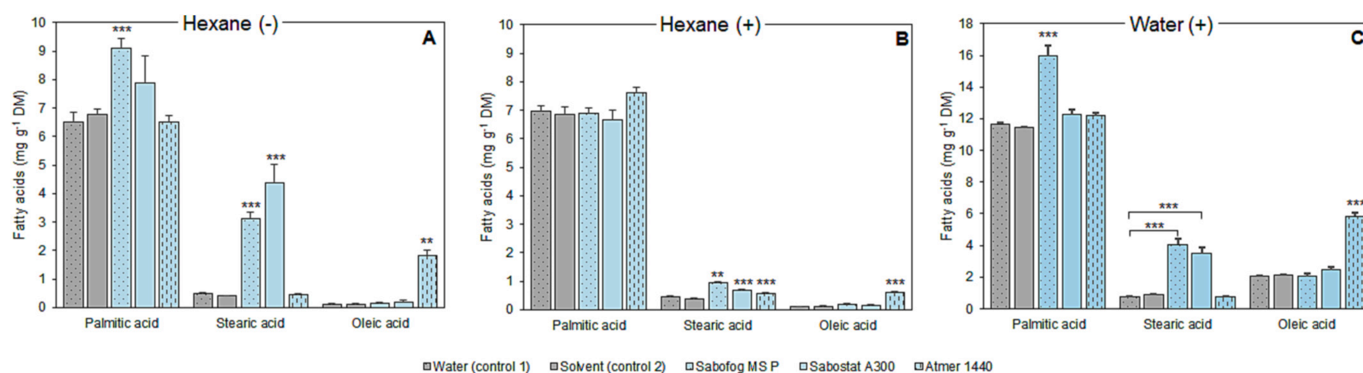

Fig. 4. Selected fatty acids (mg g^{-1} DW) in lettuce treated with water (control 1), solvent (THF/*iso*-propanol 1:1, v/v, control 2) or with dissolved antifogging additive. The main fatty acids of each antifogging additive are shown, namely Sabofog MS P (palmitic and stearic acid), Sabostat A300 (stearic acid) and Atmer 1440 (oleic acid). (A) The lettuce leaves (n = 6) were harvested and analyzed directly, or (B) were washed with hexane before analysis. (C) A subsequent set of lettuce leaves treated the same way were harvested (n = 4) then washed with water before analysis. Values show means ± SE. Asterisks indicate significant differences between the antifogging-treatment and the two controls (** $p \leq 0.005$; *** $p \leq 0.001$). The brackets show significant differences only compared to the indicated control.

directly analyzed (15.3-fold) and hexane-washed (5.7-fold) lettuces. However, this treatment also significantly affected the stearic acid in the hexane-washed lettuce, while other detected fatty acids remained unaffected.

Lettuces were washed with water to test whether adhering additives could be removed from the surface (Fig. 4). It was evident that for all three additive treatments, significantly high amounts of the main antifogging additive fatty acids were still present after the water wash, to some extent resembling the unwashed leaves (Fig. 4). However, stearic acid content was only significantly higher when comparing water controls and additive-treated lettuce for Sabofog MS P (5.4-fold) as well as Sabostat A300 (4.2-fold). Nevertheless, comparing the two controls (water and solvent treatment) and the lettuce treated with Sabofog MS P, the palmitic acid content was significantly 1.4-fold higher. Similarly, oleic acid was increased 2.8-fold in the lettuce treated with Atmer 1440 compared to the two controls. Other fatty acids were not affected for Atmer 1440 and Sabofog MS P treated lettuce; however, there was a significant difference in linoleic acid in lettuce treated with Sabostat A300 compared to both water and solvent controls (Supplemental Table S2).

The above results indicate that foliar applied fatty acids of antifogging additives can adhere to leaves. Washing with water had little effect on their removal. Even after washing the lettuce leaves with hexane to remove top layers of the cuticle, all three major fatty acids of the additives were still present in significant higher amounts compared to the controls. This may indicate residual additives in the plant cuticle. It is known that plants can take up compounds via the cuticle [36]. Since the plant cuticle is a waxy layer, the additives with their hydrophobic moiety are theoretically able to anchor or diffuse into it. The EU regulates both the substances that can be used as additives and the migration limits for food contact materials (GMOs) [37]. For the registration of these additives, toxicological and ecotoxicological data, among others, must be provided. The REACH database (Registration, Evaluation, Authorisation and Restriction of Chemicals) reveals no toxicological concerns for sorbitan ester and glycerol ester based additives, and they do not accumulate in the environment due to their biodegradability [11,12]. However, the stearyldiethanolamine based additives are not yet approved for food contact use in the EU; the evaluation by EFSA (European Food Safety Authority) is currently ongoing [38]. Furthermore, some of its ingredients are listed in the Annex III Inventory by ECHA as a risk of aquatic toxicity; however the formulated additive listed by ECHA did not show aquatic toxicity [13,39]. Moreover, this is not a tool for classification, but merely shows that there is evidence of concern. Compounds of stearyldiethanolamine based additives are not biodegradable, and one compound, which is the unreacted monomer stearyldiethanolamine, is also below the threshold of CLP regulation (Regulation on Classification, Labelling and Packaging of substances and mixtures; 500 g L^{-1}) [13]. Bioaccumulation of leached additives from plastic films should probably be taken into account. Not only the (eco-)toxicity of Sabostat A300 itself needs to be considered, as surfactant molecules, antifogging additives can potentially interact with a variety of other substances [40–43]. For example, surfactants have been used in pesticides to improve foliar uptake of these compounds [41]. Considering this, the interaction of antifogging additives and its environment could be particularly important in the case of persistent, non-removable compounds.

3.3.4. *Impact of foliar applied antifogging additives on plant physiology and metabolites*

Since antifogging additives cannot be removed by washing with water, this suggests that they are unlikely to be removed from the leaf surface by irrigation water. For this reason, the effects of adherent additives on plant physiology are important. In the present study, assimilation and transpiration rate, stomatal conductance and leaf temperature were measured before and 20 h after the additive-treatment (Fig. 5). However, no significant differences were determined. Nevertheless, tendentially increased transpiration, stomatal conductance as well as leaf temperature can be observed for all three antifogging additive treatments compared to the water control. It can be assumed that adhering additives cause an altered heat exchange, which is also indicated by an increase in leaf temperature.

Additionally, the fresh weight and selected valuable metabolites

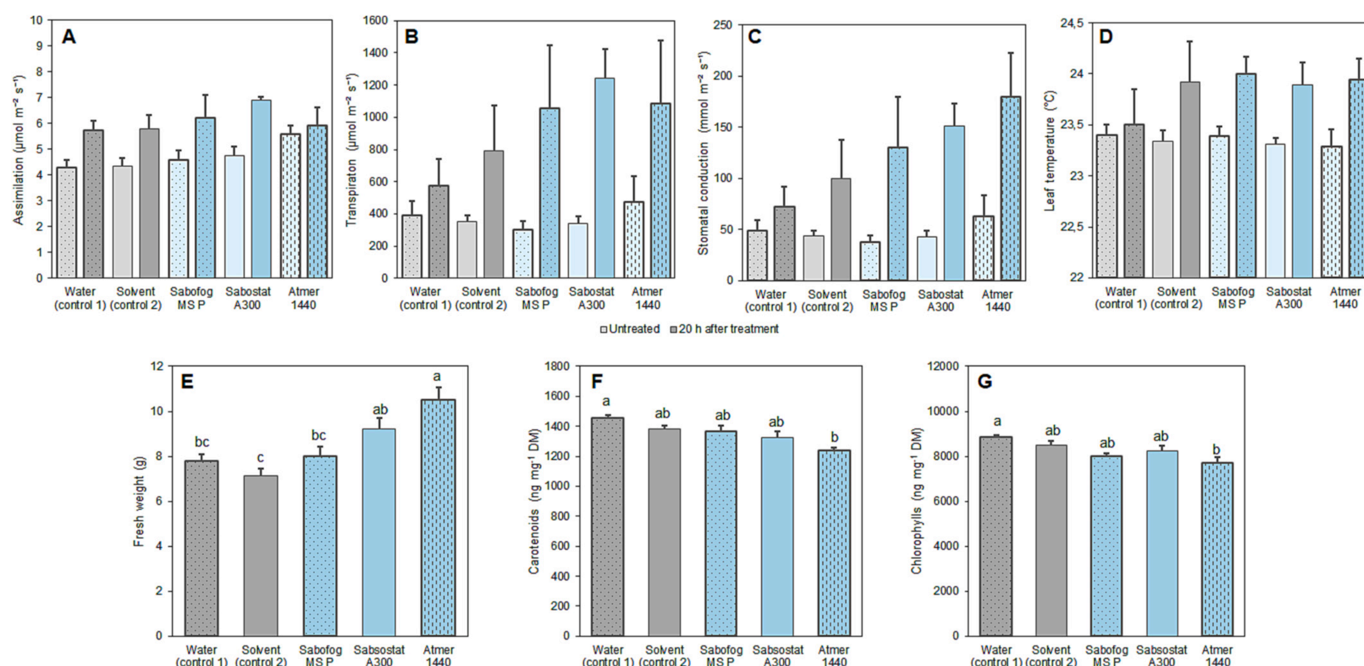

Fig. 5. Physiological characterization of lettuce leaves. (A) assimilation rate, μmol m^{-2} s^{-1}; (B) transpiration rate, μmol m^{-2} s^{-1}; (C) stomatal conductance, mmol m^{-2} s^{-1}; (D) leaf temperature, °C (all before and after 20 h treatment, first and second bar) as well as (E) fresh weight, g; (F) total carotenoids, and (G) chlorophylls, ng mg^{-1} DW, 20 h after treatment with water (control 1), solvent (THF/*iso*-propanol 1:1, v/v, control 2) or dissolved antifogging additive. Values show means ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$).

(chlorophyll and carotenoids) in additive-treated lettuce were determined. No impact on fresh weight was observed in lettuce treated with Sabofog MS P. No significant differences were found when comparing the water and solvent controls either, as might be expected after spraying with solvent. Lettuce treated with Sabostat A300 similarly showed no significant differences compared to the water control. However, Sabostat A300-treated lettuce had a significantly 1.4-fold higher fresh weight than the solvent-treated lettuce. Similary, lettuce treated with Atmer 1440 had a significantly 1.4-fold higher fresh weight compared to the two controls.

As essential compounds for plants photosynthesis, chlorophylls and carotenoids were determined. No significant differences were found between the two control groups (water- and solvent-treated) for both total carotenoids and total chlorophylls. Significantly lower total carotenoids and total chlorophylls (both 1.1-fold) were only observed in lettuce treated with Atmer 1440. The additives could change the absorption and reflection properties of the leaf surface and thus the light reaching the chloroplasts of the plants. This can lead to a reduction in photosynthetic pigments, as both light intensity and spectral quality are crucial factors in their synthesis, accumulation and degradation [44,45]. Moreover, adhering additives and applied solvents could cause stress conditions, which would contribute to the formation of reactive oxygen species [46]. As antioxidants with long conjugated double bond chains carotenoids could act as radical scavengers and be degraded [47]. Atmer 1440-treated lettuces was shown to have significantly lower levels of zeaxanthin (2.6-fold) compared to the two controls. In addition, zeaxanthin and β-carotene were significantly lower in Sabostat A300-treated lettuce (4.7-fold and 1.1-fold) compared to water control (Supplemental Fig. S3). In this short experimental period, negligible effects can be detected; however, long-term effects of adhering additives on crops could be of interest.

3.4. Limitations and analytical challenge

The method presented here provides a first indication of the fate of antifogging additives on lettuce leaves. Further research is needed to examine other less model-like conditions, as there are some limitations due to the use of the fatty acid approach and the model of additive application on leaves. First, the method does not allow measurement of intact antifogging additive molecules, and the fatty acid approach is not suitable for reproducing the original composition and structure of the additives. For example, no conclusions can be drawn about antifogging additives in unknown samples. For the advantage of determining a large variety of differently structured molecules, a loss of information must be accepted. Second, the experiment was conducted with foliar applied antifogging additives in a model setting using relatively high artificial concentrations to test the general properties. However, the amount of antifogging additives used in plastic films is usually between 0.1 and 4 wt% [26–28]. The specified migration limits of 60 mg kg^{-1} for food contact materials according to the EU regulation are also below the experimental conditions [37]. Nevertheless, adherence and non-removability could be observed within this approach. The application of a solvent could change the leaf surface. By using two controls, water and pure solvent, this problem was almost overcome in the present study. Finally, the experimental conditions were short-term, so the long-term effects of such additives covering the entire cultivation and growth period until harvest and aging processes of agriculture films may be of interest.

4. Conclusion

The method presented here is a simple and rapid method to characterize the fatty acid moieties of antifogging additives with little effort in sample preparation. Antifogging additives for polymer films present a wide structural diversity. Not only do different additives have different structural properties, but also an additive itself consists of a variety of possible compounds due to its manufacturing process. In the present study, we overcome this analytical challenge using the fatty acid approach. This approach, despite some limitations, was successfully applied to study the fate of antifogging additives leached from polymers and their impact on plants in a simulation experiment. Finally, in this study, the impact of foliar-adherent antifogging additives on plant metabolites and physiological parameters can be described as negligible under short-term conditions.

CRediT authorship contribution statement

Vanessa Harbart: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Bernd Hönig:** Resources, Writing – review & editing. **Susanne Baldermann:** Conceptualization, Validation, Investigation, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109133.

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Supplemental Information

Comprehensive profiling and quantification of antifogging additives based on fatty acid composition by GC-MS and its application in different

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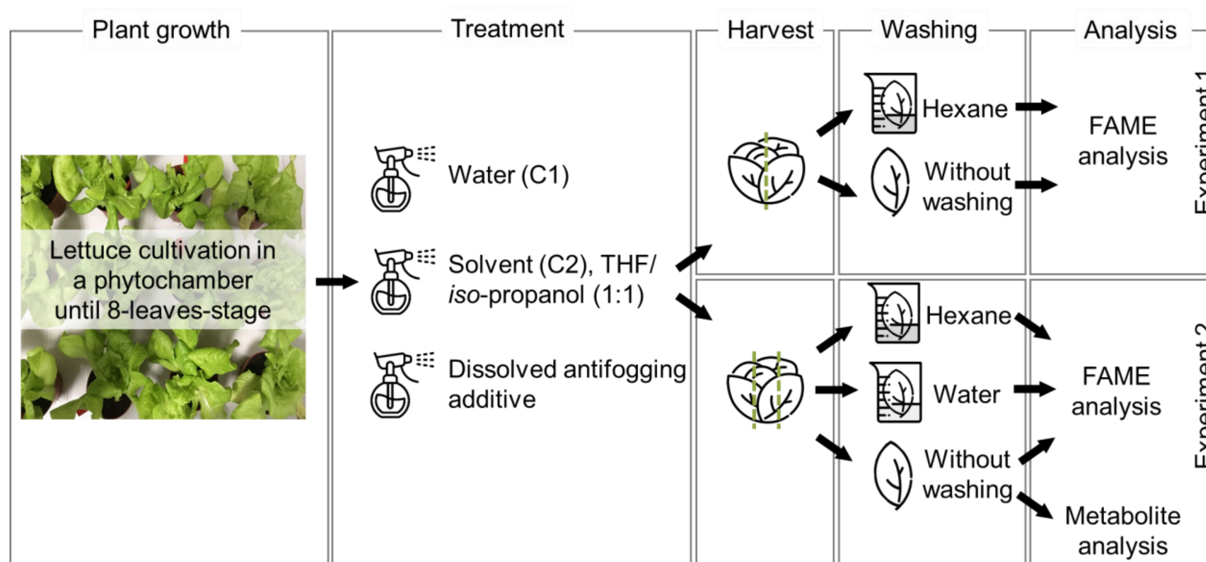
Figure S1: **Experimental procedure** for lettuce growth and treatment with water (control C1), solvent (control C2) and the dissolved antifogging additives harvested after 24 h followed by washing steps and analysis. First experiment (n = 6) and subsequent experiment (n = 4) are shown. Icons made by Freepik from www.flaticon.com.



Supplemental Information

Figure S2: **Analysis of fatty acids** of selected fatty acids (mg g^{-1} DW) in lettuce treated with water (control 1), solvent (THF/*iso*-propanol 1:1, v/v, control 2) or with dissolved antifogging additive in subsequent experiment. The main fatty acids of each antifogging additive are shown, namely Sabofog MS P (palmitic and stearic acid), Sabostat A300 (stearic acid) and Atmer 1440 (oleic acid). (A) The lettuce leaves were harvested and analyzed directly. (B) The lettuce leaves were washed with hexane before analysis. Values show means ± SE (n = 4). Asterisks indicate significant differences between the antifog-treatment and the two controls (* p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.001). The brackets show significant differences only compared to the indicated control.

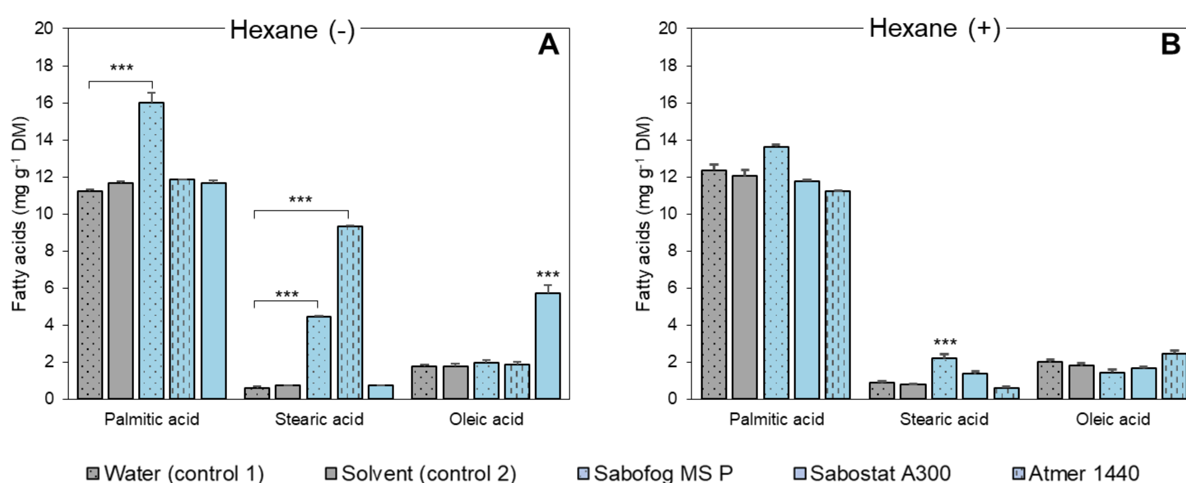
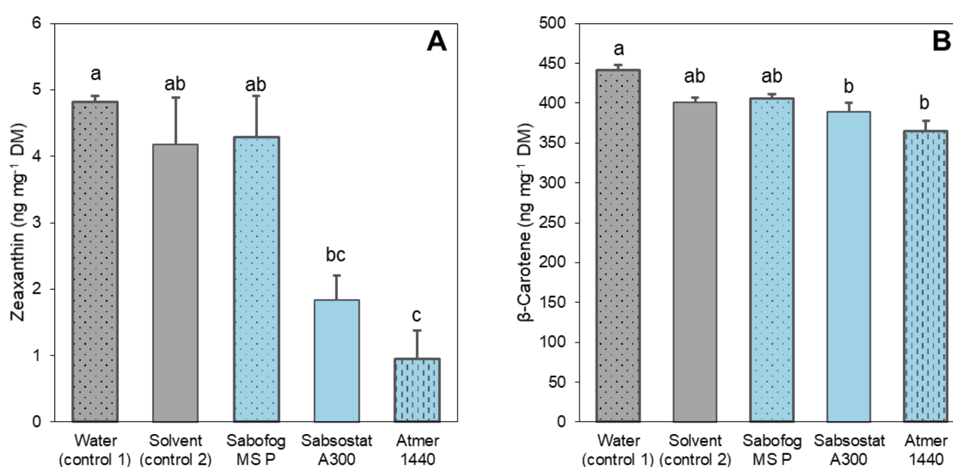


Figure S3: **Individual carotenoids in treated leaves.** (A) zeaxanthin and (B) β-carotene in lettuce treated with water (control 1), solvent (THF/*iso*-propanol 1:1, v/v, control 2) or with dissolved antifogging additives. Values show means ± SE (n = 4). Different letters indicate significant differences (p ≤ 0.05).



Supplemental Information

Table S1: **Recoveries for the two extraction protocols tested**. The recoveries for selected fatty acids were tested for acid hydrolysis in soil samples and for transesterification using standards only. Due to the better results obtained with acid hydrolysis, this protocol was used for further analysis.

	Recovery % acid hydrolysis protocol	**Recovery %** transesterification protocol
Myristic acid		151.86 ± 4.22
Palmitic acid	106.13 ± 4.00	149.79 ± 3.17
Stearic acid	116.73 ± 4.35	
Arachidonic acid		106.03 ± 2.53

Supplemental Information

Table S2: **Fatty acids in treated lettuce leaves.** Determined fatty acids (mg g^{-1} DW) in lettuce treated with water (control C1), solvent (THF/iso-propanol 1:1, v/v, control C2) or dissolved antifogging additives in both experiments. Values shows means ± SE (n = 6 (1), n = 4 (2)). Asterisks represent different levels of significant differences (*** p ≤ 0.001; ** p ≤ 0.005; * p ≤ 0.05) between the additive treatment and the controls. The brackets show significant differences only compared to one control.

	Fatty acid	Experiment 1					Subsequent experiment 2				
		Controls		Additive Treatment			Controls		Additive Treatment		
		Water (C1)	Solvent (C2)	Sabofog MS P	Sabostat A300	Atmer 1440	Water (C1)	Solvent (C2)	Sabofog MS P	Sabostat A300	Atmer 1440
No washing	*Saturated*										
	Lauric acid	1.27 ± 0.32	1.47 ± 0.17	1.09 ± 0.25	1.52 ± 0.19	1.17 ± 0.16	0.89 ± 0.12	1.40 ± 0.11	0.98 ± 0.05	1.28 ± 0.19	0.86 ± 0.03
	Myristic acid	0.21 ± 0.04	0.28 ± 0.05	0.25 ± 0.03	0.24 ± 0.03	0.30 ± 0.03	< LOD	< LOD	< LOD	< LOD	< LOD
	Palmitic acid	6.51 ± 0.34	6.78 ± 0.19	9.11 ± 0.34***	7.88 ± 0.96	6.52 ± 0.21	11.25 ± 0.09	11.65 ± 0.12	15.98 ± 0.57[***]	11.87 ± 0.01	11.69 ± 0.11
	Stearic acid	0.51 ± 0.03	0.40 ± 0.02	3.11 ± 0.23***	4.38 ± 0.63***	0.47 ± 0.01	0.62 ± 0.01	0.74 ± 0.01	4.47 ± 0.39[***]	9.34 ± 0.28[***]	0.73 ± 0.02
	Arachidonic acid	0.03 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	< LOD	< LOD	< LOD	< LOD	< LOD
	Unsaturated										
	Palmitoleic acid	0.35 ± 0.05	0.45 ± 0.03	0.30 ± 0.02[*]	0.45 ± 0.05	0.41 ± 0.05	1.54 ± 0.06	1.52 ± 0.04	1.46 ± 0.05	1.57 ± 0.04	1.41 ± 0.02
	Oleic acid	0.13 ± 0.04	0.11 ± 0.03	0.15 ± 0.03	0.20 ± 0.06	1.83 ± 0.19**	1.77 ± 0.11	1.76 ± 0.15	1.97 ± 0.15	1.88 ± 0.14	5.71 ± 0.44***
	Linoleic acid	4.80 ± 0.42	5.07 ± 0.24	4.49 ± 0.28	5.70 ± 0.69	5.37 ± 0.49	16.95 ± 0.23	17.75 ± 0.17	17.98 ± 0.44	17.73 ± 0.08	18.94 ± 0.29**
	Linolenic acid	6.97 ± 0.87	8.44 ± 0.57	6.46 ± 0.60	8.66 ± 0.98	7.94 ± 0.79	42.80 ± 1.37	42.77 ± 0.31	44.85 ± 0.60	47.11 ± 0.47[**]	43.62 ± 0.55
Hexane washing	*Saturated*										
	Lauric acid	0.58 ± 0.08	0.80 ± 0.25	0.37 ± 0.04	0.53 ± 0.09	0.73 ± 0.08	< LOD	< LOD	< LOD	< LOD	< LOD
	Myristic acid	0.22 ± 0.04	0.20 ± 0.02	0.18 ± 0.01	0.21 ± 0.04	0.30 ± 0.04	0.07 ± 0.06	< LOD	< LOD	< LOD	< LOD
	Palmitic acid	6.98 ± 0.16	6.85 ± 0.26	6.89 ± 0.21	6.65 ± 0.35	7.61 ± 0.21	12.36 ± 0.31	12.05 ± 0.34	13.62 ± 0.11	11.77 ± 0.05	11.22 ± 0.03
	Stearic acid	0.45 ± 0.03	0.38 ± 0.02	0.94 ± 0.06**	0.67 ± 0.05***	0.57 ± 0.02***	0.88 ± 0.07	0.80 ± 0.05	2.20 ± 0.24***	1.37 ± 0.15	0.62 ± 0.08
	Arachidonic acid	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	Unsaturated										
	Palmitoleic acid	0.39 ± 0.03	0.43 ± 0.04	0.37 ± 0.01	0.29 ± 0.03[*]	0.51 ± 0.06	1.55 ± 0.02	1.45 ± 0.09	1.20 ± 0.10	1.44 ± 0.08	1.24 ± 0.04

	Oleic acid	0.11 ± 0.01	0.11 ± 0.03	0.18 ± 0.04	0.14 ± 0.06	0.61 ± 0.04**	2.01 ± 0.12	1.80 ± 0.14	1.44 ± 0.15	1.68 ± 0.10	2.47 ± 0.14
	Linoleic acid	5.10 ± 0.30	5.75 ± 0.43	5.26 ± 0.40	4.89 ± 0.45	7.00 ± 0.70	19.71 ± 0.47	19.34 ± 0.83	18.02 ± 0.18	19.55 ± 0.30	17.88 ± 0.13
	Linolenic acid	8.04 ± 0.55	9.10 ± 0.74	8.05 ± 0.66	7.56 ± 0.66	11.66 ± 1.54	47.02 ± 0.55	42.38 ± 2.39	43.08 ± 0.78	47.56 ± 1.59	41.94 ± 0.62
Water washing	*Saturated*										
	Lauric acid						< LOD	< LOD	< LOD	< LOD	< LOD
	Myristic acid						0.04 ± 0.03	< LOD	< LOD	0.11 ± 0.06	0.07 ± 0.06
	Palmitic acid						11.67 ± 0.28	11.46 ± 0.12	15.95 ± 0.68***	12.26 ± 0.30	12.19 ± 0.19
	Stearic acid						0.75 ± 0.04	0.92 ± 0.07	4.07 ± 0.35[***]	3.49 ± 0.38[***]	0.77 ± 0.02
	Arachidonic acid						< LOD	< LOD	< LOD	< LOD	< LOD
	Unsaturated										
	Palmitoleic acid						1.50 ± 0.05	1.38 ± 0.06	1.45 ± 0.03	1.47 ± 0.05	1.35 ± 0.05
	Oleic acid						2.05 ± 0.10	2.14 ± 0.08	2.05 ± 0.16	2.46 ± 0.21	5.83 ± 0.25***
	Linoleic acid						18.65 ± 0.38	18.55 ± 0.29	18.23 ± 0.93	21.19 ± 0.41**	19.47 ± 0.26
	Linolenic acid						45.29 ± 1.64	40.62 ± 1.11	44.58 ± 1.34	47.71 ± 1.26	41.71 ± 1.19

Supplemental Information

Table S3: Experimentally determined response factors used for fatty acid quantification in the present study. Response factors are normalized to stearic acid (RP = 1).

Fatty acid	Response factors
Saturated	
Capric acid	1.0249
Lauric acid	1.9930
Myristic acid	0.8912
Palmitic acid	0.9200
Stearic acid	1.0000
Arachidonic acid	0.8049
Unsaturated	
Palmitoleic acid	2.1875
Oleic acid	2.1820
Linoleic acid	0.8635
Linolenic acid	0.8366

Table S4: Determined retention time indices (Kovats) of fatty acids methyl esters related to adjacent *n*-alkanes in the present study.

Fatty acid (FAME)	Carbon atoms	Kovats index	
		Experimental	NIST Database†
Saturated			
Capric acid	10	601	1581 – 1592
Lauric acid	12	1812	1770 – 1834
Myristic acid	14	2022	1990 – 2037
Palmitic acid	16	2234	2177 – 2243
Magaric acid	17	2340	2295 – 2344
Stearic acid	18	2447	2389 – 2445
Arachidonic acid	20	2660	2617 – 2646
Unsaturated			
Palmitoleic acid	16	2259	2242 – 2277
Oleic acid	18	2465	2400 – 2472
Linoleic acid	18	2514	2485 – 2523
Linolenic acid	18	2577	2590

† NIST Database: https://webbook.nist.gov/chemistry/; Data provided for Kovats' RI polar column, isothermal and temperature ramp

Publication 2

Antifogging additives for greenhouse covers – effects on phytochemicals and nutritional quality of lettuce

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SUSTAINABLE DEVELOPMENT GOALS 2 ZERO HUNGER

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Antifogging additives for greenhouse covers – effects on phytochemicals and nutritional quality of lettuce

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Summary

Antifogging additives are commercially used in greenhouse films to prevent water droplet formation on these films. This can increase light transmission, and thus, improve crop yield. However, the effect of polytunnels with antifogging additives on phytochemical content in lettuce (*Lactuca sativa var. capitata*) is currently unclear. Here, polytunnels were chosen as a model to investigate the impact of antifogging additives in a completely randomized setting. Analysis by means of chromatographic methods coupled with mass spectrometry revealed a general influence of polytunnel cultivation compared to lettuces grown without a polytunnel on the content of phenolic compounds, photosynthetic pigments and fatty acids. The use of antifogging additives does not lead to significant differences in phenolic compounds and fatty acids. However, significant differences were observed for carotenoids and chlorophylls by both polytunnel cultivation and the use of antifogging additives. These differences probably occurred predominantly due to differences in light and temperature regimes related to polytunnel cultivation. Thus, due to polytunnels in general and the use of antifogging additives in particular, environmental conditions are created that impact valuable compounds and alter nutritional quality of crops.

Keywords: Polytunnel, phenolic compounds, carotenoids, fatty acids, plastic additives, light transmission, *Lactuca sativa*

Introduction

Plastic films are widely used to cover greenhouses and polytunnels to produce horticultural crops. It is estimated that 5,630,000 ha of land was used for protected agriculture worldwide in 2019 (World Greenhouse Vegetable Statistics, 2019). In Germany, these protected agricultural area covers 1,279.3 ha (Statistisches Bundesamt, 2019). The advantages of using protected cultivation compared to open field conditions are improved yield and productivity since farmers can produce off-season or start growing ahead of the season (Gruda, 2005). Moreover, in hotter climates, it is possible to conserve water, and thus, improve the efficiency of crop production (Irusta et al., 2009). Of note is that low material costs make plastic greenhouse films more favorable than glass greenhouses and this is reflected in Southeast European countries (SEE countries) where the greenhouse surface made of glass compared to plastic is about 8,305 ha and 46,280 ha, respectively (Baudoin et al., 2017).

The benefits of using greenhouses or polytunnels result from the control of environmental factors, such as light and temperature, enabling optimal growing conditions to be created for the cultivated crops. The materials used for greenhouse covers provide different light transmittances and thermal efficiencies, so the selection of different materials can influence both crop yield and nutritional quality. Although several studies have investigated the impact of such materials on plant growth and crop production (Papadopoulos et al., 1997; Hao et al., 1999; Gruda, 2005; Cemek et al., 2006), those on how greenhouse materials affect nutritional quality are rare (Petropoulos et al., 2019; Ahmadi et al., 2019). In addition, plastic greenhouse films can be modified with various plastic additives to generate beneficial properties. For example, UV-blockers can prevent UV-light transmission and protect plants against damage (Katsoulas et al., 2020). Some studies have demonstrated that UV-blocking greenhouse films have an effect on crop yield and nutritionally valuable compounds such as plant secondary metabolites, like phenolic compounds, carotenoids and chlorophylls (reviewed by Katsoulas et al., 2020). However, research on the effect of other plastic additives, such as antifogging additives, is currently lacking. Antifogging additives are used to prevent the formation of water droplets on the inside of the greenhouse. This has several advantages, such as improved light transmission through the films, prevention of microbiological contamination as well as heat retention in the greenhouse (Ren et al., 2018).

Here, we investigated the impact of polytunnels with antifogging additives on the nutritional quality of lettuce (*Lactuca sativa var. capitate* cultivars 'Veronique' and 'Attractie'). Lettuce is often grown under protective covers. In Germany, 2,331.04 t of lettuce were produced under protected conditions on an area of 61.57 ha in 2019 (Statistisches Bundesamt, 2019). Due to its high water content, the nutritional value of lettuce has been underestimated although cultivars with favorable nutrient content are known (Kim et al., 2016). Besides essential vitamins and minerals, lettuce also provides several phytochemicals with potential health-promoting effects such as flavonoids, carotenoids and polyunsaturated fatty acids (Kim et al., 2016). Importantly, these phytochemicals are associated with positive health effects such as a reduced risk of noncommunicable diseases like cancer, cardiovascular disease or age-related functional decline (Kim et al., 2016; Clifton et al., 2017; Eisenhauer et al., 2017; Milani et al., 2017; Kim et al., 2018; Rees et al., 2018; Kopustinskiene et al., 2020).

We hypothesized that the use of antifogging additives in polytunnels will affect nutritional quality of lettuce due to changes especially in the light regime. To test this hypothesis, we cultivated lettuce under polytunnels with and without antifogging additives. To determine the general impact of microclimate induced by polytunnels, we cultivated lettuce cover-free (without a polytunnel). Climatic conditions were monitored throughout the experiment. Valuable compounds such as flavonoids and phenolic acids, carotenoids and chlorophylls as well as fatty acids were determined by chromatographic methods coupled with mass spectrometry.

A step towards Sustainable Development Goal 2 "zero hunger"

Current food production systems undergo transformation in terms of productivity, resource use and environmental impacts. Greenhouses

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and polytunnels provide favorable growth conditions for vegetables and are thought to be a possible pathway towards sustainable production (ZHOU et al., 2021). Increased productivity can be achieved e.g. by off-season production and target for instance the target 2.1 'ensure access by all people to safe and nutritious food all year around' of the SDG 2 – "end hunger, achieve food security and improved nutrition and promote sustainable agriculture". In addition, crop losses could be reduced using, for example, antifogging additives by preventing crop spoilage. Finally, the selection of useful covering materials can contribute to the improvement of nutritional quality and thus to the achievement of the SDG 2 "zero hunger".

Material and methods

Plant cultivation, preparation and covering material

The experiment was conducted from 19th September to 28th October 2019 and repeated from 16th January to 28th February 2020. A glass-coated greenhouse was used for the experiments located at the Leibniz Institute of Vegetables and Ornamental crops (Großbeeren, 52°20'5N 13°18'35.3"E). The greenhouse temperature and the relative humidity was set to 22 °C and 70%, controlled by open vents. Additional artificial light (SON T AGRO 400 W, Phillips) was applied once the outer light intensity was lower than 50 klx for a maximum of 10 h per day. Eight polytunnels (58 × 50 × 50 cm, *L × W × H*, Supplemental Figure S1) were built for a sufficient number of experimental repetitions. The covers must be completely closed to generate high humidity conditions that the antifogging additives become active. A completely randomized setup was chosen to minimize the impact due to position of plants and polytunnels. The plants under the polytunnels were randomized twice a week, and the polytunnel position was randomized once halfway through the experiment. Commercially available three-layered polyethylene film (low-density/linear low-density polyethylene/14% ethylene butyl acrylate (middle layer), 180 µm thickness, CONSTAB polyolefin additives GmbH, Rüthen, Germany) was used as covering material. Half of the films contained a mixture of antifogging additives (Sabostat A 300 and Atmer 103, 0.35%) embedded in the plant-facing side. The other half was without additives. Transmission spectra were measured for both films using a V-670 photospectrometer (Jasco Deutschland GmbH, Pfungstadt, Germany). Seven plants were grown under each polytunnel or without being covered by a polytunnel, corresponding to a total of 28 plants per treatment (84 plants in total, Supplemental Figure S2). For the first experiment, two different cultivars were chosen ('Veronique' and 'Attractie'), corresponding to 16 repetitions for 'Veronique' and 12 repetitions for 'Attractie'. The cultivars were randomly placed under the polytunnels and in the trays. The second experiment was performed with cultivar 'Veronique' only.

Lettuce seeds were germinated in a climate chamber under the following conditions: 12 °C temperature, 75% relative humidity, 12/12 h day/night period and 350 µmol m^{-2} s^{-1} light intensity. Eleven (experiment 2019) and 18 (experiment 2020) days after sowing when the plants reached the two-leaf stage, they were transplanted in 13 cm pots with soil (the pH of the soil was 5.9, N was 183 mg L^{-1}, P_2O_5 was 135 mg L^{-1}, K_2O was 212 mg L^{-1}, and salinity was 1.23 g L^{-1}, Einheitserde classic, Einheitserde Werkverband e.V., Sinntal-Altengronau, Germany) and transferred into the experimental setup. The edible part of the plants was harvested after 38 to 43 days with a fresh weight of 11.4 ± 4.2 g. Half of each plant was taken as one sample. The samples were immediately frozen in liquid nitrogen, lyophilized and stored vacuum-packed at ambient temperature in the dark until further analysis. Before analysis, samples were ground to a fine powder with a mill (Retsch® MM 400, 45 sec, 2 repetitions at 25 1 s^{-1}).

Climatic condition measurements

During the experiments, temperature, relative humidity and photosynthetic active radiation (PAR) were monitored. For this purpose, two sensors (LI-190R Quantum Sensor, LI-COR Biosciences GmbH, Germany; sensor type KPC 1/5, PT - 100 type B sensor, Galltec Mess- und Regeltechnik GmbH, Bondorf, Germany, MELA Sensortechnik GmbH Mohlsdorf-Teichwolframsdorf, Germany) were placed under two polytunnels of each treatment. To determine climatic conditions in the greenhouse chamber, an aspiration psychrometer (Type ELAU KlimaExpert, KE-PTFF-8024-OF, Elektro- und Automatisierungsanlagen Pierre Ambrozy, Gatersleben, Germany) was used. The greenhouses had PAR sensors on the roof (PAR-Quantumsensor DK-PHAR 2, deka Sensor u. Technologie, Entwicklungs u. Vertriebs GmbH, Teltow, Germany), which were used to determine the value inside. To determine the transmittance of the greenhouse roof and thus calculate the light intensity in the chamber, a light meter (Model LI-250 Light Meter, LI-COR Biosciences GmbH, Germany) was used. The measurements indicated a 50% reduction of light intensity (PAR) through the glass roof.

Analysis of flavonoid glycosides and caffeic acid derivatives by HPLC-DAD-MS/MS

The analysis was performed according to NEUGART et al. (2019). Freeze-dried and powdered samples (10 mg) were extracted with methanol/water (3:2, v/v) and analysed via HPLC-DAD-ESI-MS/MS using a series 1260 Infinity II HPLC chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an Ascentis® Express F5 column (150 mm × 4.6 mm, 5 µm, Supelco, Sigma Aldrich Chemical Co., St Louis, MO, USA), a degaser, binary pump, autosampler, column oven and a photodiode array detector (DAD). Compounds were detected in negative polarity with a Bruker amazon SL ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The tentative identification of the compounds was based on the comparison of absorption maxima, mass spectra and fragmentation pattern in MS^3 with reference compounds (when available) or with literature data (Supplemental table S1). External calibration with standards (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) was used to quantify flavonoid glycosides at wavelength 370 nm and caffeic acid derivatives at 330 nm.

Analysis of carotenoids and chlorophylls by UHPLC-DAD-ToF-MS

For the analysis, 5 mg of freeze-dried and powdered lettuce material were extracted with tetrahydrofuran/methanol (1:1, v/v), as previously described by FREDE et al. (2018) with some modifications. Analysis was performed via UHPLC-DAD-ToF-MS using an Agilent Technologies 1290 Infinity UHPLC with separation on a C30 column (YMC Co. Ltd, Kyoto, Japan, YMC C30, 100 × 2.1 mm, 3 µm). Compounds were detected with a multimode ion source in positive polarity with an Agilent Technologies 6230 ToF LC/MS. The gas temperature was set to 300 °C with a flow rate of 8 L min^{-1}, whereas the vaporizer was set to 200 °C using a nebulizer pressure of 35 psig. The voltage was set to 3500 V and a fragmentor voltage was set to 175 V, with corona current application of 4.0 µA. The (tentative) identification of the compounds was based on the comparison of retention time, absorption maxima and mass spectra with standards or with the literature (Supplemental table S2). External calibration with chlorophyll and carotenoid standards (Sigma-Aldrich, St Louis, MO, USA; CaroteNature GmbH, Münsingen Switzerland) was used for quantification at detection wavelength 450 nm.

Analysis of fatty acids as fatty acid methyl esters by GC-MS

Fatty acids were extracted and derivatized to methyl esters using a modified method by BROWSE et al. (1986). Fifteen mg of freeze-dried

and powdered material was mixed with 1 mL methanolic-hydrochloric acid reagent (3 M HCl/methanol 1:2 v/v, added with 5% 2,2-dimethoxypropane, Merck KGaA, Darmstadt, Germany). As an internal standard, 500 µL heptadecanoic acid (0.2 mg/mL, Merck KGaA, Darmstadt, Germany) was added. For the extraction and derivatization procedure, samples were shaken for 60 min at 80 °C under nitrogen atmosphere to protect unsaturated fatty acids. After samples cooled to room temperature, 750 µL hexane and 1000 µL saturated sodium chloride solution were used to extract fatty acid methyl esters in the upper phase. Samples were centrifuged for 5 min at 2560 *g* at 20 °C. A total of 500 µL of the upper hexane phase was filtered with sodium sulphate (anhydrous). Samples were immediately analyzed with GC-MS using an Agilent 6890 GC equipped with a J&W DB-23 GC Column (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany, 30 m × 0.25 mm × 0.25 µm). Samples were injected splitless at an injector temperature of 230 °C. Helium carrier gas had an initial flow of 1.2 mL min^{-1}. The following temperature program was used for elution: 80 °C for 2 min, 80 °C to 120 °C with 5 °C min^{-1}, 120 °C to 220 °C with 2 °C min^{-1}, held at 220 °C for 5 min. Compounds were detected with an Agilent 5973 mass selective detector. The source temperature was set to 230 °C, the quadrupole temperature was set to 150 °C and the voltage was set to 953 V. Analysis was performed in scan mode using a mass range between m/z 90 to 400. Fatty acids were identified as their methyl esters by comparing retention time and mass spectra with those of standards (Merck KGaA, Darmstadt, Germany, Supplemental table S3). For quantification the internal standard was used and response factors of the fatty acids of interest were determined.

Statistical analysis

The statistical analysis was performed using SigmaPlot 14 (Systat Software GmbH, Erkrath, Germany). Differences in the treatments were analyzed with a one-way ANOVA followed by Tukey's HSD *post hoc* test assuming normal distribution. In the case of non-normally distributed data, a Kruskal-Wallis test was applied. A *p*-value of $p \leq 0.05$ was considered a significant difference. Data are represented as mean ± standard error.

Results

Measurement of climatic conditions

During both experiments, the climatic conditions of temperature, relative humidity and PAR were monitored in the greenhouse chamber and under the polytunnels (Tab. 1). In 2019, the average temperature was 4.47 °C higher than in the 2020 experiment, regardless of cultivation condition. A temperature difference of 1.1 was found when comparing the temperature in the greenhouse with the temperature under the polytunnels. Between the polytunnels (with and without antifogging additives), no differences could be measured. A 1.6-fold higher relative humidity was measured under polytunnels compared with the greenhouse chamber in both experiments. No difference was detected for both polytunnels with or without additives. The measured PAR was similar in both experiments. Therefore, it is assumed that additional artificial light was able to compensate possible differences between the two experiments. Lettuce cultivated cover-free (without a polytunnel) in the greenhouse chamber were exposed to a 1.9-fold higher light intensity followed by lettuce grown under polytunnel with additives (1.2-fold), both compared to lettuce grown under additive-free polytunnels.

Determination of the lettuce fresh weight

The fresh weight of each lettuce (edible part, including leaf and stem) was determined directly after the harvest (Fig. 1). In 2019, the fresh weight of polytunnel-grown lettuce was significantly higher compared to lettuce grown without a polytunnel for both cultivars 'Attractie' (1.4-fold) and 'Veronique' (1.9-fold). Incorporated antifogging additives did not lead to differences in the fresh weight of both cultivars. However, there was a significant difference (1.2-fold) in fresh weight of lettuce grown under polytunnels with and without antifogging additives, while there is no difference comparing cover-free grown lettuce and lettuce grown under additive-containing polytunnels in 2020. For the experiment conducted in 2020, the overall fresh weight of the lettuce was 2.0-times lower compared with the experiment in 2019.

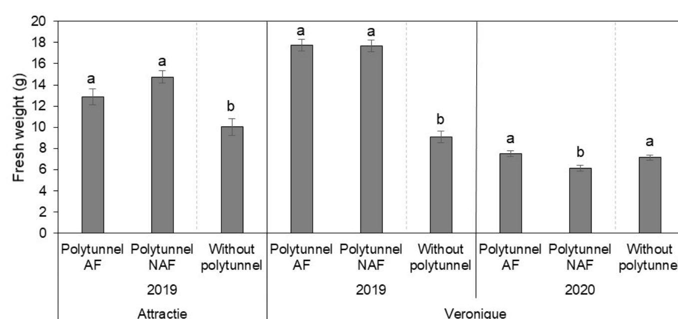

Fig. 1: Lettuce fresh weight (g) grown under polytunnel with (AF) and without antifogging additives (NAF) and without polytunnel. The fresh weight is expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) for each experiment and cultivar are indicated by different letters.

Flavonoid glycosides and caffeic acid derivatives

In both cultivars, three flavonoid glycosides and five caffeic acid derivatives were tentatively identified (Supplemental table S1) and quantified (Fig. 2 and 3). Quercetin and luteolin flavonoids, both conjugated with glucuronide moieties and a quercetin glucoside bound with a malonylic acid moiety were found in lettuce. Notably, the individual flavonoid glycosides as well as total flavonoid glycosides had

Tab. 1: Monitored climatic conditions in the greenhouse chamber (without polytunnel), under polytunnels with (AF) and without antifogging additives (NAF). Temperature (°C) and relative humidity (%) are expressed as daily averages ± SD. The light intensity (photosynthetic active radiation PAR) is expressed as averaged daytime ± SD (6 AM to 6 PM, µmol m^{-2} s^{-1}) and daily light integral ± SD (mol m^{-2} d^{-1}). Calculated values are marked by †.

	2019			2020		
	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel
Temperature	22.93 ± 1.44	22.96 ± 1.38	20.82 ± 0.67	18.56 ± 1.04	18.27 ± 1.03	16.60 ± 0.56
Relative humidity	94.12 ± 4.55	95.46 ± 2.45	61.15 ± 6.05	95.76 ± 3.71	96.21 ± 3.58	60.62 ± 4.81
Averaged photosynthetic active radiation (PAR)	87.96 ± 32.25	68.61 ± 23.56	131.74 ± 45.50†	84.14 ± 32.00	70.34 ± 24.20	127.80 ± 35.72†
Daily light integral (DLI)	4.04 ± 1.43	3.17 ± 1.06	6.39 ± 1.94†	3.78 ± 1.38	3.15 ± 1.03	4.42 ± 1.59†

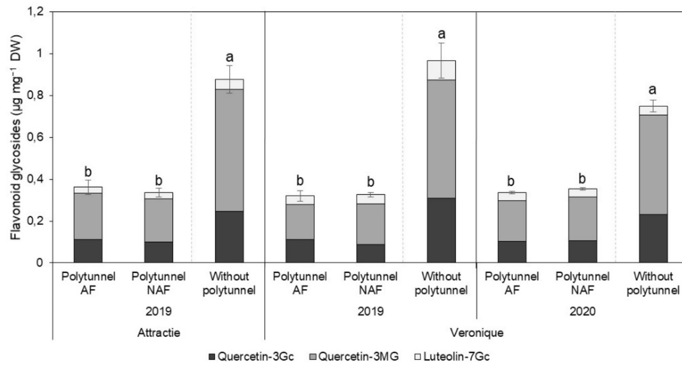

Fig. 2: Content of flavonoid glycosides (µg mg^{-1} DW) in lettuce grown under polytunnel with (AF) and without antifogging additives (NAF) and without polytunnel. The total flavonoid glycoside content is expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) of total flavonoid glycosides for each experiment and cultivar are indicated by different letters. Abbreviations, Gc: glucuronide, MG: malonyl glucoside.

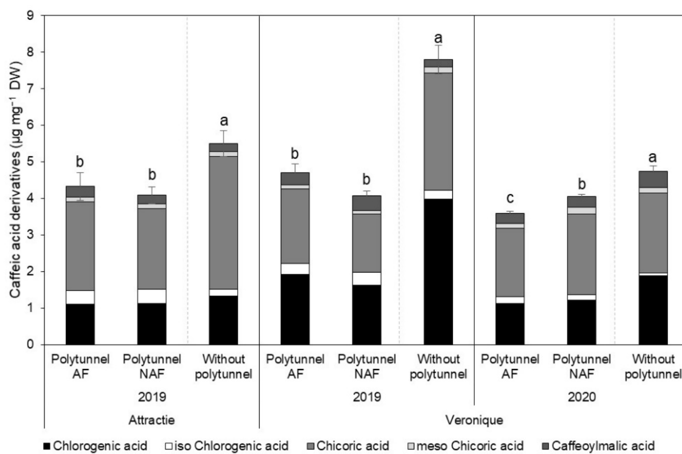

Fig. 3: Content of caffeic acid derivatives (µg mg^{-1} DW) in lettuce grown under polytunnel with (AF) and without antifogging additives (NAF) and without polytunnel. The total caffeic acid content is expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) of total caffeic acids for each experiment and cultivar are indicated by different letters.

a 2.4-fold higher content in the lettuce grown cover-free compared with the lettuce grown under polytunnels, regardless of the content of antifogging additives. However, no significant differences were detected for flavonoid glycosides in lettuce grown under polytunnels with antifogging additives compared with the additive-free polytunnels. This pattern was found in both experiments conducted in 2019 and 2020.

In both cultivars, the two main caffeic acid derivatives, chlorogenic acid and chicoric acid, were tentatively identified (Supplemental table S1). They also contained three derivatives namely *iso*-chlorogenic acid, *meso* chicoric acid and caffeoylmalic acid – albeit in minor amounts. Total caffeic acid content was highest for cover-free lettuce (1.4 fold compared to polytunnel-grown lettuce), whereas no significant differences were observed for lettuce grown under polytunnels with additives compared to without additives in 2019. In contrast, for the 2020 experiment a significant 1.1-fold higher content was found in lettuce under additive-free polytunnels compared those with antifogging (Fig. 3). However, a closer look at the content of individual caffeic acid derivatives revealed some differences. In detail, the chlorogenic acid content in cultivar 'Veronique' was 1.9-fold higher in cover-free lettuce, but in cultivar 'Attractie' no differences were observed. There were also no significant differences between both polytunnel cultivation conditions. Moreover, in both cultivars grown in 2019, the chicoric acid content in the cover-free lettuce was also 1.6-fold higher compared to the lettuce grown under polytunnels. In cultivar 'Veronique' grown under polytunnels with additives, a significant 1.3-fold higher content was detected compared to those grown additive-free. The lettuce grown in 2020 also showed a significant difference for both polytunnel cultivation conditions. What is remarkable, is the high content of chicoric acid in lettuce grown under polytunnels without additives, which was comparable to the cover-free grown lettuce. The minor-content caffeic acids showed predominantly lower contents in the cover-free lettuce compared to polytunnel cultivation. Finally, significant differences were detected for both polytunnel cultivation conditions (1.2-fold) for all minor-content caffeic acids in lettuce in the 2020, but not the 2019 experiment.

Carotenoids and chlorophylls

The analysis revealed chlorophyll a, chlorophyll b and lutein as the main pigments in both cultivars. The lettuces also contained the xanthophylls neoxanthin as well as zeaxanthin in small amounts. Beta-carotene and a lettuce-specific carotenoid lactucaxanthin were also identified (Supplemental table S2, Fig. 4 and 5). A significantly lower amount of total carotenoids occurred in cover-free grown lettuce of 'Veronique' compared with lettuce grown under additive-free polytunnels for both experiments in 2019 (1.4-fold) and 2020 (1.1-fold). In 2019, this is also reflected in the individual carotenoids neoxanthin (1.7-fold), lactucaxanthin (1.5-fold), lutein (1.3-fold) and β-carotene (1.3-fold) as well as the chlorophylls (1.5-fold). In the 2020 experiment, for the individual carotenoids lutein (1.2-fold), neoxanthin (1.1-fold) and chlorophyll b (1.2-fold) such differences were detected. Moreover, in the 2020 experiment, zeaxanthin and

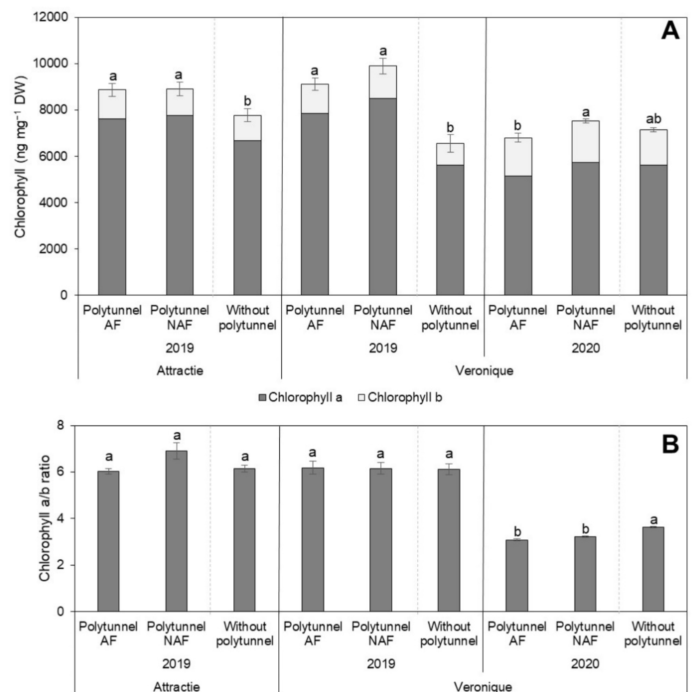

Fig. 4: (A) Chlorophyll content (ng mg^{-1} DW) and (B) chlorophyll a/b ratio of lettuce grown without polytunnel and lettuce grown under polytunnel with (AF) and without antifogging additives (NAF). Ratios and total chlorophylls are expressed as mean ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$) of total chlorophyll content and chlorophyll a/b ratios for each experiment and cultivar.

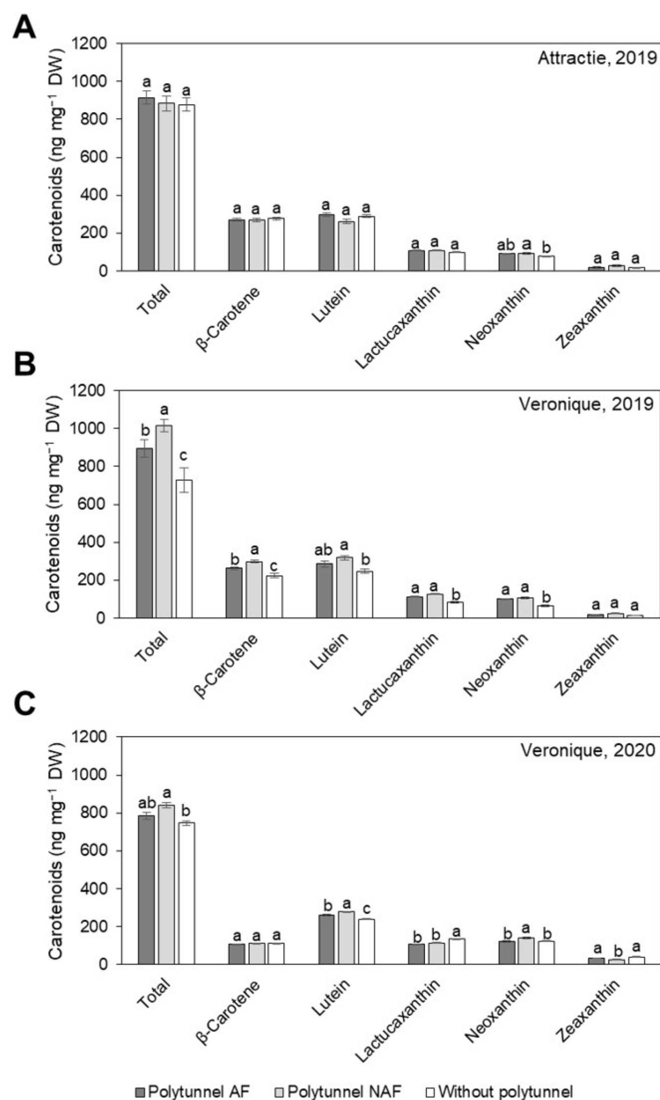

Fig. 5: Carotenoid content (ng mg^{-1} DW) of cultivar 'Attractie' (A) and 'Veronique' (B) from the 2019 experiment and 'Veronique' from the 2020 experiment (C) grown under polytunnel with (AF) and without antifogging additives (NAF) and without polytunnel. Values show means ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$) of individual carotenoids for each experiment and cultivar.

lactucaxanthin were significantly higher in cover-free lettuce than in the additive-free polytunnel grown lettuce by 1.5-fold and 1.2-fold, respectively. Here, the β-carotene content was not affected at all. When comparing total carotenoids in cultivar 'Veronique' for both polytunnel treatments, a significant 1.1-fold higher amount was observed in additive-free polytunnel grown lettuce, only in the 2019 experiment. Additionally, a significant effect due to the use of additives in the polytunnels occurred for β-carotene (1.1-fold) in 2019 and for zeaxanthin (1.3-fold), lutein (1.1-fold), neoxanthin (1.1-fold) and chlorophyll a (1.1-fold) in 2020. In general, the use of antifogging additives in polytunnels leads to lower carotenoid contents in the cultivar 'Veronique' compared to lettuce grown under additive-free polytunnels.

For cultivar 'Attractie', some differences to 'Veronique' were found. At first, no significant differences were observed for total and individual carotenoids, except neoxanthin. Cover-free grown lettuce had a 1.2-fold significantly lower neoxanthin and chlorophyll a content compared to lettuce grown under additive-free polytunnels. No effect due to the use of additives were detected for both pigments. No differences in chlorophyll b content between cover-free lettuce and additive-free polytunnel grown lettuce were observed. However, lettuce grown under polytunnels with additives showed a significant 1.1-fold higher chlorophyll b content compared with both. Thus, the differences between cultivars indicate a cultivar-specific effect, both through the use of polytunnels in general and antifogging additives in particular.

Fatty acids

Palmitic acid followed by linolenic acid and linoleic acid were the main fatty acids determined in lettuce extracts. Furthermore, palmitoleic, stearic and oleic acid were identified in both cultivars (Supplemental table S3). Although there are few differences in total fatty acid content for all cultivation conditions, closer examination revealed some differences (Tab. 2). In cultivar 'Veronique', the amounts of total fatty acids were 2.9-fold higher in 2020 than in 2019. Lower palmitic acid content was observed in cover-free grown lettuce compared with both polytunnel treatments for cultivar 'Veronique' (1.2-fold) and 'Attractie' (1.1-fold). In addition, in 'Veronique', cover-free grown lettuces had significantly lower amounts of stearic acid (1.1-fold) and oleic acid (3.0-fold) in the 2019 experiment and linoleic acid (1.4-fold) in the 2020 experiment compared to polytunnel cultivation. Nevertheless, most individual fatty acids were unaffected and were present in similar amounts, regardless of cultivation conditions. No effect was detected for usage of antifogging additives.

Tab. 2: Composition of saturated and unsaturated fatty acids (µg mg^{-1} DW) in lettuce grown without a polytunnel and lettuce grown under polytunnels with (AF) and without antifogging additives (NAF). Values shows means ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$) for each experiment and cultivar.

	Attractie 2019			Veronique 2019			Veronique 2020		
	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel
Total	21.15 ± 0.65^{a}	22.40 ± 1.52^{a}	21.16 ± 1.01^{a}	18.46 ± 1.36^{ab}	22.47 ± 1.37^{a}	18.03 ± 1.07^{b}	60.75 ± 3.43^{a}	59.05 ± 3.55^{a}	49.80 ± 4.69a
Saturated									
Palmitic acid	12.15 ± 0.13^{a}	11.99 ± 0.30^{a}	10.72 ± 0.19^{b}	11.90 ± 0.22^{a}	12.24 ± 0.22^{a}	9.66 ± 0.27^{b}	11.50 ± 0.16^{a}	11.59 ± 0.03^{a}	10.72 ± 0.08^{b}
Stearic acid	0.87 ± 0.03^{a}	0.85 ± 0.08^{a}	0.91 ± 0.02^{a}	0.83 ± 0.04^{a}	0.89 ± 0.03^{a}	0.76 ± 0.02^{b}	0.87 ± 0.29^{a}	0.57 ± 0.01^{a}	0.60 ± 0.04^{a}
Unsaturated									
Palmitoleic acid	1.03 ± 0.04^{a}	0.98 ± 0.06^{a}	1.04 ± 0.04^{a}	0.95 ± 0.04^{a}	1.10 ± 0.04^{a}	0.89 ± 0.05^{a}	1.64 ± 0.17^{a}	1.49 ± 0.01^{a}	1.46 ± 0.03^{a}
Oleic acid	0.32 ± 0.04^{a}	0.35 ± 0.06^{a}	0.28 ± 0.04^{a}	0.20 ± 0.03^{ab}	0.38 ± 0.05^{a}	0.10 ± 0.03^{b}	1.35 ± 0.22^{a}	1.06 ± 0.08^{a}	0.86 ± 0.11^{a}
Linoleic acid	3.04 ± 0.24^{a}	3.68 ± 0.53^{a}	3.15 ± 0.32^{a}	2.55 ± 0.18^{a}	3.35 ± 0.42^{a}	2.22 ± 0.31^{a}	15.57 ± 1.11^{a}	15.05 ± 1.10^{a}	11.03 ± 1.26^{b}
Linolenic acid	3.16 ± 0.32^{a}	4.08 ± 0.67^{a}	4.02 ± 0.47^{a}	2.70 ± 0.24^{a}	3.99 ± 0.65^{a}	2.68 ± 0.46^{a}	28.36 ± 2.40^{a}	28.01 ± 2.38^{a}	23.66 ± 3.18^{a}

Discussion

Experimental setup and general restrictions of the study

Several studies indicate an impact of greenhouse covering materials on plant yield, however, few have focused on nutritionally important metabolites. PAPADOPOULOS et al. (1997) showed differences in marketable tomato yields among three greenhouse covering materials, namely D-poly, acrylic and glass. Furthermore, PETROPOULOS et al. (2019) investigated the impact of three different polyethylene greenhouse covering materials with differently structured layers on tomato fruit yield and quality. They found that yield and valuable compounds such as tocopherols, carotenoids and chlorophylls were affected by different cultivation conditions. A difference of the polytunnel films with and without incorporated antifogging additives was only observed for the experiment in 2020, but not in 2019. Polytunnel cultivation generally resulted in significantly higher fresh weights of the lettuce plants in the 2019 experiment compared to cover-free grown lettuce, while no differences in fresh weight of lettuce grown under polytunnel with antifogging additives compared to cover-free grown lettuce were observed in the 2020 experiment. This is probably more an effect of the temperature difference of 4.47 °C between the 2019 and 2020 experiments and would reflect the overall 2.0-fold higher fresh weight of lettuce in the 2019 experiments compared to 2020.

However, it is difficult to reconcile the dimensions of a greenhouse with the necessary number of replicate greenhouses to generate statistically significant results. In this context, PETROPOULOS et al. (2019) used three separate greenhouses covered with three different materials albeit for one repetition. In contrast, in this study, we used multiple polytunnels due to their reduced size (58 × 50 × 50 cm, $L \times W \times H$). CEMEK et al. (2006) highlighted the problem of greenhouse size with repetitions. To overcome it, they built greenhouses in smaller dimensions (9 × 3 × 2.5 m, $L \times W \times H$) with two replicates per treatment. Moreover, PAPADOPOULOS et al. (1997) reduced the greenhouses size to 6.2 × 7.2 × 3 m ($L \times W \times H$), in order to ensure three replicates per covering material. However, not only the number of repetitions, but also the placement of plants could cause bias.

To overcome this experimental challenge, we used polytunnels. Lettuce under the polytunnel were randomized regularly and the polytunnels themselves were randomized halfway through the experiment to prevent spatial influences. CEMEK et al. (2006) and PETROPOULOS et al. (2019) pointed out that as a solution they used a randomized complete block experimental design to ensure reproducibility of subsequent experiments.

It must be noted that in this study, the selected polytunnel sizes might have had an impact on the microclimate since the temperature inside the polytunnels was 1.1-fold higher compared with the greenhouse chamber in both experiments. These differences, however, are comparable with the results of CEMEK et al. (2006). In their study, the temperatures varied from 15.9 °C (outside greenhouses) to 21.3 °C (D-Poly greenhouse). HAO et al. (1999) did not observe significant differences for the temperature inside greenhouses with different covering materials (D-Poly, acrylic and glass). The optimal temperature for lettuce cultivation is between 16 °C and 18 °C (SANDERS, 2019). This temperature range corresponds to the conditions in the 2020 experiment. In contrast, in 2019, temperatures were about 4 °C above this optimum, which was not due to the polytunnel microclimate but rather due to the general climate in that month. However, high relative humidity was monitored under the polytunnels compared with the greenhouses used in the study by CEMEK et al. (2006). Therefore, lettuce was selected for this study because it is a crop with high water demand (SANDERS, 2019). Nevertheless, some caution should be exercised in interpreting the results since the microclimate and greenhouse conditions may also affect the plant response.

FADEL et al. (2016) highlights temperature and light as the most important greenhouse controlled environmental factors. Plants respond to these changing environments by adapting their metabolite profiles. Of note is that such a response could result in altered nutritional value of plants grown in greenhouses or polytunnels. Temperature varied in both experiments, light intensity was similar.

Flavonoid glycosides and caffeic acid derivatives

In this study, flavonoid glycosides as well as main caffeic acid derivatives showed the highest content in cover-free grown lettuce. No significant differences were observed for flavonoid glycosides in lettuce ('Attractie' and 'Veronique') grown under polytunnels with and without additives in both experiments and main caffeic acid derivatives in the 2019 experiment. However, a significant difference was detected for most caffeic acid derivatives in the 2020 experiment. Flavonoids provide several health-promoting effects for humans. As free-radical scavenging antioxidants, they have been associated with a lower risk of cardiovascular disease, various types of cancer and in addition, they tend to have anti-inflammatory and immunomodulatory properties (REES et al., 2018, KOPUSTINSKIENE et al., 2020). This study demonstrates a negative effect on the content of these phenolic compounds in lettuce by using polytunnels, independent of whether antifogging additives were used.

AHMADI et al. (2019) found that only individual but not total flavonoids and phenolic acids in tomato fruits varied due to growth under different polyethylene-covered greenhouses. In contrast to the present results, where no differences were observed between the two cultivars, they showed cultivar-specific differences. In agreement with our study, lettuces grown in greenhouses showed lower levels of flavonoids compared to open-field conditions (ROMANIA et al., 2002). Moreover, the use of UV-blocking covering materials for greenhouses had a negative effect on phenolic compounds in leafy vegetables, including rocket and lettuce (KATSOULAS et al., 2020). In this study, UV-light transmission was partly reduced by the polyethylene films with and without antifogging additives (Supplemental Figure S3), whereas the cover-free lettuce was grown in a UV-transmissible greenhouse chamber, which resulted in the highest contents of flavonoid glycosides and caffeic acid derivatives.

BECKER et al. (2013) observed significantly higher levels of flavonoid glycosides in lettuce treated with higher light (410 µmol m^{-2} s^{-1}) compared with lower light intensities (225 µmol m^{-2} s^{-1}). Although the light intensities were slightly higher in polytunnels with antifogging additives compared to polytunnels without additives, no significant differences were observed for flavonoid glycosides and few differences were observed for some caffeic acid derivatives in both cultivars in the 2019 experiment. This observation might be due to the differences in the light regime being too small to cause significant effects. Interestingly in the study of BECKER et al. (2013), the caffeic acid derivatives were not affected at all, which is in contrast to the findings of the 2020 experiment. However, chlorogenic acid as well as *meso* chicoric acid content in lettuce 'Attractie' was not altered due to polytunnel cultivation. The high content of minor caffeic acid derivatives in lettuce grown under polytunnels without additives in general and the high amount of chicoric acid in lettuce grown under polytunnels in particular cannot be currently explained. However, it should be borne in mind that an increase in temperature from 25 °C to 33 °C can cause higher flavonoid accumulation in lettuce (SUBLETT et al., 2018). Thus, a possible reason for the previous observation would be the different temperature regime.

Carotenoids and chlorophyll

While carotenoids are largely unaffected by polytunnel cultivation with and without antifogging additives in the cultivar 'Attractie', differences are evident in 'Veronique'. This suggests a cultivar-specific response to different cultivation conditions, which AHMADI et al. (2019) also found for greenhouse-grown tomato fruits covered with

different polyethylene materials. In the same study, they found that lycopene, but not lutein or total carotenoids were affected by different covering materials. This is in contrast with this study, where individual and total carotenoids differ due to the use of antifogging additives in polytunnels. Finally, Petropoulos et al. (2019) showed a possible impact of polyethylene covering materials on carotenoids and also chlorophyll degradation associated with tomato fruit ripening.

Light is an important factor for biosynthesis of carotenoids and chlorophylls as they are photosynthetic pigments. Light regime differs when comparing open-field conditions and greenhouses and greenhouse covering materials can also affect light transmission and light quality. For example, Ohashi-Kaneko et al. (2007) showed that different light qualities using colored LEDs resulted in altered levels of carotenoids and chlorophylls in spinach and lettuce. Cozzolino et al. (2020) compared how clear and diffuse greenhouse films affect valuable compounds in lamb's lettuce and observed no significant differences for carotenoids and chlorophylls. However, it is known that light intensity can influence the content of photosynthetic pigments in plants. For example, Kosma et al. (2013) detected a positive correlation between total chlorophyll content and reduced PAR intensities (26, 47 and 73% of incident light intensity) in hydroponically cultivated lettuce. In the present study, differences in light intensities (PAR) were detected for all three cultivation conditions. In agreement with the aforementioned studies, chlorophyll contents were higher in lettuce grown under polytunnels compared to cover-free grown lettuce. Furthermore, significantly higher chlorophyll a and b contents of lettuce grown under additive-free compared to additive-containing polytunnels were observed in the 2020 experiment. In their review, Shafiq et al. (2021) showed that the behavior of plants in terms of chlorophyll content seems to be different under low light conditions (shade), while some studies showed lower chlorophyll contents in shade-grown plants, some also found the opposite. Therefore, Shafiq et al. (2021) hypothesized that chlorophyll content tends to increase in shade tolerant cultivars in response to enhanced light harvesting. In fact, comparable photosynthetic rates in lettuce grown under polytunnels with and without additives compared to cover-free grown lettuce were observed for the 2020 experiment (Supplemental Figure S4). Presumably, the adaptation of the photosynthetic pigments in the lettuce led to an efficient light harvesting and did compensate the lower light intensities under the polytunnels. In addition, a significantly higher photosynthesis rate was measured in lettuce grown under polytunnels with additives than in additive-free polytunnels, which is probably also related to the different light intensity.

The influence of different light intensities, ranging from 125 to 620 $\mu mol\ m^{-2}\ s^{-1}$, on major carotenoids (β-carotene and lutein) and chlorophylls was examined by Lefsrud et al. (2006) in kale and spinach. The highest pigment contents tended to be found at 335 $\mu mol\ m^{-2}\ s^{-1}$ in kale and at 200 $\mu mol\ m^{-2}\ s^{-1}$ in spinach. Song et al. (2020) treated lettuce with different light intensities (150 to 450 $\mu mol\ m^{-2}\ s^{-1}$) and nutrient solution concentrations. Comparing the carotenoids in lettuce at different light intensities and same nutrient solution treatments (1/4 and 3/4 nutrient solution level), no significant differences for carotenoid contents between these treatments were found, which is in line with our findings for 'Attractie'. Interestingly, the chlorophyll a/b ratio of their treated lettuce was highest under higher light intensities (at same nutrient solution levels). This is consistent with the results of the 2020 experiment, but not with 2019, where no differences in chlorophyll ratios were determined. It should be noted that the 2019 and 2020 experiments were conducted in different months of the years and some differences in the content of photosynthetic pigments were observed. This could possibly be due to the different spectral qualities of the light in these months. In detail, the plant photosystems PSI and PSII exhibit different absorption maxima due to their carotenoid and chlorophyll compositions, resulting in differing responses depending on the light quality (Caffarri et al., 2014). In addition, not only the light quality but also the light quantity may stimulate the two photosystems differently. This could also have led to a different adaptation of the photosystems during the experiments (Ballottari et al., 2007). Thus, the altered chlorophyll a/b ratio in 2019 compared to 2020 might be an indication of this altered adaptation of the photosystems.

However, not only light, but also other factors can impact the photosynthetic pigments in lettuce grown under polytunnels. For example, temperature can potentially affect the adaptation of the photosystems (Ballottari et al., 2007). In this context, the 4.47 °C temperature difference in the 2019 and 2020 experiments is remarkable. Lefsrud et al. (2005) cultivated kale and spinach at different air temperatures (from 10 to 30 °C) and showed that β-carotene, lutein and chlorophyll contents for both vegetables tended to be the highest at 30 °C, when calculated on a dry weight basis. This observation could also explain the differences in carotenoid and chlorophyll content comparing the 2019 and 2020 experiments. In particular, carotenoids showed the highest levels in polytunnel-grown lettuce in 2019, while these differences were observed only for a few carotenoids in the 2020 experiment. This might be a result of the 4.47 °C higher temperatures in 2019 than in 2020. The changing spectrum of sunlight and photoperiod in the different months of the experiments could also have an influence on the carotenoids. The changes in zeaxanthin indicate a temperature-dependent difference in accumulation in both experiments. Zeaxanthin protects plant membranes against reactive oxygen species under high light and high temperature conditions (Davison et al., 2002). Under lower light conditions, zeaxanthin decreases and converts to violaxanthin, as part of the violaxanthin-zeaxanthin cycle (Jahns et al., 2009). This is consistent with the observations in both experiments. While in 2019, zeaxanthin tended to have higher amounts in lettuce under polytunnels, the opposite was found in the 2020 experiment. There were significantly lower amounts of zeaxanthin in lettuce grown under polytunnels compared to cover-free grown lettuce. It therefore appears that the effect of greenhouse covering materials on the plants grown below is a complex interaction of various environmental factors to which the plant adapts, presumably to optimize the photosynthetic process under the given environmental conditions. Such adaptations also affect the nutritional quality of cultivated vegetables, which has implications on human health since carotenoids and chlorophylls have potential health-promoting effects. Carotenoids and chlorophylls as well as derivatives exert antioxidant activities that have been associated with a reduced risk of cardiovascular disease and cancer, as well as eye disease (Ferruzzi et al. 2007; Milani et al., 2017). Especially, the carotenoids lutein and zeaxanthin have shown preventive effects against age-related macular degeneration, while other carotenoids can act as provitamin A (Eisenhauer et al., 2017; Milani et al., 2017). As shown in this study, the use of polytunnels (protected cultivation) revealed an accumulation on these compounds in lettuce.

Fatty acids

In general, it can be seen that polytunnel cultivation and the incorporation of antifogging additives have a negligible effect on the fatty acid profiles of lettuce. The differences found for fatty acids in lettuce when comparing growing conditions might be caused by the higher temperatures generated under the polytunnels. Falcone et al. (2004) investigated changes in membrane fatty acid profiles of *Arabidopsis thaliana* due to elevated temperatures (from 17 to 36 °C) and found increased levels of some unsaturated fatty acids (oleic and linoleic acids) and saturated palmitic acid, a finding that is consistent with the present study. In contrast, they also showed decreased levels of linolenic acid, which was not observed in this study. Based on their

finding they hypothesized that plant membranes might require certain levels of distinct fatty acids for photosynthetic thermostability and acclimation. PETROPOULOS et al. (2019) found some variations in the polyunsaturated/saturated fatty acid ratios of tomatoes grown under different polyethylene cover materials. They emphasized the good nutritional value of the polyunsaturated fatty acids present in tomatoes. KIM et al. (2016) studied the nutritional value of different lettuce cultivars and detected the essential fatty acids linolenic and linoleic acid as the main fatty acids in both cultivars. Notably, cultivars 'Veronique' and 'Attractie' also contained both essential fatty acids in predominant amounts.

Conclusion

The impact of antifogging additives from greenhouse covering materials and polytunnel cultivation (protected cultivation) on valuable phytochemicals in lettuce was investigated in this study. Both, the polytunnel cultivation and the additives can alter the phytochemical content. This is due to a complex interaction of different environmental conditions, especially light and temperature. Since, antifogging additives slightly alter the light transmission through the polytunnels compared to those without additives, differences were presumably only detected for pigments related to photosynthesis. Nevertheless, the highest levels of these phytochemicals were detected under polytunnels without additives. A negative effect on flavonol glycosides as well as main caffeic acid derivatives was shown by the utilization of polytunnels, probably due to the shielding effect of such films. However, the use of antifogging additives did not cause any changes in these compounds. Antifogging additives are not only used to improve light transmission, but also to prevent plant damage and microbiological contamination by condensed water. In this study, the lettuce had a short growing period, and thus, such factors are of less importance within the experimental time. Even though the use of antifogging additives in greenhouse films did not have an overall positive impact on phytochemicals, they do protect crops with a longer growing period from spoiling. In addition, the effect of polytunnel cultivation and additive use on plant metabolite profiles was shown to be cultivar-specific. To conclude, with regard to the nutritional value of plants, the selection of a greenhouse covering material and the incorporation of useful additives could be a factor to improve the quality of horticultural crops and thus contributes to the implementation of SDG2 "zero hunger". However, as a limitation of this study remains the size of the polytunnels, future studies should therefore address non-model conditions.

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Conflict of interest
No potential conflict of interest was reported by the authors.

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Correction
Although every effort was made to ensure the accuracy of the information, an unintentional error was overlooked during the rigorous review process and incorrect data for Daily light integral (DLI) was printed in Tab. 1 of the published version from Jun 15, 2022. The authors sincerely apologize for any confusion or inconvenience caused by this oversight. The corrected data for Daily light integral (DLI) is published in this corrected version. The change does not affect other data in this article or its overall conclusions.

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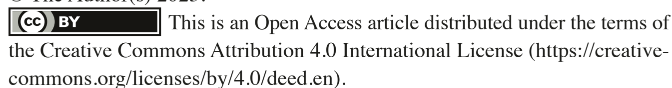
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Supplemental material

Figure S1: Pictures of the polytunnels and lettuce grown without a polytunnel in the greenhouse chamber used in this study.

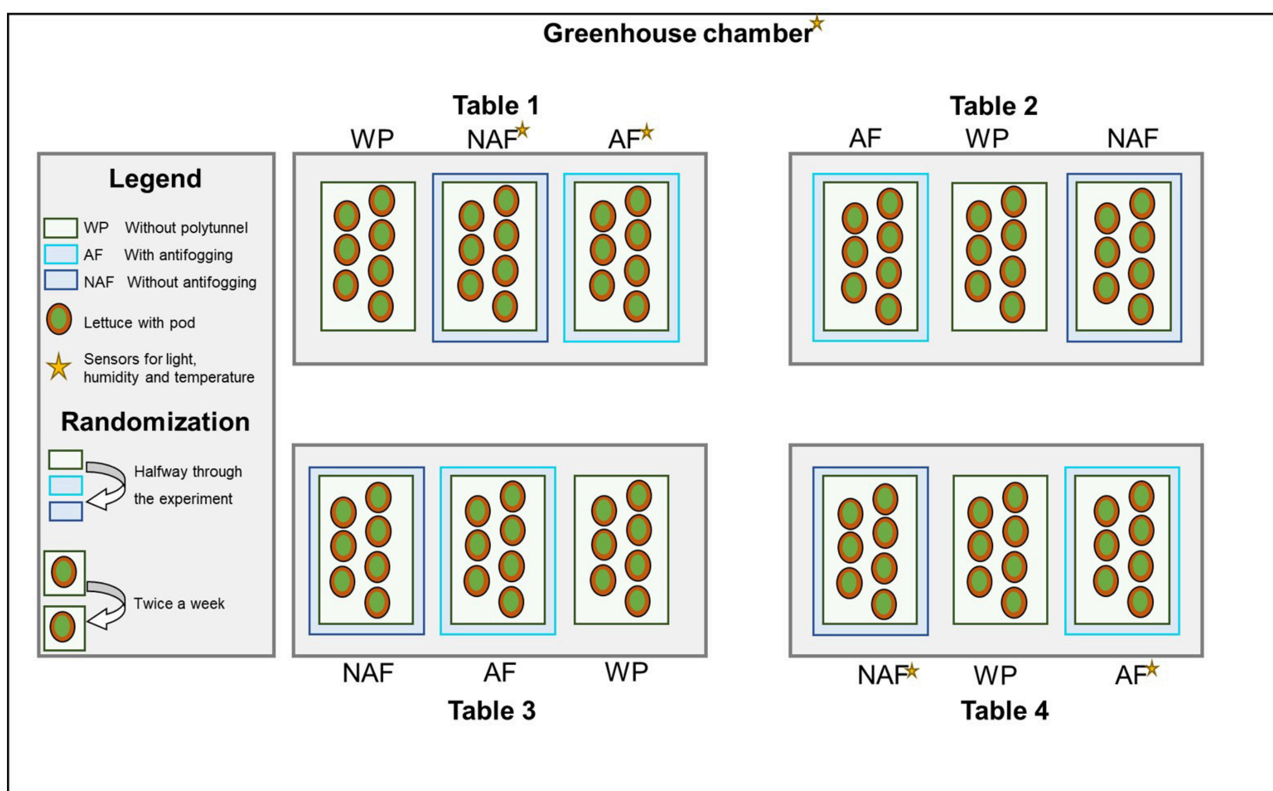

Figure S2: Experimental setup and randomization procedure of the experiments in 2019 and 2020.

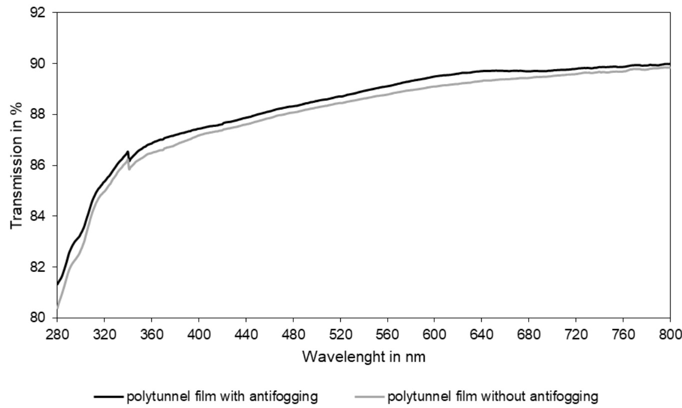

Figure S3: Light transmission (%) of polytunnel films with and without antifogging additives before use.

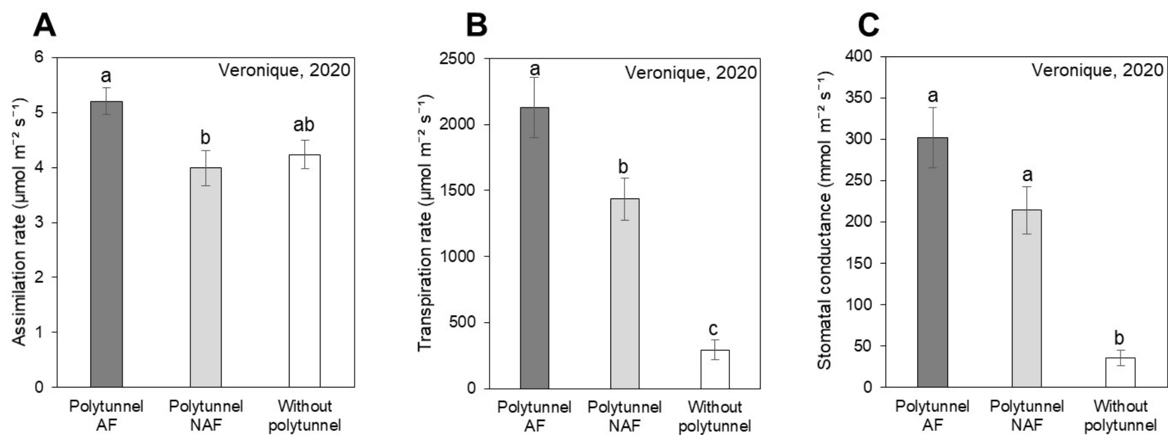

Figure S4: Physiological measurements (assimilation rate A, µmol m^{-2} s^{-1}; transpiration rate B, µmol m^{-2} s^{-1} and stomatal conductance C, mmol m^{-2} s^{-1}) of cultivar 'Veronique' from the 2020 experiment grown under polytunnel with (AF) and without antifogging additives (NAF) and without polytunnel. Values show means ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$) between the cultivation conditions.

Measurement of physiological plant parameters

The measurements were performed with the LI-6800 gas exchange system (LI-COR Biosciences GmbH, Germany) for lettuce only in the 2020 experiment in the afternoon, one day before the harvest. The measurements were conducted at eight plants of each cultivation condition (two plants per table, Figure S2). The measurement conditions were PAR 290 µmol m^{-2} s^{-1}, temperature at 22 °C and 70 % relative humidity. The carbon dioxide concentration in the chamber was set to 400 µmol mol^{-1} with a flow rate of 500 µmol s^{-1}. Fan speed was set to 8000 rpm.

Table S1 Identification parameters for phenolic acids and flavonoid glycoside compounds in lettuce based on the literature. Compounds verified with authentic standard compounds are marked by †.

Compound	Retention time in min	MS[1] *m/z* [M-H]-	MS[2] *m/z* [M-H]-	MS[3] *m/z* [M-H]-	Absorption maxima in nm
Chlorogenic acid†	7.36	354	191 179		246, 300, 340
iso-Chlorogenic acid†	23.21	515	191, 179		242, 326
Chicoric acid†	13.77	473	311	149, 179	244, 300, 342
meso Chicoric acid	13.01	473	311	149, 179	242, 328
Caffeoylmalic acid	9.60	295, 133	353, 179		242, 328
Quercetin-3-glucuronide†	17.88	477	301		256, 350
Quercetin-3-malonylglucoside†	21.58	549, 505	463, 301		256, 352
Luteolin-7-glucuronide†	20.49	461	285		222, 252, 342

Tentative identification based on the literature by: BECKER, C. et al., 2015: PLoS One 10, 11 e0142867, LLORACH R et al., 2008: Food. Chem. 108(3), 1028-1038.

Table S2 Identification parameters for chlorophylls and carotenoids in lettuce based on the literature. Compounds verified with authentic standard compounds are marked by †.

Compound	Retention time in min	Ion	MS *m/z*	Absorption maxima in nm
β-Carotene†	48.39	$[M+H]^+$	537.45	424 452 480
Lutein†	18.65	$[M+H-H2O]^+$	551.43	420 444 472
Lactucaxanthin	16.55	$[M+H-H2O]^+$	551.43	414 438 468
Neoxanthin (9-*Z*)†	12.16	$[M+H-H2O]^+$	583.42	410 434 464
Zeaxanthin†	20.60	$[M+H]^+$	569.43	426 452 478
Chlorophyll a†	22.47	$[M+H]^+$	893.54	432
Chlorophyll b†	18.02	$[M+H]^+$	907.52	468

569.43 426 452 478

Tentative identification based on the literature by: DIOP NDIAYE, N. et al., 2011: J. Agric. Food Chem. 59(22), 12018-12027, BRITTON, G. et al., 2004: Carotenoids: Handbook, Birkhäuser, GOPAL et al., 2017: Food Funct. 8, 1124.

907.52

Table S3 Identification parameters for fatty acids in lettuce. All compounds were verified with authentic standard compounds.

25.18

Compound	Retention time in min	MS *m/z* methylated fatty acid
Palmitic acid	25.18	270
Palmitoleic acid	25.38	268
Stearic acid	32.30	298
Oleic acid	32.82	296
Linoleic acid	34.44	294
Linolenic acid	36.44	292

Table S4: Determined phenolic compounds (µg mg^{-1} DW) in lettuce grown without a polytunnel and lettuce grown under polytunnels with (AF) and without antifogging additives (NAF). Values shows means ± SE (n = 4). Different letters indicate significant differences (p ≤ 0.05) for each experiment and cultivar. Abbreviations, Gc: glucuronide, MG: malonyl glucoside.

	Attractie 2019			Veronique 2019			Veronique 2020		
	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel
Caffeic acid derivatives									
Total	4.327 ± 0.379^{b}	4.086 ± 0.230^{b}	5.499 ± 0.347^{a}	4.700 ± 0.243^{b}	4.068 ± 0.135^{b}	7.796 ± 0.383^{a}	3.825 ± 0.071^{c}	4.329 ± 0.060^{b}	4.905 ± 0.160^{a}
Chlorogenic acid	1.110 ± 0.056^{a}	1.121 ± 0.113^{a}	1.329 ± 0.102^{a}	1.917 ± 0.152^{b}	1.628 ± 0.068^{b}	3.975 ± 0.264^{a}	1.122 ± 0.021^{b}	1.212 ± 0.024^{b}	1.888 ± 0.089^{a}
Iso chlorogenoic acid	0.359 ± 0.056^{a}	0.400 ± 0.031^{a}	0.178 ± 0.027^{b}	0.305 ± 0.040^{a}	0.342 ± 0.020^{a}	0.239 ± 0.024^{b}	0.182 ± 0.008^{a}	0.144 ± 0.006^{b}	0.069 ± 0.003^{c}
Chicoric acid	2.431 ± 0.254^{b}	2.201 ± 0.125^{b}	3.629 ± 0.240^{a}	2.029 ± 0.095^{b}	1.608 ± 0.051^{c}	3.212 ± 0.123^{a}	1.872 ± 0.053^{b}	2.221 ± 0.037^{a}	2.195 ± 0.064^{a}
Meso chicoric acid	0.133 ± 0.016^{a}	0.127 ± 0.009^{a}	0.132 ± 0.026^{a}	0.110 ± 0.005^{a}	0.082 ± 0.004^{b}	0.159 ± 0.016^{a}	0.143 ± 0.005^{b}	0.176 ± 0.006^{a}	0.150 ± 0.005^{b}
Caffeoylmalic acid	0.293 ± 0.020^{a}	0.238 ± 0.013^{ab}	0.229 ± 0.017^{b}	0.339 ± 0.023^{a}	0.408 ± 0.022^{a}	0.210 ± 0.015^{b}	0.263 ± 0.008^{c}	0.300 ± 0.005^{b}	0.429 ± 0.008^{a}
Flavonoid glycosides									
Total	0.362 ± 0.034^{b}	0.336 ± 0.020^{b}	0.876 ± 0.066^{a}	0.320 ± 0.025^{b}	0.326 ± 0.010^{b}	0.966 ± 0.084^{a}	0.336 ± 0.007^{b}	0.354 ± 0.006^{b}	0.750 ± 0.029^{a}
Quercetin-3-Gc	0.113 ± 0.012^{b}	0.101 ± 0.006^{b}	0.245 ± 0.018^{a}	0.111 ± 0.007^{b}	0.088 ± 0.003^{b}	0.309 ± 0.023^{a}	0.104 ± 0.002^{b}	0.107 ± 0.002^{b}	0.231 ± 0.010^{a}
Quercetin-3-MG	0.221 ± 0.022^{b}	0.205 ± 0.015^{b}	0.584 ± 0.061^{a}	0.169 ± 0.023^{a}	0.196 ± 0.006^{a}	0.564 ± 0.088^{a}	0.194 ± 0.004^{b}	0.208 ± 0.004^{b}	0.475 ± 0.019^{a}
Luteolin-7-Gc	0.029 ± 0.002^{b}	0.030 ± 0.002^{b}	0.047 ± 0.005^{a}	0.040 ± 0.004^{b}	0.042 ± 0.001^{b}	0.093 ± 0.007^{a}	0.038 ± 0.001^{b}	0.038 ± 0.001^{b}	0.044 ± 0.001^{a}

Table S5: Carotenoids, chlorophylls (ng mg^{-1} DW) and chlorophyll a/b ratio in lettuce grown without a polytunnel and lettuce grown under polytunnels with (AF) and without antifogging additives (NAF). Values shows means ± SE (n = 4). Different letters indicate significant differences (p ≤ 0.05) for each experiment and cultivar.

	Attractie 2019			Veronique 2019			Veronique 2020		
	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel	Polytunnel AF	Polytunnel NAF	Without polytunnel
Carotenoids									
Total	914.03 ± 23.37^{a}	883.92 ± 22.22^{a}	877.91 ± 19.39^{a}	894.20 ± 32.43^{b}	1015.76 ± 22.72^{a}	726.98 ± 39.51^{c}	783.53 ± 18.77^{b}	840.87 ± 12.70^{a}	745.96 ± 11.06^{b}
Beta-carotene	271.08 ± 7.08^{a}	269.37 ± 7.83^{a}	276.42 ± 5.94^{a}	262.64 ± 8.35^{b}	299.07 ± 7.06^{a}	223.82 ± 11.96^{c}	107.90 ± 2.53^{a}	111.13 ± 1.61^{a}	112.10 ± 1.58^{a}
Lutein	295.54 ± 8.57^{a}	261.27 ± 12.81^{a}	288.72 ± 6.36^{a}	286.85 ± 16.48^{ab}	317.98 ± 13.16^{a}	247.79 ± 12.85^{b}	262.38 ± 5.14^{b}	277.60 ± 3.75^{a}	239.55 ± 3.74^{c}
Lactucaxanthin	109.12 ± 3.10^{a}	108.16 ± 3.14^{a}	99.01 ± 3.21^{a}	113.81 ± 3.86^{a}	126.52 ± 2.67^{a}	84.88 ± 5.78^{b}	106.54 ± 2.92^{b}	113.83 ± 1.68^{b}	135.55 ± 2.17^{a}
Neoxanthin	91.00 ± 3.33^{ab}	93.30 ± 4.50^{a}	78.64 ± 2.67^{b}	100.55 ± 3.62^{a}	107.74 ± 4.66^{a}	64.94 ± 5.12^{b}	123.21 ± 3.55^{b}	141.00 ± 3.04^{a}	124.29 ± 2.61^{c}
Zeaxanthin	18.18 ± 3.08^{a}	27.92 ± 4.63^{a}	17.53 ± 2.50^{a}	17.25 ± 2.02^{a}	25.90 ± 3.84^{a}	14.47 ± 1.43^{a}	34.82 ± 1.70^{a}	26.04 ± 1.87^{b}	40.03 ± 1.83^{a}
Chlorophylls									
Total	8876.83 ± 931.91^{a}	8902.02 ± 942.97^{a}	7776.72 ± 826.78^{b}	9119.07 ± 839.53^{a}	9893.03 ± 944.88^{a}	6557.33 ± 634.76^{b}	6801.80 ± 190.98^{b}	7524.05 ± 97.55^{a}	7151.17 ± 96.72^{ab}
Chlorophyll a	7613.32 ± 235.79^{ab}	7776.01 ± 275.76^{a}	6688.05 ± 245.00^{b}	7849.85 ± 230.91^{a}	8510.29 ± 301.48^{a}	5634.00 ± 330.86^{b}	5139.95 ± 155.82^{b}	5741.45 ± 77.64^{a}	5605.56 ± 76.56^{a}
Chlorophyll b	1263.51 ± 36.14^{a}	1126.01 ± 38.31^{b}	1088.67 ± 26.99^{b}	1269.22 ± 55.00^{a}	1382.74 ± 36.22^{a}	923.33 ± 53.97^{b}	1661.85 ± 37.56^{b}	1782.60 ± 21.86^{a}	1545.61 ± 21.50^{b}
Chlorophyll a/b ratio	6.03 ± 0.11^{a}	6.91 ± 0.34^{a}	6.14 ± 0.15^{a}	6.18 ± 0.27^{a}	6.15 ± 0.24^{a}	6.10 ± 0.23^{a}	3.09 ± 0.04^{b}	3.22 ± 0.02^{b}	3.63 ± 0.02^{a}

Publication 3

Regulation of carotenoid and flavonoid biosynthetic pathways in *Lactuca sativa var capitate* L. in protected cultivation

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Regulation of carotenoid and flavonoid biosynthetic pathways in *Lactuca sativa var capitate* L. in protected cultivation

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In the face of a growing world population and limited land, there is an urgent demand for higher productivity of food crops, and cultivation systems must be adapted to future needs. Sustainable crop production should aim for not only high yields, but also high nutritional values. In particular, the consumption of bioactive compounds such as carotenoids and flavonoids is associated with a reduced incidence of non-transmissible diseases. Modulating environmental conditions by improving cultivation systems can lead to the adaption of plant metabolisms and the accumulation of bioactive compounds. The present study investigates the regulation of carotenoid and flavonoid metabolisms in lettuce (*Lactuca sativa var capitate* L.) grown in a protected environment (polytunnels) compared to plants grown without polytunnels. Carotenoid, flavonoid and phytohormone (ABA) contents were determined using HPLC-MS and transcript levels of key metabolic genes were analyzed by RT-*q*PCR. In this study, we observed inverse contents of flavonoids and carotenoids in lettuce grown without or under polytunnels. Flavonoid contents on a total and individual level were significantly lower, while total carotenoid content was higher in lettuce plants grown under polytunnels compared to without. However, the adaptation was specific to the level of individual carotenoids. For instance, the accumulation of the main carotenoids lutein and neoxanthin was induced while the β-carotene content remained unchanged. In addition, our findings suggest that the flavonoid content of lettuce depends on transcript levels of the key biosynthetic enzyme, which is modulated by UV light. A regulatory influence can be assumed based on the relation between the concentration of the phytohormone ABA and the flavonoid content in lettuce. In contrast, the carotenoid content is not reflected in transcript levels of the key enzyme of either the biosynthetic or the degradation pathway. Nevertheless, the carotenoid metabolic flux determined using norflurazon was higher in lettuce grown under polytunnels, suggesting posttranscriptional regulation of carotenoid accumulation, which should be an integral part of future studies. Therefore, a balance needs to be found between the individual environmental factors, including light and temperature, in order to optimize the carotenoid or flavonoid contents and to obtain nutritionally highly valuable crops in protected cultivation.

KEYWORDS

greenhouse, bioactive compounds, lettuce, flavonoid, carotenoid, metabolism, UV, crop cultivation

1 Introduction

Even small environmental changes can alter a plant's metabolome; in this respect, plant metabolism is still a black box. To improve cultivation systems and nutritional quality of horticultural crops, it is crucial to understand the regulation of a plant's metabolism. By 2050, the human population is predicted to reach 9.75 billion (Prb, 2022), therefore, efficient production of horticultural crops is important to ensure food security. Moreover, not only crop yields should be addressed, but also new approaches to producing nutrient-rich crops.

Protected cultivation systems, which is the term used for growing crops such as vegetables, in greenhouses, polytunnels, or row covers, can be one approach to increasing yields as well as improving the nutritional quality of crops. In 2019, an area of 5,630,000 ha of land was used for protected agriculture worldwide (World Greenhouse Vegetable Statistics, 2019), while in Europe (2020), 1,140,913 ha of agricultural land was under protective covers compared to 288,051,555 ha of cropland [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), (2020)]. This represents an increase of around 5% in agricultural land under protective covers in six years (since 2014) [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), (2020)]. Certainly, greenhouse area is constantly increasing for vegetable production, and certain greenhouse cultivars achieve much higher yields (e.g. tomato cultivars with 40% higher yields compared to old-cultivars). Thus, much higher vegetable greenhouse production is estimated in the future (Marcelis and Heuvelink, 2019). Protected cultivation of crops improves yields and enables higher productivity due to extending seasonal production times compared to open fields (Gruda, 2005). Furthermore, such crop production offers opportunities for sustainable cultivation, which is fundamental for future food production. For example, strategic location of greenhouses with short transportation distances to reduce food miles or in areas unsuitable for open-field cultivation could be considered (Zhou et al., 2021), as well as resource-efficient water use, particularly in hotter climates (Irusta et al., 2009).

Another important aspect of sustainable crop production is the choice of covering material. Different materials generate different radiometric and physical properties and thus individual selection that depends on regions, seasons, or crop species may be most beneficial (Maraveas, 2019). For polymer-based greenhouse covers, the incorporation of additives offers additional possibilities for desired properties. For example, UV blockers are added to polymers to reduce the transmission of UV light, which can cause plant damage due to pests and diseases (Katsoulas et al., 2020), whereas antifogging additives can improve light transmission and avoid microbiological contamination due to the prevention of water droplets on the plant facing side (Irusta et al., 2009). The use of protected cultivation also enables control of temperature and light regimes, in particular, to trigger adaptations in the metabolic response of plants. Thus, the selection of different greenhouse covers, including incorporating property-improving additives, can provide another often-neglected possibility for improving the nutritional quality of horticultural crops (Ahmadi et al., 2018; Petropoulos et al., 2019; Harbart et al., 2022). Therefore, understanding the metabolic responses and regulation of bioactive compounds is crucial for developing and selecting suitable materials.

Bioactive compounds such as plant secondary metabolites are ubiquitously distributed in plants. Although not essential for human health, they are associated with several beneficial properties when included in human nutrition. For example, epidemiological studies have revealed that both carotenoids and flavonoids have beneficial effects on non-transmissible diseases such as cardiovascular diseases or cancer (Hertog et al., 1993; Kim and Je, 2017; Milani et al., 2017; Rees et al., 2018). In addition, certain carotenoids are precursors of vitamin A, and the xanthophylls lutein and zeaxanthin have been shown to affect the development and progression of age-related macular degeneration (Eisenhauer et al., 2017). *In planta*, both bioactive compounds have protective functions against photoinhibition, and carotenoids in particular are involved in photosynthesis (Agati and Tattini, 2010; Cazzonelli, 2011).

The biosynthesis of flavonoids follows the shikimate pathway, while carotenoids are synthesized through mevalonate and non-mevalonate pathways (KEGG pathway database, Figure 1), both biosynthetic pathways are well studied and highly conserved in the plant kingdom. However, it is less well understood how plants regulate the biosynthesis, accumulation, and degradation of these compounds. In addition, many studies have been conducted in model organisms such as *Arabidopsis thaliana*; however, many mechanisms, particularly in carotenoid pathway, seem to be species- and tissue-specific (Ohashi-Kaneko et al., 2007; Shumskaya et al., 2012; Lätari et al., 2015). Consequently, there is a need for studies in horticultural crops in order to effectively transfer knowledge from plant model systems.

Light and temperature were identified as the most important factors controlled by greenhouses (Fadel et al., 2016). Frequently, solar radiation is the only source of light in low-tech protected cultivation, such as in simple polytunnels. Plants respond to light using different pathways. For example, plants detect blue, red, or UV light through various photoreceptors that trigger transcriptional cascades for metabolite adaptation such as carotenoid and flavonoid levels (Rizzini et al., 2011; Morales et al., 2013; Toledo-Ortiz et al., 2014; Yang et al., 2018; Stanley and Yuan, 2019; Tissot and Ulm, 2020). In addition, light modulating processes such as shade avoidance are also part of the physiological response (Bou-Torrent et al., 2015). Plants need to adjust their metabolites to a given environment and direct their metabolic flux and energy input towards synthesizing the most favorable compounds. Thus the regulation of bioactive compounds such as carotenoids and flavonoids, which share some functions in the plant such as protection or attraction (anthocyanins), is probably interrelated (Cao et al., 2015). Understanding such crosstalk between pathways can help improve the nutritional quality of horticultural crops and such knowledge can contribute to targeted decision making about cultivation conditions.

The aim of this study was to advance our understanding of the metabolic regulation of carotenoids and flavonoids as nutritionally valuable bioactive compounds in lettuce. We grew lettuce without or under polytunnels, testing two different covering materials, with and without incorporated antifogging additives. Climatic conditions were monitored during the experiments, and carotenoid and flavonoid profiles as well as the phytohormone (ABA) content were determined by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Promising target gene candidates encoding key biosynthetic enzymes of both metabolic pathways as well as light-depended transcription factors were analyzed by

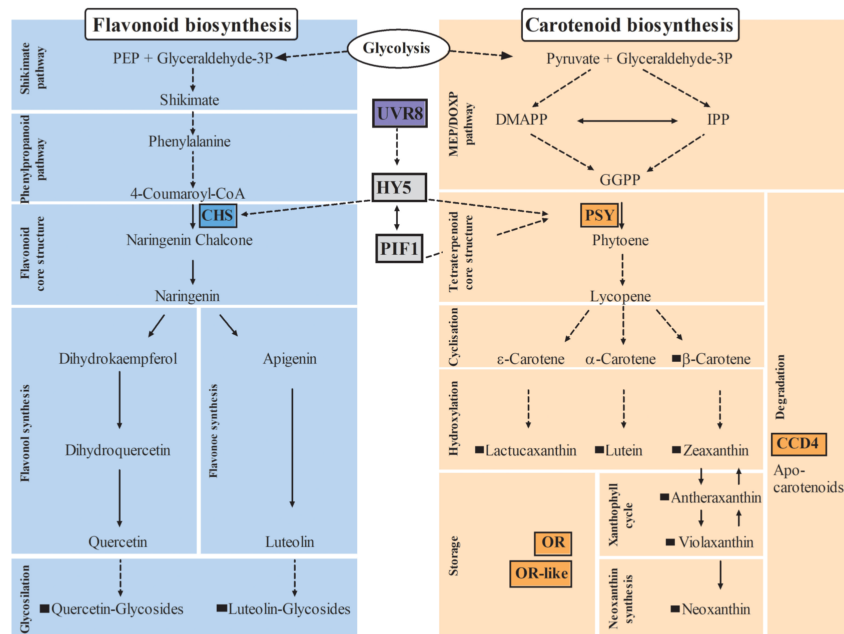

FIGURE 1
Impact on carotenoid and flavonoid biosynthetic pathways in plants. Arrows with dashed lines indicate more than one reaction, arrows with continuous lines indicate one reaction; key enzymes of flavonoid and carotenoid pathways are highlighted by colored boxes; black squares indicate metabolites identified in lettuce in this study. CHS, Chalcone synthase; PSY, Phytoene synthase; CCD4, Carotenoid cleavage dioxygenase4; OR, Orange protein; UVR8, Ultraviolet resistance locus8; HY5, Elongated hypocotyl5; PIF1, Phytochrome interacting factor1; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; MEP/DOXP, mevalonate/non-mevalonate pathway.

RT-*q*PCR. We demonstrate that different mechanisms, at both the transcriptional (flavonoids) and posttranscriptional (carotenoids) levels, influence the accumulation of these bioactive compounds in lettuce cultivated under polytunnels. The inverse relationship between flavonoid and carotenoid levels is probably caused by physicochemical mechanisms rather than a shared transcriptional signaling pathway.

2 Material and methods

2.1 Plant growth and cultivation

Lettuce (*Lactuca sativa var capitata* L., cultivar 'Veronique') seeds were obtained from Samenhaus Müller GmbH (Wildeck-Bosserode, Germany) and were sown in trays with soil (substrate type P, pH 5.9, N 120 mg L^{-1}, PO_4^{2-} 120 mg L^{-1}, K 170 mg L^{-1}, Mg 120 mg L^{-1}, Einheitserde classic, Einheitserde Werkverband e.V., Sinntal-Altengronau, Germany). After reaching two-leaf-stage, seedlings were transplanted into pots (diameter 13 cm) filled with soil (substrate typ T, pH 5.9, N 183 mg L^{-1}, P_2O_5 135 mg L^{-1}, K_2O 212 mg L^{-1}, salinity 1.23 g L^{-1}, Einheitserde classic, Einheitserde Werkverband e.V., Sinntal-Altengronau, Germany). The pots were then placed in a cabin in a glasshouse located at Leibniz Institute of Vegetable and Ornamental Crops (Grossbeeren, 52°20'5N 13° 18'35.3"E). Three experimental repetitions were performed in April, May and September 2021. The glasshouse cabin temperature and the relative humidity was set to 22°C and 70%, controlled by open vents. No supplemental artificial light was used.

Polytunnels, placed in the glasshouse, were covered using three-layered polyethylene films (low-density polyethylene/linear low-density polyethylene/14% ethylene butyl acrylate (middle layer), 180 µm thickness, CONSTAB polyolefin additives GmbH, Rüthen, Germany), four films contained antifogging additives (Sabostat A 300 and Atmer 103, 0.35%) whereas the other four were additive free (Figure S1). Thus, in total, each experiment was performed with four biological replicates per cultivation condition (polytunnel with antifog, antifog-free polytunnel and without polytunnel). Five lettuce plants were cultivated under each polytunnel as well as 20 lettuce plants without. To avoid an influence due to position, the lettuce plants were randomized twice a week and the positions of the polytunnels were randomized once halfway through the experimental period. Harvesting was carried out about 20 days after transplanting. The five largest leaves per plant were cut off, the midrib was removed, and a sample containing leaves from four lettuce plants was immediately frozen in liquid nitrogen. Two samples were taken per biological replicate, since one was used for metabolite analysis and the other for RNA extraction. Samples for metabolite analysis were lyophilized and stored vacuum-packed at ambient temperature in the dark until further analysis. Homogenization was performed using a mill (Retsch® MM 400, 45 s, 2 repetitions at 25 1 s^{-1}). The fresh material was homogenized for RNA extraction using a mortar and pistil under liquid nitrogen and stored at -80°C.

2.2 Monitoring of climatic conditions

The temperature, relative humidity and the PPFD (photosynthetic photon flux density) were monitored during the experiments in the

greenhouse cabin as well as under the polytunnels in the greenhouse. To detect temperature and relative humidity, sensors (PT - 100 type B sensor, Galltec Mess- und Regeltechnik GmbH, Bondorf, Germany, MELA Sensortechnik GmbH Mohlsdorf-Teichwolframsdorf, Germany). were placed under four polytunnels Monitoring in the greenhouse cabin was performed by an aspiration psychrometer (Type ELAU KlimaExpert, KE-PTFF-8024-OF, Elektro- und Automatisierungsanlagen Pierre Ambrozy, Gatersleben, Germany). Five PAR sensors (photosynthetic active radiation, LI-190R Quantum Sensor, LI-COR Biosciences GmbH, Germany) were used, four placed under the polytunnels and one in the greenhouse cabin. Furthermore, the UVA and UVB transmittances inside the polytunnels were determined once during the experiment using a spectrometer (Optic Spectrometer, Ocean Optics Inc., Ostfildern, Germany), UV/VIS transmission spectra of both films were also determined before use (Figure S2) using photospectrometer (Lambda 365, PerkinElmer, Inc., Waltham, USA).

2.3 Chemicals and standards

Ethanol (≥ 99.9%, LiChrosolv®), tetrahydrofuran (THF, ≥ 99.9%, LiChroSolv®), n-hexane (SupraSolv®), dichloromethane (< 99.8%, SupraSolv®), chlorophyll a and b (analytical standards) and norflurazone (Pestanal®, analytical standard) were obtained from Merck KGaA (Darmstadt, Germany). *Tert*-butyl methyl ether (≥ 99.9%, Rotisolv®), 2-propanol (≥ 99.9%, Rotisolv®), ammonium acetate (≥ 98%) and acetic acid (100%, Supra Quality) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Methanol (Chemsolute®) and acetonitrile (Chemsolute®) were purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). Carotenoid standards were obtained from CaroteNature GmbH (Münsingen, Switzerland) and flavonol glycosides from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Abscisic acid (ABA) standard was purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) and (+)-abscisic acid-d6, ≥ 98%) from Toronto Research Chemicals (North York, Canada). All solvents were of LC-MS quality and the water was of ultra-pure quality.

2.4 Analysis of carotenoid metabolic flux with norflurazon treatment

Lettuce was grown in September and October 2021 in two independent experiments under the same conditions as mentioned above. Norflurazon treatment was performed at the lettuce 8-leaf-stage after 10 days of polytunnel cultivation, as described previously for leaves of *Arabidopsis thaliana* (Koschmieder and Welsch, 2020). In detail, two leaves per plant were transferred in an aqueous norflurazon solution (70 µM in aqueous 0.125% 2-propanol) and two leaves in water (aqueous 0.125% 2-propanol) as controls. The leaves were incubated in darkness for 2 h. Afterwards, the norflurazon solution was changed to 10 µM and the leaves were incubated for another 4 h in daylight (PPFD: approximately 140 µmol s^{-1} m^{-2}) under each cultivation condition. Lettuce leaves from a total of five plants were collected as one sample (pool sample per polytunnel or without polytunnel), the midrib was removed and samples were frozen in liquid nitrogen and stored at -60°C until further analysis. Sample preparation was performed as described above.

2.5 Analysis of flavonoid glycosides *via* HPLC-DAD-MS/MS

10 mg of homogenized sample was extracted three times with methanol/water (3:2, v/v) as previously described (Neugart et al., 2019). Combined supernatants were subsequently dried using a Speedvac (SPD111V, Thermo Scientific). The dried samples were redissolved in 200 µL methanol/water (1:9, v/v) and filtered through Spin-X cellulose acetate filters (0.22 µM) tubes. The extracts were analyzed by Agilent 1260 Infinity II HPLC equipped with an Ascentis® Express F5 column (150 mm × 4.6 mm, 5 µm, Supelco, Sigma Aldrich Chemical Co., St Louis, MO, USA). The flavonoid glycosides were detected using a photodiode array detector at wavelength 370 nm. Compounds were eluted using solvent A: 0.5% acetic acid and solvent B: acetonitrile in gradient mode. A Bruker amazon SL ion trap mass spectrometer was used to determine mass spectra and perform fragmentation of the separated compounds. Ionization was performed by ESI (electrospray ionization) in negative polarity. The flavonoid glycosides were identified based on their absorption maxima, mass spectra and fragmentation pattern either comparing with authentic standards and with literature data (Table S1). Quantification was performed using external calibration at 370 nm. Quercetin derivatives were quantified using quercetin-3-glucoside and luteolin derivatives as luteolin-7-glucoside.

2.6 Analysis of carotenoids *via* HPLC-DAD-ToF-MS

For carotenoid analysis, 5 mg of homogenized samples were weighed out, followed by three times extraction with 500 µL tetrahydrofuran/methanol (1:1, v/v) as previously described (Harbart et al., 2022). The collected and combined supernatants were dried under a nitrogen stream and redissolved in 250 µL dichloromethan/2-propanol (1:5, v/v). After filtration through PTFE filters (0.2 µm) the extracts were analyzed by HPLC-DAD-ToF-MS using an Agilent Technologies 1290 Infinity UHPLC coupled with an Agilent Technologies 6230 ToF LC/MS. Briefly, the separation was performed in gradient mode on a C30 column (YMC Co. Ltd, Kyoto, Japan, YMC C30, 100 × 2.1 mm, 3 µm) with eluents containing A: methanol/water (96:4, v/v) and B: methanol/*tert*-butyl methyl ether/water (6:90:4, v/v/v) both added with ammonium acetate (20 mM) to enhance the ionization. Ionization was achieved using a multimode ion source in positive polarity. Carotenoids were (tentatively) identified based on their specific absorption and mass spectra, in comparison with the literature or authentic standards (Table S2). The quantification of carotenoids was calculated *via* external calibration with authentic standards at wavelength 450 nm.

2.7 Analysis of phytoene *via* HPLC-QToF-MS

Phytoene was extracted from 5 mg homogenized samples by the modified method of Frede and Baldermann (2022). At first, 200 µL ethanol and 100 µL water were added followed by 1 min sonication. Phytoene was then extracted twice with 500 µL and 300 µL hexane. The collected supernatants were evaporated to dryness under a

nitrogen stream. Finally, samples were redissolved in 375 µL dichloromethane/2-propanol (1:5, v/v) and filtered through PTFE filters (0.2 µm). The instrument settings were applied in general as for carotenoids, but with the following modifications. A 1290 Infinity HPLC-DAD coupled with a 6546 QToF-MS (Agilent Technologies, Waldbronn, Germany) was used for the measurements. In contrast to the other carotenoids, phytoene was detected using a QToF system equipped with an APCI source (atmospheric pressure chemical ionization). Ions were detected in positive polarity with a gas temperature of 325°C, vaporized at 350°C, drying gas with a flow of 8 L min^{-1} and nebulizer at 35 psi. Corona voltage was set to 3500 V and a current of 6.5 µA. Phytoene was identified comparing mass spectra with an authentic standard (Table S2). Quantification was performed *via* external calibration with the authentic standard using the extracted masse of the m/z = 545.5081 $[M+H]^+$ ion. Phytoene was quantified as the sum of the isomers present.

2.8 Analysis of abscisic acid *via* HPLC-MS/MS

Abscisic acid (ABA) extraction was performed according to the method by Errard et al. (2016) with modifications. ABA was extracted from 10 mg homogenized sample using methanol/water (3:2, v/v). Deuterated ABA was added as an internal standard followed by sonication in cold. Combined supernatants were filtered through PTFE filters (0.2 µm) and diluted with 0.1% acetic acid in ultrapure water (1:1, v/v). The extracts were analyzed using an Agilent Technologies 1260 Infinity HPLC coupled with a triple quadrupole, Q-Trap® 6500-MS/MS system (AB Sciex LLC, Framingham, USA) equipped with a Zorbax Eclipse Plus C18 column (1.8 µm, 2.1 mm x 50 mm; Agilent Technologies, Waldbronn, Germany). Elution was performed in gradient mode using solvent A: 0.1% acetic acid and solvent B: acetonitrile and 0.1% water. Ionization was performed in negative mode using ESI (electrospray ionization) at 500°C with the following settings: ionization voltage, -4,500 V; curtain gas, 50 psi; drying gas, 50 psi; nebulizer gas, 50 psi; auxiliary gas, 65 psi; and multi reaction monitoring (MRM) at a dwell time of 0.3781 s. Identification and quantification were based on MRM transitions (263→153 quantifier, 263→203 and 263→122 qualifier). ABA was quantified using external calibration with internal standard.

2.9 Gene expression analysis *via* RT-*q*PCR

Approximately 50 mg of powdered fresh tissue was weighed out for RNA extraction using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, with an on-column DNAse I digestion. RNA concentration was determined spectrophotometrically with Nanodrop at 260 nm (ND1000, Thermo Fisher Scientific, Waltham, MA, USA) with a desired ratio of 260/280 ~ 2.0 and 260/230 ~ 2.0-2.3. Additionally, the quality of selected RNA samples was checked using the bioanalyzer (2100 bioanalyzer, Agilent Technologies). A RIN value of ≥ 7.3 was accepted for further usage. The cDNA was synthesized with the SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and oligo (dT)12–18 primers as described by the manufacturer using 250 ng total RNA. Primers for target and reference genes were designed using sequences available at Phytozome or NCBI (National Center for Biotechnology Information; Table S4). The primer amplification efficiencies were determined with cDNA dilution analysis. Detailed information about primer sequences and efficiencies can be found in Table S3. The stability of selected reference genes (actin 7, ubiquitin-conjugating enzyme E2 A and elongation factor 1-alpha) was checked (M value <0.5; coefficient variance <0.25). The RT-*q*PCR experiments were performed in triplicates using 3 µL diluted cDNA (1:10), 5 µL 2× SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany) and 2 µL of 2 µM primer. Experiments were conducted with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following thermal cycling conditions: 95°C for 10 min, 39 cycles of 95°C for 15 s, 58°C for 15 s followed by 72°C for 30 s and a subsequent melting curve analysis. For the analysis of *CCD4* and *OR* family genes, an adjusted annealing temperature of 60°C was used. Data were evaluated using the ΔΔCq method according to Vandesompele et al. (2002); Pfaffl (2004) with the geometric mean of the three reference genes. The expression of genes of interest were calculated as *n*-fold changes relative to gene expression in the lettuce samples grown without polytunnels.

2.10 Statistical analysis

The SigmaPlot 14.0 software (Systat, Erkrath, Germany) was applied for statistical analysis. Data were compared by one-way ANOVA followed by Tukey HSD *post hoc* test assuming normal distribution and variance homogeneity. If the assumption did not apply, a Kruskal-Wallis one way ANOVA on ranks was performed. Significant differences were considered at $p \leq 0.05$ and are indicated by different letters or asterisk. Outlier identification was performed by a Grubbs test, assuming outliers with a $G \geq 1.4925$ for a representative sample size of $n = 4$. Data are presented as mean ± standard error unless otherwise stated.

3 Results

3.1 Changing climate conditions caused by protected cultivation

To assess the different light transmittance, reflectance, scattering and heat absorption properties of the materials used in protected cultivation, climatic conditions were evaluated for each experimental setup. We found that regardless of the experimental repetition (in April, May or September), similar differences when comparing cultivation with and without polytunnels were observed for all factors examined (Table 1). In detail, a 1.31-fold lower daily light integral (DLI), 1.1-fold higher temperature, and 1.89-fold higher relative humidity were determined under polytunnels (with and without antifogging additives). However, absolute values varied between experimental repetitions due to seasonal changes. Interestingly, temperature in general was not significantly different between repetitions, but DLI, photoperiod, and relative humidity were affected, with higher DLI and photoperiod and lower relative humidity in May and September compared to April. Although no temperature differences due to seasonal changes were determined for lettuce without polytunnels, significant differences in temperature were observed due to the use of polytunnels. Notably, the temperature

TABLE 1 Lettuce cultivation characteristics of three independent experimental repetitions in April (1), May (2) and September (3).

Lettuce cultivation characteristics	Experimental repetition	Lettuce cultivation condition		
		Without polytunnel	Polytunnel with antifog	Polytunnel without antifog
Daily light integral (mol m^{-2} d^{-1})	1	7.23 ± 1.91^{AB,a}	5.81 ± 1.63^{AB,b}	5.33 ± 1.41^{B,b}
	2	9.01 ± 3.34^{A,a}	7.10 ± 2.36^{A,b}	7.07 ± 2.22^{A,b}
	3	5.97 ± 3.41^{B,a}	4.30 ± 0.31^{B,ab}	2.10 ± 0.21^{C,b}
Photoperiod (daylight, h)	1	13.37 ± 0.41^{B}		
	2	15.14 ± 0.55^{A}		
	3	12.67 ± 0.60^{C}		
Temperature (°C)	1	22.61 ± 0.54^{a}	25.29 ± 1.61^{AB,b}	25.19 ± 1.43^{AB,b}
	2	23.04 ± 1.17^{a}	26.06 ± 2.26^{A,b}	26.28 ± 2.38^{A,b}
	3	22.57 ± 0.80^{a}	24.07 ± 0.78^{B,b}	24.05 ± 0.77^{B,b}
Relative humidity (%)	1	42.98 ± 3.90^{C,a}	86.30 ± 8.77^{B,b}	91.79 ± 8.89^{c}
	2	46.67 ± 5.76^{B,a}	90.43 ± 5.75^{AB,b}	92.13 ± 5.14^{b}
	3	56.52 ± 5.67^{A,a}	95.48 ± 1.82^{A,b}	95.48 ± 1.67^{b}

Lettuce was grown without or under polytunnels with and without antifogging additives. Data are shown as averaged values (mean ± SD) monitored continuously over the experimental period. Capital letters indicate significant differences ($p \leq 0.05$) between the three repetitions within similar cultivation conditions. Lower case letters indicate significant differences ($p \leq 0.05$) between cultivation conditions within one experimental repetition; no letters indicate absence of significance.

differences under polytunnels were consistent with changes in DLI. Hence, there is a close relationship between temperature and DLI, particularly under the polytunnels, which is a phenomenon known as the greenhouse effect (Baudoin et al., 2013).

The material of the polytunnels reduced certain light transmission (Figure S2), which led to differences in light intensity and light quality among the polytunnels, compared to cultivation without polytunnels (Figure 2). A 1.43-fold lower UVA and 1.50-fold lower UVB transmittance were determined due to either covering material, while light intensity (PAR) was 1.25-fold lower when comparing cultivation without polytunnels and polytunnels without antifog. Interestingly, no difference in PAR light intensity was observed for cultivation without polytunnels and polytunnels with antifog. Additionally, the use of polytunnels did not affect UVA to UVB ratios as well as far-red to red light ratio (Figure S3), but the PAR to UV ratio was 1.17-fold higher under polytunnels.

We assume that light is the most crucial factor here likely to affect the metabolic processes in the protected cultivation of lettuce, firstly, due to the strong direct correlation between higher DLI and increasing temperature under the polytunnels compared to without (Figure S7), and

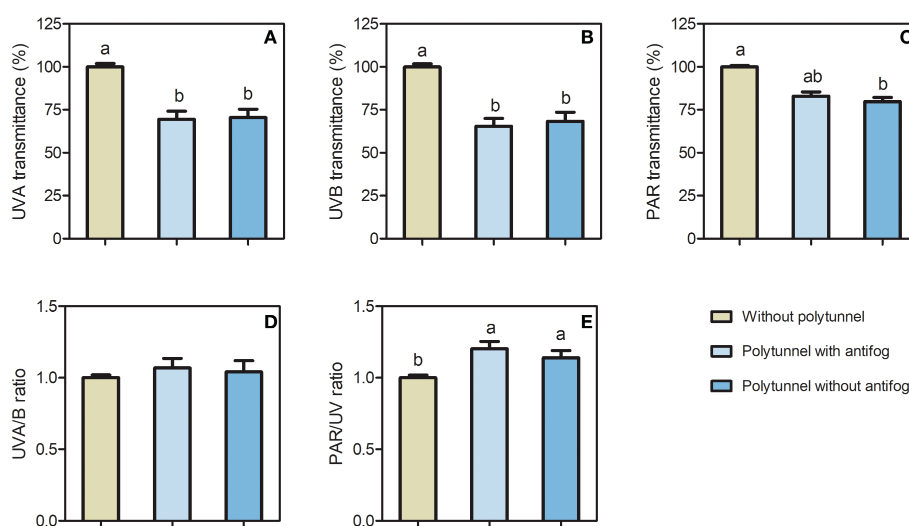

FIGURE 2
Differences in light intensity and light quality without and under polytunnels. **(A, B)** UV, and **(C)** PAR transmittance (%) of polytunnel materials with and without antifogging additives compared to without polytunnels, **(D)** UVA/B ratio, and **(E)** UV/PAR ratio. Measurements were conducted within the second experimental repetition (in May). Data represent mean ± SD (n = 4). Different letters indicate significant differences ($p \leq 0.05$); no letters indicate no significance. UV, ultraviolet; PAR, photosynthetic active radiation.

secondly, due to altered light quality as a consequence of the transmission properties of the films. As a result, we decided to present the data obtained from experimental repetition 1, since there were no significant differences in either DLI or photoperiod in experimental repetitions 2 and 3.

Since light and temperature are key factors that differ through protected cultivation, these require consideration for evaluating the regulation of metabolic pathways of bioactive compounds in horticultural crops. Notably, the two factors are interrelated when using covers and it is difficult to evaluate them separately.

3.2 Polytunnel cultivation affects the flavonoid glycoside content

Light intensity, quality, and temperature, among other environmental factors, can modulate the flavonoid content in horticultural crops. To unravel changes in flavonoids caused by altering climate conditions in protected cultivation, we analyzed flavonoid glycosides in lettuce cultivated under polytunnels (with and without antifog) or without. Two flavonols (quercetin derivatives) and a flavone (luteolin derivative) were identified in lettuce as glycosylated and acylated with sugar and organic acid moieties. Quercetin was present as glucuronide and malonyl-glucoside and luteolin was detected as a glucuronide derivative (Table S1 and Figure S6). The cultivation of lettuce under polytunnels resulted in 3.87-fold lower amounts of total flavonoids, as well as reduced amounts of individual compounds (quercetin glucuronide 3.10-fold, quercetin malonyl-glucoside 4.61-fold, luteolin glucuronide 1.72-fold; Figure 3). This was not dependent on the antifogging additives. Furthermore, lower flavonoid content in lettuce grown under polytunnels compared to without were measured in all experimental repetitions (Figure S8).

3.3 Polytunnel cultivation affects the carotenoid content

The bioactive carotenoids act as photosynthetic pigments, and, in particular, are responsive to differences in the light regime and temperature as well as other environmental factors. Polytunnel cultivation affected carotenoid content of lettuce significantly. Major carotenoids such as β-carotene and lutein were (tentatively) identified in lettuce, besides phytoene, violaxanthin, and neoxanthin and other minor carotenoids (Table S2, Figures S4 and S5). In addition, the lettuce-specific carotenoid lactucaxanthin was (tentatively) identified. Phytoene, violaxanthin and neoxanthin contents are represented as the sum of their detected isomers. Besides carotenoids as photosynthetic pigments, chlorophyll a and b were also detected (Figures S13-15).

Total carotenoid content was affected due to polytunnel cultivation (Figure 4). In particular, lettuces grown under polytunnels showed 1.08-fold higher total carotenoid content compared to growth without polytunnels. At the individual level, lutein (1.13-fold) and phytoene (2.05-fold) content were higher with polytunnel cultivation than without. However, neoxanthin content was 1.55-fold higher in lettuces grown under antifog-free polytunnels than without polytunnels. Albeit not significant, the xanthophylls lactucaxanthin and violaxanthin showed similar tendencies in polytunnel grown lettuces. Trends of higher carotenoid levels were also evident in each experimental repetition (1 to 3), however except for lutein, changes at the level of the individual carotenoids also occur between the repetitions (Figure S9).

In summary, the use of polytunnels for lettuce cultivation resulted in lower overall flavonoid glycoside content and higher contents of carotenoids, although differences occur at the level of individual carotenoid compounds.

3.4 RNA transcript levels of carotenoid and flavonoid pathway genes and transcription factors

Transcription factors and regulatory genes potentially affecting lettuce metabolism under varying light and temperature regimes were identified based on the literature (Li et al., 2010; Stracke et al., 2010; Toledo-Ortiz et al., 2014; Stanley and Yuan, 2019). Selected genes encoding key enzymes of the core biosynthesis for carotenoid

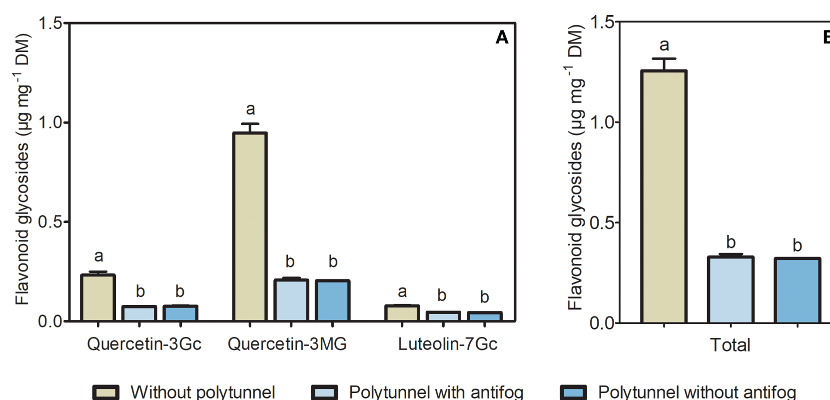

FIGURE 3
Flavonoids in lettuce cultivated without or under polytunnels. **(A)** Individual, and **(B)** total content of flavonoid glycosides (µg mg^{-1} DM) in lettuce grown without or under polytunnels with and without antifogging additives. The first experimental repetition in April is shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) between treatment of individual compounds and total content are indicated by different letters. Gc, glucuronide; MG, malonyl glucoside.

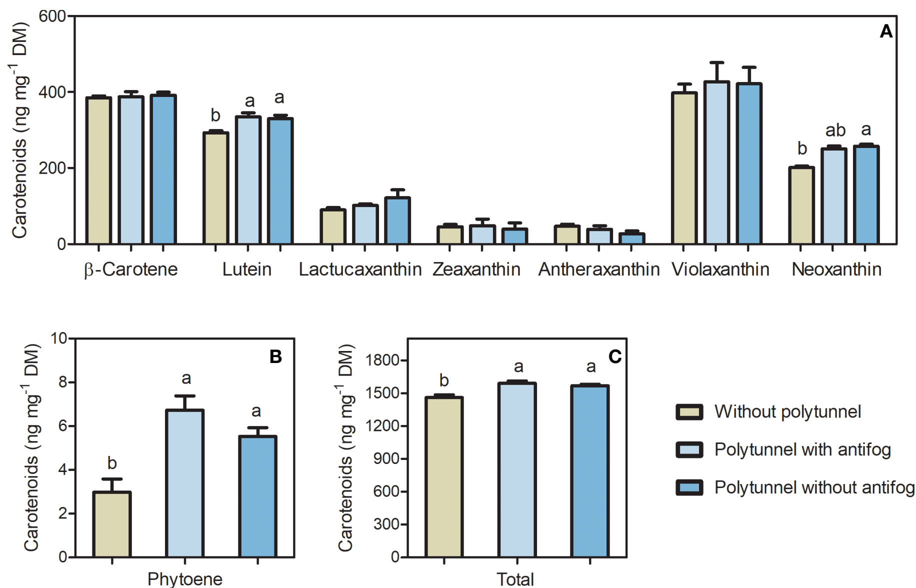

FIGURE 4
Total carotenoid content affected by polytunnel cultivation. Carotenoid content (ng mg^{-1} DM) in lettuce grown without or under polytunnels with and without antifogging additives. **(A)** Individual carotenoids β-/ε-branch and downstream, **(B)** upper pathway metabolite phytoene, and **(C)** total carotenoids. First experimental repetition in April is shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) between treatment of individual compounds and total content are indicated by different letters; no letters indicate absence of significance.

(Phytoene synthase, *PSY*) and flavonoid pathways (Chalcone synthase, *CHS*) were analyzed as *n*-fold expression based on lettuce cultivation without polytunnels (Figure 5) to obtain further insights into their metabolic regulation.

The *CHS* gene encodes an enzyme early in the flavonoid pathway (Figure 1) that catalyzes the condensation of cinnamic acid (derivative) CoA-ester with malonyl-CoA yielding naringenin chalcone (Dao et al., 2011). It is thus the hub for the synthesis of a wide diversity of flavonoids in plants. The expression of *CHS* was 14.47-fold lower in lettuce grown under polytunnels with antifog, and 11.34-fold lower under polytunnels without antifog than lettuce grown without polytunnels. However, only a trend can be detected in the lower *CHS* expression under polytunnels without antifog. In agreement with this observation, the transcripts of *UVR8* (UV resistance locus 8) are also 2.05-fold lower in both polytunnels compared to cultivation without polytunnels. This gene encodes the *Arabidopsis* UVB photoreceptor, which is known to induce UVB light-triggered metabolic responses (Rizzini et al., 2011).

The enzyme encoded by the *PSY* gene is the first committed step in carotenoid biosynthesis (Von Lintig et al., 1997). *PSY* expression in lettuce was 1.52-fold lower when grown under polytunnels (independently of antifog) than without polytunnels. This is a relevant observation, since the amounts of carotenoids are significantly higher in lettuce grown under polytunnels. In contrast, the transcripts of *CCD4* (Carotenoid dioxygenase 4), a gene encoding a cleavage enzyme that forms apocarotenoids, were 5.02-fold higher. Taken together, fewer transcripts for the *PSY*-based biosynthesis, and more abundant transcripts for the *CCD4*-based degradation, together with contrasting higher amounts of carotenoids suggest that additional mechanisms are important for regulating the carotenoid pool in lettuce. *OR* and *OR-like* encoding proteins, known as posttranscriptional regulators (Zhou et al., 2015), interact with PSY. In lettuce grown without or under polytunnels, no differences were observed for either *OR* or *OR-like* transcripts.

The transcription factors *HY5* (Elongated hypocotyl 5) and *PIFs* (Phytochrome interacting factors), are closely related to light and temperature signaling and impact metabolic responses of both carotenoids and flavonoids (Lee et al., 2007a; Toledo-Ortiz et al., 2014; Stanley and Yuan, 2019). Here, *HY5* and *PIF1* (Phytochrome interacting factor 1) are antagonistic: while *HY5* acts as transcriptional activator and is able to bind to *CHS* and *PSY* promotors, *PIF1* acts as transcriptional repressor and is able to bind the *PSY* promotor. This highlights *HY5* and *PIF1* as promising candidates to be investigated. In lettuce grown without or under polytunnels, *HY5* transcripts were similar and no significant differences were evident, whereas *PIF1* transcripts were 1.30-fold higher in lettuce under polytunnels. The experimental repetitions also showed predominantly similar patterns, although in some cases tendencies are present (Figures S10 and S11). For example, *PSY* expression is lower in repetitions 2 and 3 in polytunnel grown lettuce, albeit not significantly; however, the accumulation of carotenoids in such lettuce still cannot be explained.

In summary, the transcripts of flavonoid-related biosynthetic enzymes were associated with flavonoid content in lettuce. Transcripts of UVB photoreceptor *UVR8* were lower in lettuce grown under polytunnels, and thus related to flavonoid content. However, the transcripts for carotenoid-related biosynthetic enzymes did not show this kind of association with carotenoid content. Focusing on the transcription factors, *HY5* transcription did not seem to differ in any experimental repetitions, whereas the transcription level of *PIF1* was higher in lettuce grown under polytunnels than without polytunnels.

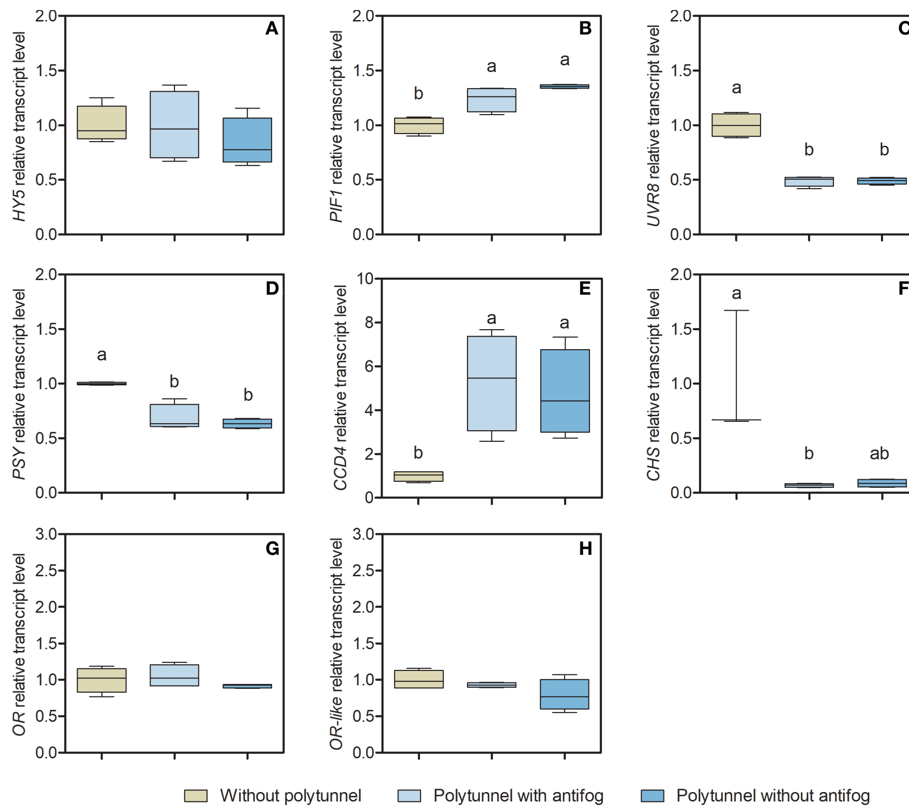

FIGURE 5
Gene transcripts for key enzymes of the core carotenoid and flavonoid biosynthesis pathways. Transcript levels of **(A)** *HY5*, **(B)** *PIF1*, **(C)** *UVR8*, **(D)** *PSY*, **(E)** *CCD4*, **(F)** *CHS*, **(G)** *OR*, and **(H)** *OR-like* in lettuce grown without or under polytunnels with and without antifogging additives. The first experimental repetition in April is shown. The data are expressed as Box-Whisker-Plots (n = 4); Whiskers show maximal and minimal values. Data was normalized to lettuce grown without polytunnels. Different letters indicate significant differences ($p \leq 0.05$) of transcripts under different cultivation conditions; no letters indicate absence of significance. *HY5*, Elongated hypocotyl5; *PIF1*, Phytochrome interacting factor1; *UVR8*, Ultraviolet resistance locus8; *PSY*, Phytoene synthase; *CCD4*, Carotenoid cleavage dioxygenase4; *CHS*, Chalcone synthase; *OR*, Orange protein; *OR-like*, Orange-like protein.

3.5 Polytunnel cultivation affects the phytohormone abscisic acid

Since phytohormones act as signal transducers and are responsive to changing environmental conditions, we analyzed the content of the phytohormone ABA (Figure 6). A 4.01-fold lower ABA content was determined in lettuce under polytunnels without antifog compared to without polytunnels. A tendency of lower ABA content (3.11-fold) was observed when comparing polytunnels with antifog to without polytunnel. Similarly, in the third, but not the second repetition, ABA in lettuce was significantly lower due to polytunnel cultivation (Figure S12).

3.6 Polytunnel cultivation alters carotenoid metabolic flux

Since neither transcript levels of *PSY*-based carotenoid synthesis nor *CCD4* pathway-based degradation can explain the carotenoid accumulation in lettuce cultivated under polytunnels, we looked more closely at the carotenoid biosynthetic pathway. Therefore, we investigated the metabolic flux of the upper carotenoid pathway using the bleaching herbicide norflurazon. This inhibits carotenoid pathway enzyme PDS and those downsteam, resulting in phytoene accumulation, and indicating metabolic flux (Koschmieder and Welsch, 2020). Overall, norflurazon treatment led to higher amounts of phytoene in lettuces than in the water treated controls, independently of cultivation conditions (Figure 7). In lettuce grown without polytunnels, phytoene amounts were 8.53-fold higher, whereas in lettuce under polytunnels with antifog they were 13.45-fold higher, and without antifog 11.87-fold higher than the water controls. Notably, under norflurazon treatment higher (1.69-fold) phytoene contents were determined in lettuce grown under polytunnels than without, indicating higher carotenoid metabolic flux. This provided a possible explanation for the higher carotenoid contents.

4 Discussion

Growing crops under protected cultivation leads to differing climatic conditions compared to open-field production. Furthermore, the covering materials used can affect these conditions. The polytunnels used in this study influenced UV and PAR light regimes as well as temperature. Although reduced transmission was determined in this range, there were no changes in the ratio of far-red and red light. The generated greenhouse effect resulted in temperature increases closely related to the modulated

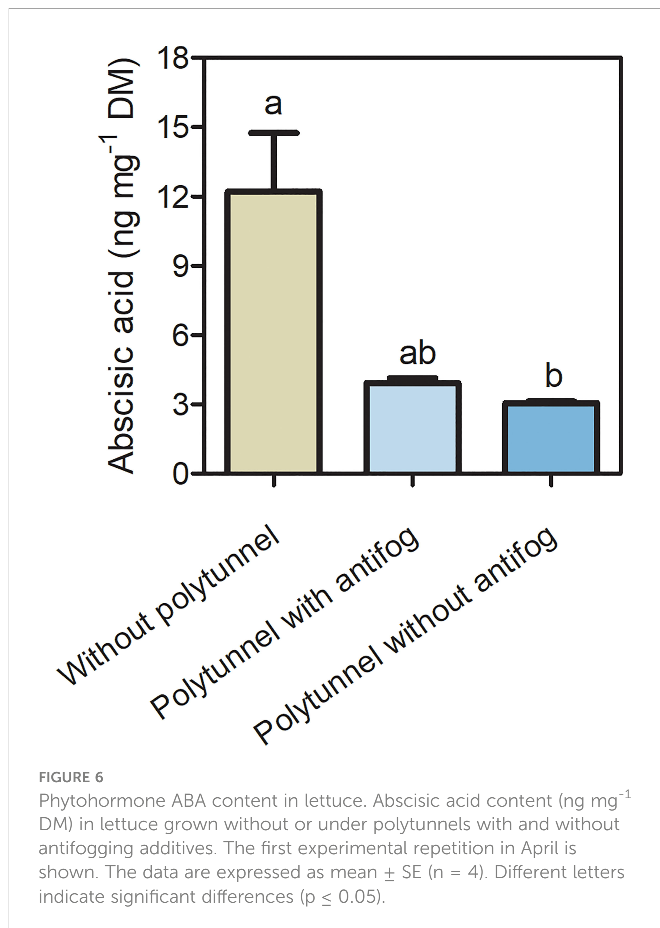

FIGURE 6
Phytohormone ABA content in lettuce. Abscisic acid content (ng mg^{-1} DM) in lettuce grown without or under polytunnels with and without antifogging additives. The first experimental repetition in April is shown. The data are expressed as mean ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$).

light conditions. Polytunnels with and without antifogging additives showed no differences in temperature nor UV transmittance, and only minor differences in PAR intensity. For both polytunnel covering materials, with and without antifog, similar differences in metabolite profiles, transcripts, phytohormones, and metabolic fluxes were observed. Consequently, the results are not discussed individually but are summarized below under the general term 'polytunnel'.

4.1 Regulation of flavonoid biosynthesis in protected cultivation

Flavonoids are bioactive compounds from the diverse group of plant phenols and based on structural properties are divided into subgroups such as the flavonols and flavones, both detected in lettuce. In terms of human consumption, epidemiological studies have shown that flavonoid-rich foods have beneficial properties, for instance against chronic metabolic or cardiovascular diseases (Hertog et al., 1993; Kim and Je, 2017; Rees et al., 2018). In plants, they are known as antioxidants capable of scavenging reactive oxygen species (ROS) produced for example under solar UV light exposure (UVA and UVB) or plant stress (Agati and Tattini, 2010). Thus, the UV-shielding flavonoids are mainly found in the plant epidermis and act as UV protectants – a plant's sunscreen. Studies have reported that flavonoid contents increase in lettuce grown with additional UVA, UVB and UVC light (Lee et al., 2014; Assumpção et al., 2019).Using covered systems for crop cultivation, changes in UV light occur due to the reduced transmittance of the materials used. Indeed, UVA and UVB light are reduced by using polytunnels in this study, resulting in lower total and individual flavonoids than seen in cultivation without polytunnels. This is in accordance with Katsoulas et al. (2020), who reviewed that UV-blocking materials, defined as complete or partial solar UV absorbing materials, have negative effects on plant phenolic compounds such as flavonoids, including in lettuce.

Transcription of the *CHS* gene, encoding a key enzyme in the flavonoid pathway, is reported to be induced by UVA and UVB light (Jenkins et al., 2001; Dao et al., 2011). Accordingly, lettuce cultivated under polytunnels showed lower *CHS* expression than cultivation without polytunnels. The *CHS* promotor contains multiple light regulatory units, and photoreceptor signaling may play a role in the interaction with such units. In *Arabidopsis*, there is evidence that solar UV in particular is able to trigger changes in the flavonoid biosynthetic pathway *via* UVR8 photoreceptor regulation together with other photoreceptors, where UVB specifically triggers a UVR8 response (Morales et al., 2013). We assume the UVR8 photoreceptor contributes to flavonoid regulation in lettuce grown under polytunnels; however, the UVB signal transduction pathway from UVR8 to flavonoid biosynthesis is not well understood (Yang et al., 2018). The UVB/*HY5* pathway has been well studied. *HY5* is known to bind in promotor regions of several genes, such as *CHS* (Lee et al., 2007a; Toledo-Ortiz et al., 2014) and so can act as a transcriptional activator. Stracke et al. (2010) showed that *HY5* is required in light and UVB responsive gene regulation. However, in this study, lettuce grown without or under polytunnels showed no significant differences in *HY5* transcripts in any experimental repetition. Consequently, regulation may occur at the protein level, which should be clarified in further studies. Furthermore, interactions with other transcription factors, for example from the MYB family, have been described before (Cloix and Jenkins, 2008; Luo et al., 2008; Qian et al., 2020)

In addition to transcriptional induction of flavonoid accumulation, studies suggest that ABA has an impact on regulating flavonoid biosynthesis. ABA is a phytohormone that affects plant development and growth, among other things, and also acts as signaling molecule in response to abiotic and biotic environmental conditions (Vishwakarma et al., 2017). For example, Berli et al. (2010) showed increased flavonoid levels in leaves of *Vitis Vinifera* L. when exogenous ABA was applied, whereas another study also shows involvement of *PAL* (Phenlylalanine ammonia-lyase) enzyme activity (Hao et al., 2010). In the present study, higher amounts of flavonoids related to higher ABA content were found in lettuce grown without polytunnels than grown in polytunnels. Altered ABA levels are in particular discussed in response to high light, UVB irradiation or changing temperatures (Vishwakarma et al., 2017; Brunetti et al., 2019). Furthermore, it has been shown that in leaves of *Vitis Vinifera* L. less ABA and flavonoids accumulated when UVB light is filtered by a polyester covering (Berli et al., 2010). It is worth noting that *HY5* transcriptional activation is also discussed as contributing to ABA signaling (Chen et al., 2008). Thus, the role of *HY5* in lettuce without and with polytunnels remains uncertain.

Neugart et al. (2019) demonstrated a priming effect of the PAR light regime in the response of *Arabidopsis* to additional UVA and UVB light. Therefore, the accumulation of flavonoids could be influenced by both light regimes in this study.

In addition to the light regime, temperature is another regulating factor related to flavonoid content. Studies on the effect of

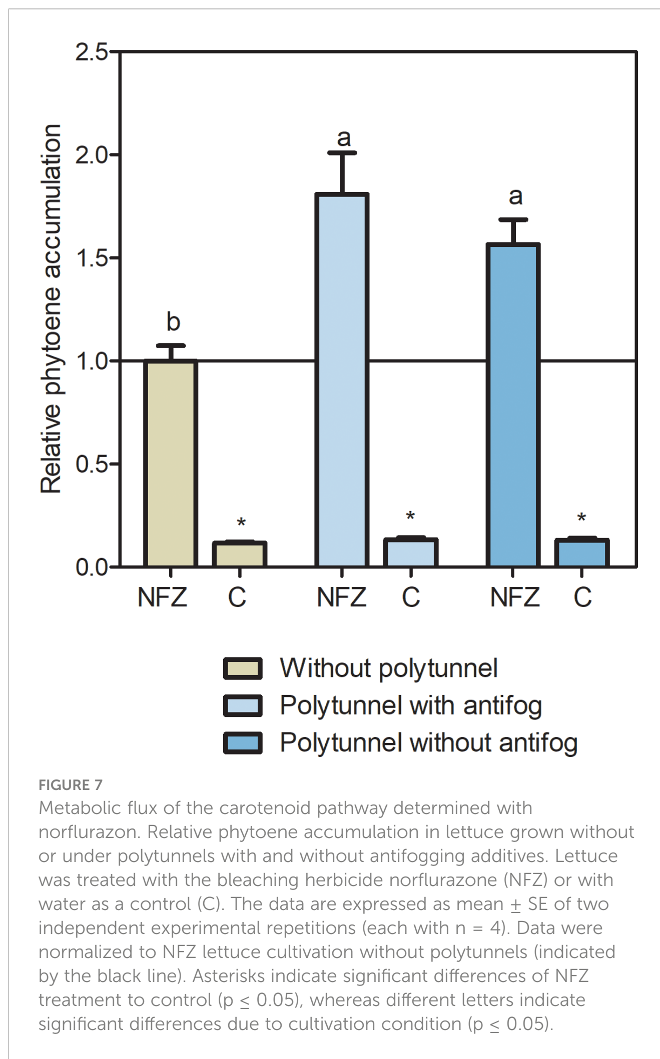

FIGURE 7
Metabolic flux of the carotenoid pathway determined with norflurazon. Relative phytoene accumulation in lettuce grown without or under polytunnels with and without antifogging additives. Lettuce was treated with the bleaching herbicide norflurazone (NFZ) or with water as a control (C). The data are expressed as mean ± SE of two independent experimental repetitions (each with n = 4). Data were normalized to NFZ lettuce cultivation without polytunnels (indicated by the black line). Asterisks indicate significant differences of NFZ treatment to control ($p \leq 0.05$), whereas different letters indicate significant differences due to cultivation condition ($p \leq 0.05$).

temperature on flavonoids in lettuce are contrasting; while most studies show higher flavonoid accumulation in lettuce grown at lower temperatures (Boo et al., 2011; Sytar et al., 2018), one study shows the opposite (Sublett et al., 2018). In other plant species such as *Arabidopsis thaliana*, *Ginkgo biloba* L. or *Angelica sinensis*, the flavonoid accumulation was promoted at lower temperatures (Leyva et al., 1995; Guo et al., 2020; Dong et al., 2022). This supports the higher flavonoid accumulation in this study, in which temperatures were between 1.49°C to 3.13°C lower when lettuce was grown without polytunnels. In addition, transcript levels of key biosynthetic enzymes like *PAL* or *CHS* as well as enzyme activity were shown to be highest at lower temperatures (Leyva et al., 1995; Guo et al., 2020; Dong et al., 2022). This could lead to the assumption that the *CHS* transcripts and flavonoid content in lettuce in this study were possibly also related to the temperature regime. However, the study by Sytar et al. (2018) showed that this should be taken with caution. They observed that UV light, but not cultivation temperature, had the major impact on flavonoid accumulation in lettuce grown in greenhouses and outdoors (Sytar et al., 2018).

Lettuce grown under polytunnels contains lower total and individual flavonoid compounds, mainly due to the reduced solar UV (UVA and UVB) transmissibility of the covering material. This is also reflected in the transcript levels of biosynthetically active genes, which are regulated at the transcriptional level.

4.2 Regulation of carotenoid biosynthesis in protected cultivation

As accessory pigments, carotenoids are involved in light harvesting and contribute to effective photosynthesis. Additionally, they are also involved in non-photochemical quenching (NPQ) and protect plants from adverse environmental conditions such as excessive light or high temperatures (Cazzonelli, 2011). In human nutrition, carotenoids are associated with several health-promoting properties, such as a lower risk of non-transmissible diseases or protection against age-related macular degeneration (Eisenhauer et al., 2017; Milani et al., 2017). Thus, there is consumer interest in vegetables rich in these bioactive compounds, and understanding their metabolic regulation is important for targeting their enhancement.

The carotenoid pathway in plants is highly conserved and well understood (Stanley and Yuan, 2019); however, regulatory mechanisms are still the subject of research. Since the regulation of carotenoids appears to be species- and tissue-specific (Ohashi-Kaneko et al., 2007; Shumskaya et al., 2012; Lätari et al., 2015), and plant research mainly focuses on model plants, such as *Arabidopsis thaliana*, little is known about its regulation in horticultural crops. In lettuce grown under polytunnels, we observed higher total and individual carotenoids compared to cultivation without polytunnels.

The PSI and PSII photosystems have different absorption maxima due to their carotenoid and chlorophyll composition, possibly leading to different adaptations of their composition depending on both light quality and quantity (Ballottari et al., 2007; Caffarri et al., 2014). A carotenoid steady-state has been suggested as a balance between biosynthesis and turnover in photosynthetic leaf tissue (Lätari et al., 2015). The adaptation of carotenoid content and profile in leaves probably results from an imbalance in the photosystem's excitation, as suggested by Frede et al. (2019) for pak choi (*Brassica rapa* subsp. *chinensis*) sprouts illuminated with different LED light qualities. Changing environmental conditions can cause the photosynthetic pigments to adapt and achieve effective light harvesting that contributes to photosynthesis. Altered light quality due to the reduction in the UV/PAR and to a lesser extent far-red/red wavelength by the covering material, as well as altered light intensity, occur in polytunnel cultivation. As part of the photosynthetic apparatus, carotenoid changes are concomitant with changes in chlorophylls, and co-expression of chlorophyll- and carotenoid-related genes is also evident (Meier et al., 2011; Stange and Flores, 2012).

In this study, increased carotenoid and chlorophyll contents were observed in polytunnel grown lettuce compared to without polytunnels for all three experimental repetitions (Figures S13-15). In particular, the decrease in chlorophyll a/b ratio indicates an adaptation of the photosystems and alteration in chlorophyll metabolism to achieve effective light harvesting under polytunnels, since chlorophyll b acts as an accessory pigment in the antenna (Lee et al., 2007b; Caffarri et al., 2014). This is also reflected in individual carotenoids. Depending on their localization and function in the photosystems, β-carotene and zeaxanthin in core structure, and lutein, violaxanthin and neoxanthin in light harvesting antenna (Caffarri et al., 2014), one can suggest that the lower light intensity and altered spectral quality under polytunnels leads to adaptation of

accessory carotenoids. Besides the light regime, it is discussed that carotenoids protect plants at high temperatures by scavenging resulting in reactive oxygen species (ROS) generated in PSII and thylakoid membranes (Stanley and Yuan, 2019). Toledo-Ortiz et al. (2014) determined higher carotenoid contents in *Arabidopsis* grown at higher temperatures (17°C to 27°C), while there was only a positive trend in kale and spinach (from 10°C to 30°C) (Lefsrud et al. (2005). This is consistent with the higher carotenoids in lettuce and the prevailing polytunnel conditions in this study, although the major antioxidant carotenoids in the photosystem core structure, β-carotene and zeaxanthin, appear to be less responsive in lettuce.

Studies demonstrate that higher *PSY* transcription is related to carotenoid accumulation induced by changes in light regimes (Von Lintig et al., 1997; Frede and Baldermann, 2022). Furthermore, *PSY* transcription was shown to be lower at elevated temperatures in maize leaves, while carotenoid accumulation was higher (Li et al., 2008). For this reason, it is assumed that the *PSY* transcript is not responsible for regulating carotenoid metabolism at higher temperatures (Stanley and Yuan, 2019). The synthesis of phytoene *via* PSY is a key and rate-limiting step in carotenoid biosynthesis. Surprisingly in this study, the *PSY* transcripts in lettuce predominantly showed no major differences or decreases in polytunnel cultivation, which are not reflected in the lettuce carotenoid contents and may indicate temperature-dependent regulation. How plants regulate the carotenoid biosynthetic pathway, particularly under different light and temperature regimes, is still largely unknown. Nevertheless, the involvement of some essential transcription factors has been confirmed (Stanley and Yuan, 2019). *HY5* functions as an intermediate control point downstream of photoreceptor signal transduction (Toledo-Ortiz et al., 2014). In light, *HY5* can bind to the *PSY* promotor and *HY5* accumulation is stabilized at lower temperatures favoring the binding to the Arabidopsis *PSY* promoter (Toledo-Ortiz et al., 2014). However, in this study, involvement of *HY5* in the regulation of carotenoid biosynthesis is unlikely since *HY5* transcripts showed no differences in lettuce grown without or with polytunnels. Moreover, the *HY5* antagonist *PIF1* showed higher transcript levels in the investigated lettuce.

PIF1 antagonizes *HY5* by binding to the same target at *PSY* promotor and repressing its transcription in the dark and elevated temperatures (Toledo-Ortiz et al., 2014). In de-etiolated leaves of *Arabidopsis* and *Sinapis alba*, *PSY* transcription seems to be phytochrome mediated *via PIF1* (Von Lintig et al., 1997). Moreover, *PIF1* is involved in shade-triggered reduction of carotenoid accumulation through *PSY* modulation in a *HY5* independent manner (Bou-Torrent et al., 2015). Both processes are triggered by far-red to red light, among others. Apart from carotenoid regulation, *PIF1* is found to interact with chlorophyll biosynthetic genes (Moon et al., 2008), and thus the regulation of carotenoids and chlorophylls are closely linked to photosynthetic efficiency. Furthermore, carotenoid and chlorophyll accumulation in *Arabidopsis* pif1 mutants were most affected at elevated temperature (Toledo-Ortiz et al., 2014). Therefore, the observed *PIF1* accumulation in this study results from low light, altered light qualities and elevated temperatures in polytunnels (Toledo-Ortiz et al., 2014). We assume that lettuce in polytunnels accumulates less *PSY* transcripts through *PIF1* triggered repression related to light and temperature regimes. In contrast, carotenoid contents are increased, suggesting additional mechanisms beyond the transcriptional level. Therefore, further research should aim to elucidate the regulation by the HY5/PIF1 network at the protein level.

The OR protein family is known from yellow cauliflower and its involvement in carotenoid accumulation (Lu et al., 2006). OR family members *OR* and *OR-like* are known to act in posttranscriptional regulation with PSY in leaves (Zhou et al., 2015). In this study, lettuce transcript levels of both were elevated in polytunnel cultivation in the second but not the first or third experimental repetition (Figures 5, S10, S11). Thus, *OR*-derived posttranscriptional regulation based on *OR* and *OR-like* transcripts do not explain carotenoid accumulation.

The carotenoid content is not only altered by biosynthesis, but also by degradation. CCDs (carotenoid cleavage dioxygenases), characterize a group of carotenoid-degrading enzymes. Recent studies indicate an effect of light quality on *CCD4* transcription Frede et al. (2018). Furthermore, a more recent study by Frede and Baldermann (2022) showed that a combination of blue and white light leads to both increased *CCD4* transcript levels and carotenoid content. We observed increased transcription levels of *CCD4*, which indicates higher carotenoid turnover as suggested by Frede and Baldermann (2022).

Since carotenoid metabolic flux was found to be higher in lettuce grown under polytunnels than without, carotenoid accumulation is likely independent of *PSY* transcription. Therefore, PSY protein levels or PSY enzyme activity could be different due to cultivation conditions. Here, different mechanisms are known in other species. Firstly, posttranscriptional regulation of PSY involving phytochrome photoreceptors seems reasonable. This has been discussed in tomato fruit: specifically, PSY enzyme activity but not transcription levels were different in tomatoes grown in red light grown compared to red/far-red and the dark (Schofield and Paliyath, 2005). Secondly, PSY localization appears to be important for its enzyme activity, particularly observed with regard to light qualities (Welsch et al., 2000). The membrane-bound PSY protein is active and contributes to metabolic flux, whereas the soluble PSY protein in the stroma is inactivate (Shumskaya et al., 2012; Lätari et al., 2015). Thirdly, light and temperature differences can cause changes in membrane fluidity, which were discussed for light or heat stress by Yamamoto (2016), and might contribute to the solubility of PSY protein. In order to shed light on posttranscriptional mechanisms of lettuce carotenoid accumulation, it is essential to analyze protein levels and enzyme activities in future research. Since PSY is affected by light in the red and far-red regions, special emphasis should also be given on greenhouse films in these light regions and their effects on the regulation of these bioactives.

Finally, as discussed above for flavonoids, ABA might be involved in mediating carotenoid steady-state levels. In *Arabidopsis*, induction of *PSY* transcription at post-germination under continuous light is negatively regulated by ABA (Meier et al., 2011). However, in the present study, *PSY* transcripts are reduced or unaffected and are probably not predominantly affected by ABA. Exogenous application of ABA shows contrasting effects depending on plant tissue and species (Baldermann et al., 2013; Barickman et al., 2014; Liu et al., 2020). Overall, no conclusion can be made about ABA being involved in mediating carotenoid accumulation.

Polytunnel cultivation of lettuce leads to higher total and individual carotenoids, mainly related to altered spectral quality

and light intensity due to the covering material and higher temperatures. *PIF1* transcripts are associated with biosynthetically active genes, suggesting an influence of phytochromes. Nevertheless, the higher carotenoid content seems more likely to be the result of higher metabolic flux due to posttranscriptional regulation of PSY. Further research should aim to elucidate the underlying mechanism influencing PSY but also CCD4 protein amounts as well as their activities.

4.3 Co-regulation of carotenoid and flavonoid pathways

Carotenoids and flavonoids both share similar functions in plants as protective compounds, as antioxidants scavenging ROS to protect the photosynthetic apparatus from photoinhibition (Agati and Tattini, 2010; Cazzonelli, 2011). Hence, they are involved in maintaining efficient plant photosynthesis even under unfavorable environmental conditions. However, in contrast to flavonoids, carotenoids are directly involved in the light harvesting process of photosynthesis, which is reflected in their location in plants. Carotenoids are bound to membranes in the chloroplast, whereas flavonoids primarily accumulate in the epidermis but also in chloroplasts' envelope to quench ROS (Agati and Tattini, 2010; Cazzonelli, 2011). For this purpose, these bioactive compounds have light-absorbing structures; however, their structural properties result in different light absorption characteristics. Carotenoids absorb predominantly in the blue light region (about 450 nm), whereas flavonoids absorb in the UV region (about 350 nm). Thus, by filtering UV light, flavonoids shield chloroplasts and the photosynthetic apparatus from UV-induced ROS production. Quercetin and luteolin flavonoids, both predominant in lettuce, in particular are discussed to efficiently protect plants due to their catechol structure in their B-ring, although the glycosylation pattern also affects these properties (Agati and Tattini, 2010; Neugart et al., 2019).

In the presented study, we observed an inverse relationship between carotenoids and flavonoids. Lettuce cultivated under polytunnels has higher carotenoid but lower flavonoid content, whereas in lettuce cultivated without polytunnels the findings are the opposite. This was also seen in the individual experiments, with the highest correlation found for lettuce grown in May (Spearmans: 0.832, repetition 2) followed by moderate correlations in April (Spearmans: 0.464, repetition 1) and September (Spearmans: 0.459, repetition 3), although no significance was found for the latter two (Figure S16). In all experiments, similar effects were found by growing lettuce without or under polytunnels, although the total amounts of both metabolites varied. A moderate negative correlation (Spearmans: 0.406) between carotenoids and flavonoids was also observed when all experimental repetitions were included.

The observed inverse relationship between carotenoids and flavonoids is in accordance with literature, where several studies revealed antagonistic occurrence of carotenoid and flavonoid content within different treatments (Ngwene et al., 2017, Ben Cao et al., 2015; Abdallah et al., 2016; Neugart et al., 2018). For instance, Becker et al. (2015) studied the impact of nitrogen deficiency on different metabolites in red and green lettuce. Here, carotenoids and chlorophylls decreased, while flavonoids increased under a lack of nitrogen.

Despite our initial assumption that *HY5* is involved in the inverse regulation of carotenoids and flavonoids, we hypothesize that there is no underlying regulatory mechanism based on transcriptional control of the biosynthetic pathways, but rather this phenomenon of inverse levels of carotenoid and flavonoid content is physicochemical in nature. Under higher light intensities without polytunnels, the light shielding properties of flavonoids are crucial to protect the plant from excessive UV light, while at lower light intensities under polytunnels the light must be used as efficiently as possible, which can lead to an increase in carotenoids as light harvesting compounds. The inverse relationship between carotenoids and flavonoids could thus be explained by their differing relative importance under different light intensities: high light protection/flavonoids and low light harvest/carotenoids. This is supported by our data, since the correlation is weaker for experiments conducted in months with lower solar radiation, leading to less flavonoid but higher carotenoid accumulation for effective light harvesting.

Further research could aim to elucidate the inverse relationship between the carotenoid and flavonoids by using a metabolic flux analysis with isotopically labeled compounds. Since mechanisms are species specific not only model plants, but also crop plants should be targeted.

4.4 Limitations and potential

There are certain limitations when studying the impact of protected cultivation systems. A balance must be found between greenhouse size and replicates as we discussed previously (Harbart et al., 2022). In this study, we evaluated the effects of solar radiation on selected bioactive compounds. We did not use supplementary artificial light, but this is likely to affect the performance of the antifogging additives: in this study no differences were observed when comparing both greenhouse covering materials, in contrast to our previous study using artificial light (Harbart et al., 2022). Moreover, the polytunnel and greenhouse conditions are not, or only semi-controlled cultivation conditions. This is reflected in the climatic conditions we recorded in experimental repetitions due to seasonal changes (Table 1). Photoperiods, DLIs and temperatures differed to some extent. Despite the experimental variations, similar results (tendencies) were found for most metabolites and changes in transcripts. Nevertheless, there were some differences. In particular, ABA content was different in the second experimental repetition: here no differences were observed between lettuce grown without or under polytunnels. We assume this is likely due to a rapid temperature increase a few days before harvesting (Figure S17). Conducting such experiments under controlled conditions as in a phytochamber is hardly possible due to the required size and number of polytunnels. However, limiting slightly varying conditions in the experimental repetitions would potentially make the observed effects more consistent and robust.

5 Conclusion

Bioactive compounds such as carotenoids and flavonoids have health-beneficial properties when integrated into the human diet.

Understanding the regulatory mechanisms of their biosynthetic pathways in differently cultivated horticultural crops is crucial for optimizing conditions for nutrient-rich crops. The aim of this study was to examine the effects of varying climatic conditions in protected cultivation in lettuce. Covering materials impacted light quality and quantity in close relationship to the temperature determined under the polytunnels compared to without polytunnels. Flavonoid contents decreased whereas carotenoid contents increased, showing an inverse correlation, although all antioxidants, the regulatory mechanisms responsible for their accumulation were found to be different. Flavonoid accumulation in lettuce appears to be predominantly regulated by solar UV light detection at a transcriptional level, whereas the carotenoid steady-state levels are regulated posttranscriptionally. In conclusion, the production of nutrient-rich horticultural crops has to be balanced between various influential factors favoring accumulation of health-beneficial compounds under protected cultivation, such as season, location or type and composition (e.g. UV- or red/far-red- blocking/UV- or red/far-red-transmissible) of agricultural films.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

VH and SB designed the research. VH accomplished experiments. HPLC analysis of ABA and evaluation was performed by VH with the help MF and RT-*q*PCR analysis was performed by VH with the help of KF. VH drafted the manuscript. Revision of the manuscript was done by MF, KF and SB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1124750/full#supplementary-material

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Supplementary Material

Supplementary Table S1. Parameters for flavonoid glycoside identification in lettuce. Compounds verified with authentic standards are marked with †.

Compound name	Retention time (min)	Ion	MS[1] *m/z*	MS[2] *m/z*	MS[3] *m/z*	Absorption maxima (nm)
Quercetin-3-glucuronide†	16.10	$[M-H]^-$	477	301	257 179	222 256 352
Quercetin-3-malonylglucoside†	19.56	$[M-H]^-$	549 505	463 301	257 179	256 354
Luteolin-7-glucuronide†	18.53	$[M-H]^-$	461	285		222 252 346

Identification based on the literature: Becker, C. et al., 2015: PLoS One 10, 11 e0142867, Llorach R et al., 2008: Food. Chem. 108(3), 1028-1038.

Supplementary Table S2. Parameters for carotenoid and chlorophyll identification in lettuce. Compounds verified with authentic standards are marked with †. Unless otherwise stated, carotenoids have *all-trans* stereochemistry.

Compound name	Retention time (min)	Ion	MS *m/z*	Absorption maxima (nm)
β-Carotene†	44.21	$[M+H]^+$	537.44	426 452 478
Lutein†	18.30	$[M+H-H_2O]^+$	551.43	420 445 472
Lactucaxanthin	16.21	$[M+H-H_2O]^+$	551.43	414 438 468
Zeaxanthin†	20.02	$[M+H]^+$	569.43	424 450 478
Antheraxanthin†	15.93	$[M+H]^+$	585.43	420 442 470
Violaxanthin†	9.52	$[M+H]^+$	601.42	414 438 468
Violaxanthin unidentified isomer	12.27	$[M+H]^+$	601.43	422 447
Neoxanthin (9-*Z*)†	10.74	$[M+H-H_2O]^+$	583.41	412 434 464
Neoxanthin unidentified isomer 1	7.94	$[M+H-H_2O]^+$	583.42	412 436 466
Neoxanthin unidentified isomer 2	8.59	$[M+H-H_2O]^+$	583.42	398 420 446
Chlorophyll a†	21.12	$[M+H]^+$	893.54	432
Chlorophyll b†	17.03	$[M+H]^+$	907.52	468

Phytoene (*E*/*Z*)†	20.59	$[M+H]^+$	545.51	n.d.

Identification based on the literature: Diop Ndiaye, N. et al., 2011: J. Agric. Food Chem. 59(22), 12018-12027, Britton, G. et al., 2004: Carotenoids: Handbook, Birkhäuser, Gopal et al., 2017: Food Funct. 8, 1124.

Supplementary Table S3. Primer pairs, amplicon size and experimentally determined amplification efficiency (E) used for the gene expression analysis with RT-qPCR.

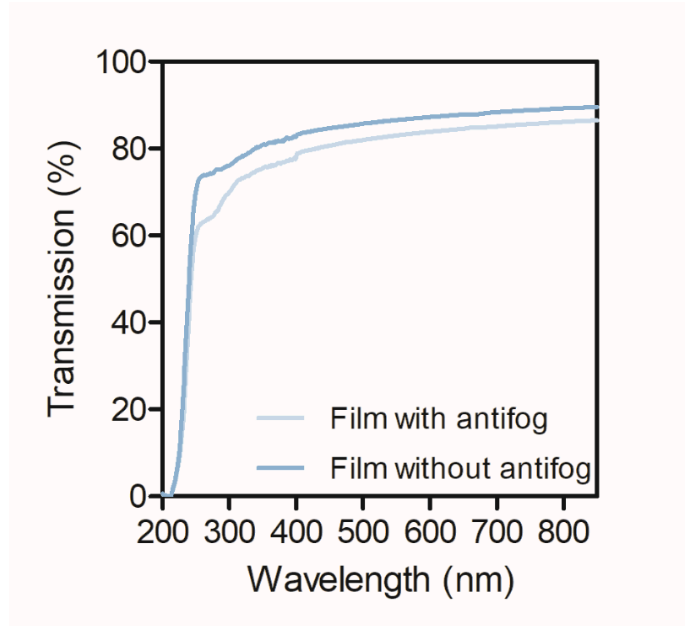
Protein (gene name)	**Primer pairs (5' to 3')**		**E (%)**	**Amplicon size (bp)**
Target genes				
Phytoene synthase (*PSY*)	F: TATTGTTATTATGTGGCTGGAAC	R: GCATCTTCTCCCACATCTCT	100	161
Carotenoid dioxygenase 4 (*CCD4*)	F: TGGTTTGAGGTGCCTGGATT	R: GATTGATCACAGGAAACTCCAA	100	241
Chalcone synthase (*CHS*)	F: TCAATGATAACAAAGCGATACATG	R: TGCTCCGGGCATGTCAAC	100	234
UVB-resistance 8 (*UVR8*)	F: CAGCAATCACTACTCCTCC	R: CAATTTGCCTTATTCGCAACC	92	356
Elongated hypocotyl 5 (*HY5*)	F: TTAAAGAAGGAATGGAAAGTGATG	R: CTCTCTCTTGCTTGTTGAGC	76	235
Phytochrome interacting factor 1 (*PIF1*)	F: GAAATCAGCAATGGGCGATG	R: CGTTATTATGAAGGTAGAGGTC	96	263
ORANGE protein (*OR*)	F: GGCTTGTGCTAGGTGTGCT	R: CTCGCCATAGCCATTCCTG	88	174
ORANGE-like Protein (*OR-like*)	F: TTCAGATGATTCAGCCGCGT	R: GTCAGGCATCTCGTTGTCC	100	253
Reference genes				
Actin 7 (*ACT7*)	F: AAGGATGCTTATGTAGGAGATG	R: TCTGTTTTCCTTGGGATTTAATG	77	201
Ubiquitin-conjugating enzyme E2 2 (*UBC2*)	F: ATGACACTCCTTGGGATGG	R: CAACATCATAGATTGGACTCC	81	177
Elongation factor 1-alpha (*EF1α*)	F: TGAACAAACGTTCATTCAAATACGC	R: CGATGACTGTGCAGTAGTAC	86	126

Supplementary Table S4. Gene Bank accession numbers of nucleotide sequences used for the primer design.

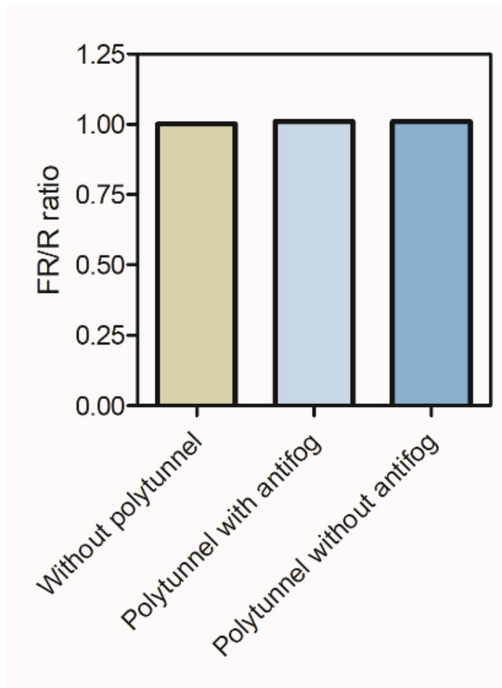
Gene name	Phytozome gene ID	GenBank accession number
PSY	Lsat_1_v5_gn_2_71880 (PAC:38961715)	XM_023911676.1
	Lsat_1_v5_gn_3_67861 (PAC:38918955)	XM_023907827.1
	Lsat_1_v5_gn_6_52200 (PAC:38938321)	XM_023886351.1
CHS	Lsat_1_v5_gn_2_76880 (PAC:38960353)	XM_023879789.1
	Lsat_1_v5_gn_2_42860 (PAC:38957201)	XM_023878437.2
UVR8	-	XM_023886847.2
HY5	Lsat_1_v5_gn_6_22420 (PAC:38939429)	XM_023879513.2
	Lsat_1_v5_gn_5_30441 (PAC:38970748)	XM_023903859.2
PIF1	Lsat_1_v5_gn_9_11161 (PAC:38926882)	XM_023877721.2
CCD4	Lsat_1_v5_gn_5_64880 (PAC:38963506)	XM_023895898.2
	Lsat_1_v5_gn_1_6181 (PAC:38942668)	XM_023877528.2
	Lsat_1_v5_gn_6_71300 (PAC:38940698)	XM_023876919.2
OR	-	XM_023888191.2
OR-like	-	XM_023878966.2
ACT7	Lsat_1_v5_gn_5_41421 (PAC:38969943)	XM_023911194.2
	Lsat_1_v5_gn_5_42341 (PAC:38970681)	XM_023911199.2
	Lsat_1_v5_gn_5_41281 (PAC:38964955)	XM_023878534.2
UBC2	Lsat_1_v5_gn_2_88820 (PAC:38961340)	XM_023917173.2
	Lsat_1_v5_gn_4_177020 (PAC:38934583)	XM_023902571.2
	Lsat_1_v5_gn_3_102101 (PAC:38915680)	XM_023888250.2
EF1α	Lsat_1_v5_gn_2_97861 (PAC:38962797)	XM_023895734.2
	Lsat_1_v5_gn_1_53981 (PAC:38947268)	XM_023910869.2
	Lsat_1_v5_gn_9_67180 (PAC:38923710)	XM_023891343.2
	Lsat_1_v5_gn_2_109480 (PAC:38962554)	XM_042899440.1

Phytozome database (https://phytozome-next.jgi.doe.gov/info/Lsativa_V8)

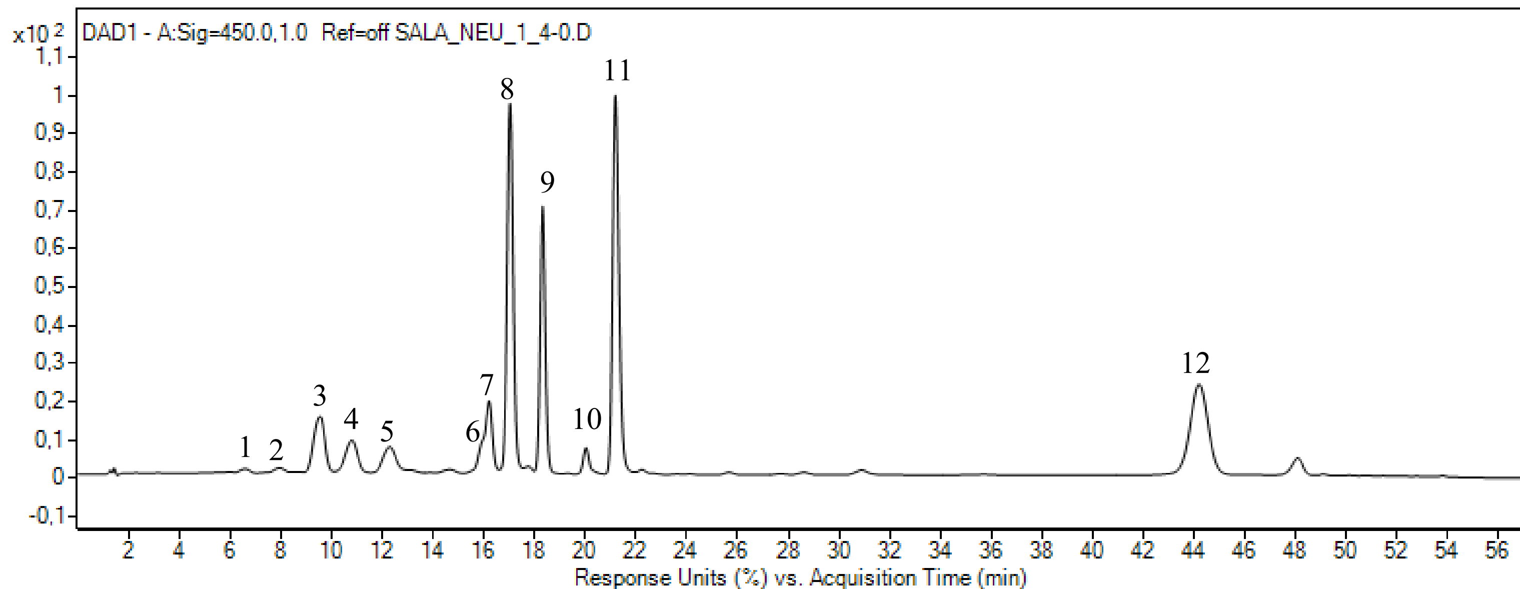
Supplementary Figure S1 Polytunnel with and without antifogging additives containing lettuce. (A) Lettuce grown without polytunnel; (B) Polytunnel with antifog; (C) Polytunnel without antifog.



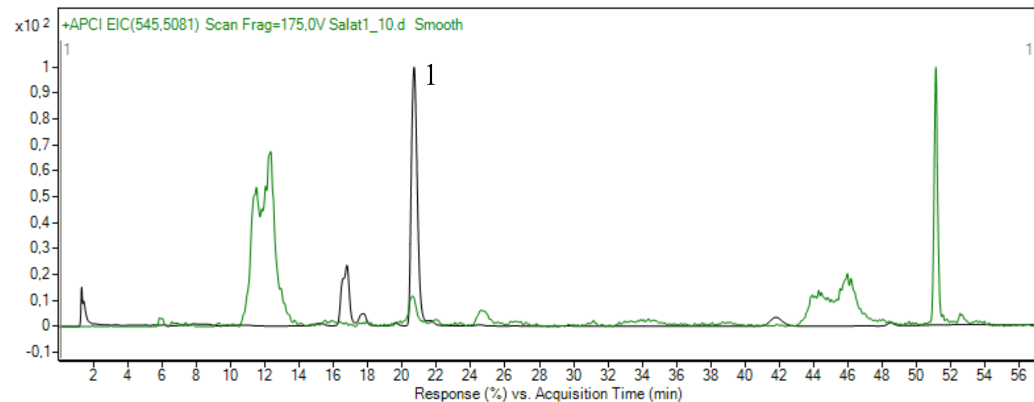
Supplementary Figure S2 The polytunnel material reduced certain light transmission. Light transmission spectra of polytunnel films with and without antifogging additives before use.



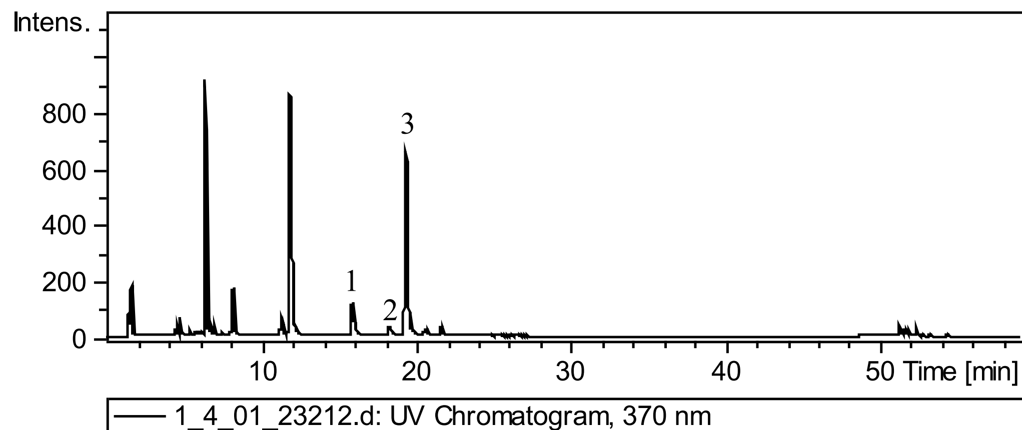
Supplementary Figure S3 The polytunnel material did not affect far-red to red light ratios. Far red (FR) to red (R) light ratio of polytunnel films were calculated based on UV/VIS spectral information before use (Figure S2, 655-665 nm and 725-735 nm).



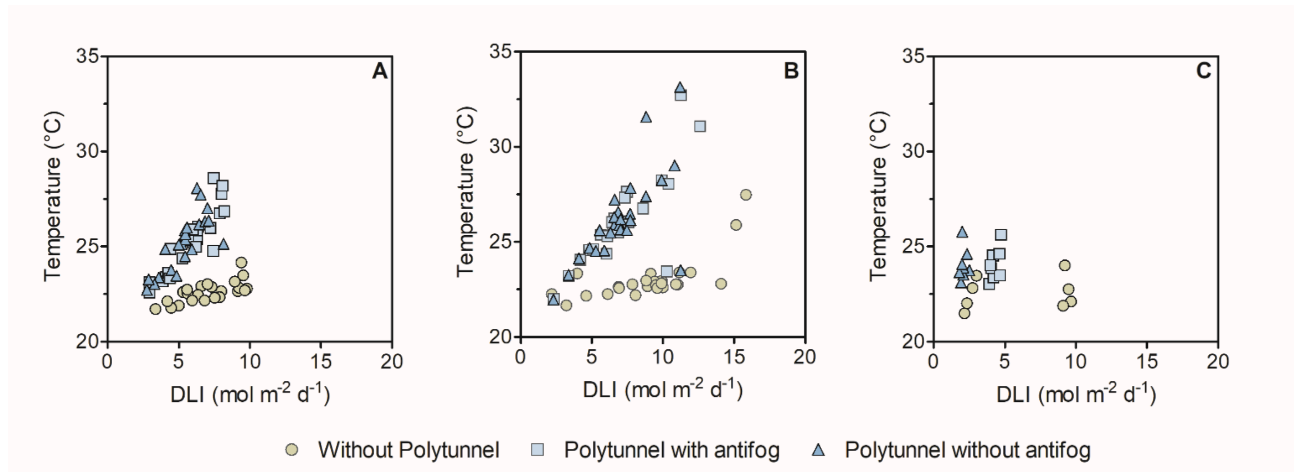
Supplementary Figure S4 Chromatogram of carotenoids and chlorophylls. Characteristic chromatogram (450 nm) of carotenoids and chlorophylls in lettuce grown without polytunnels detected with HPLC-DAD-ToF-MS. Unless otherwise stated, carotenoids have *all-trans* stereochemistry. (1) Unidentified neoxanthin isomer 1; (2) Unidentified neoxanthin isomer 2; (3) violaxanthin; (4) neoxanthin (9-*Z*); (5) violaxanthin unidentified *cis*-isomer; (6) antheraxanthin; (7) lactucaxanthin; (8) chlorophyll b; (9) lutein; (10) zeaxanthin; (11) chlorophyll a; (12) β-carotene.



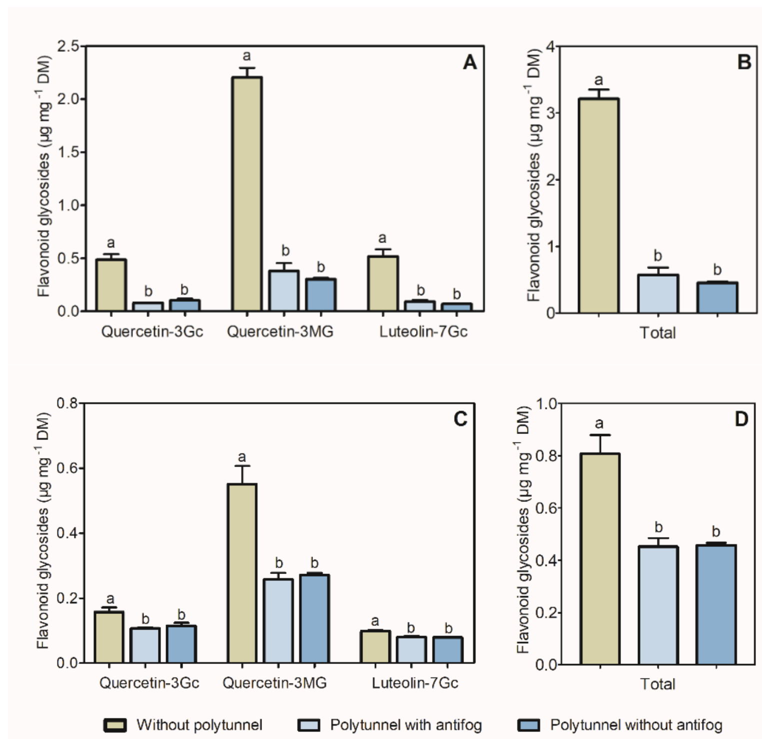
Supplementary Figure S5 Chromatogram of phytoene. Characteristic DAD- (280 nm; black) and EIC (*m/z* 545.5081; green) chromatogram of phytoene in lettuce grown without polytunnels detected with HPLC-DAD-QToF-MS. (1) Phytoene isomer.



Supplementary Figure S6 Chromatogram of flavonol glycosides. Characteristic chromatogram (370 nm) of flavonol glycosides in lettuce grown without polytunnels detected with HPLC-DAD-MS^3. (1) Quercetin-3-glucuronide; (2) Luteolin-7-glucuronide 2; (3) Quercetin-3-malonylglucoside.

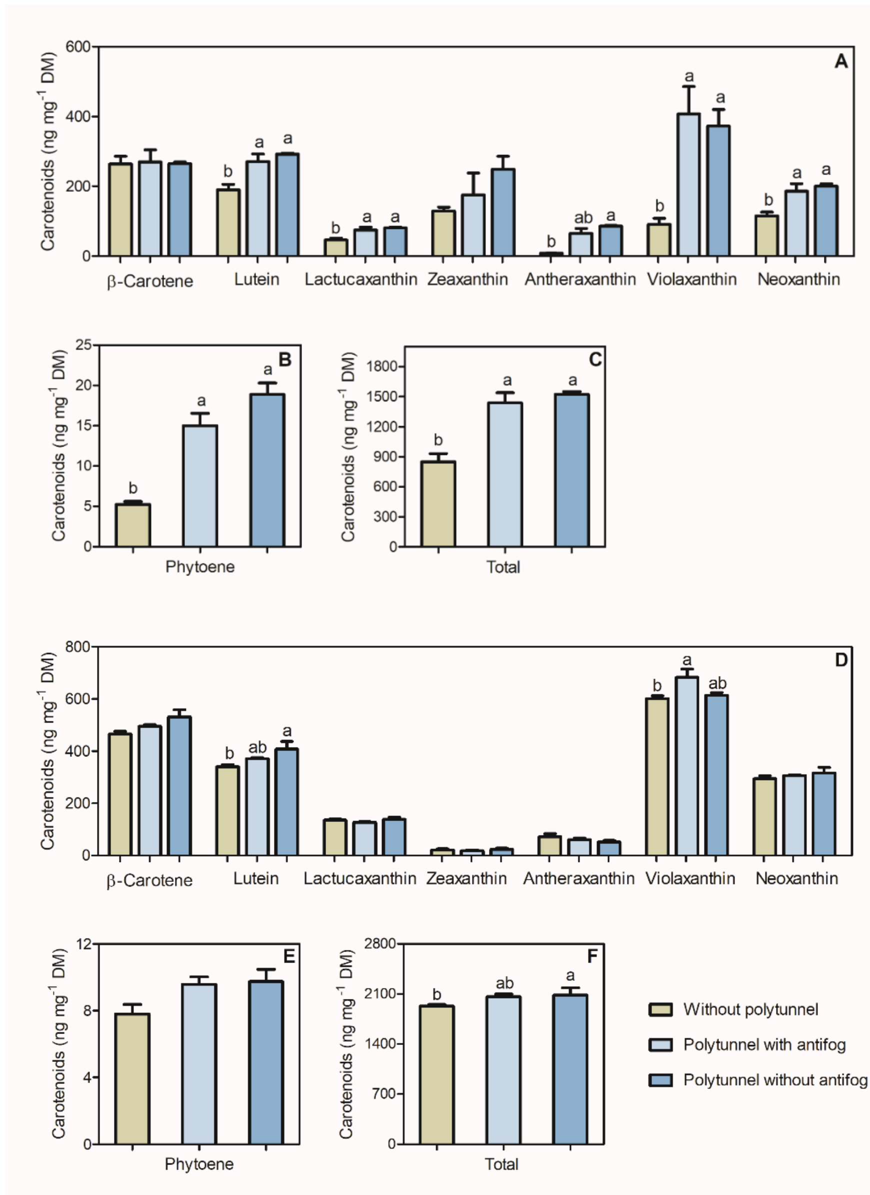


Supplementary Figure S7 Relationship between light and temperature due to protected cultivation. Comparisons between cultivation without and with polytunnels. Shown are daily averaged values of the three independent experimental repetitions in (A) April, (B) May, and (C) September.

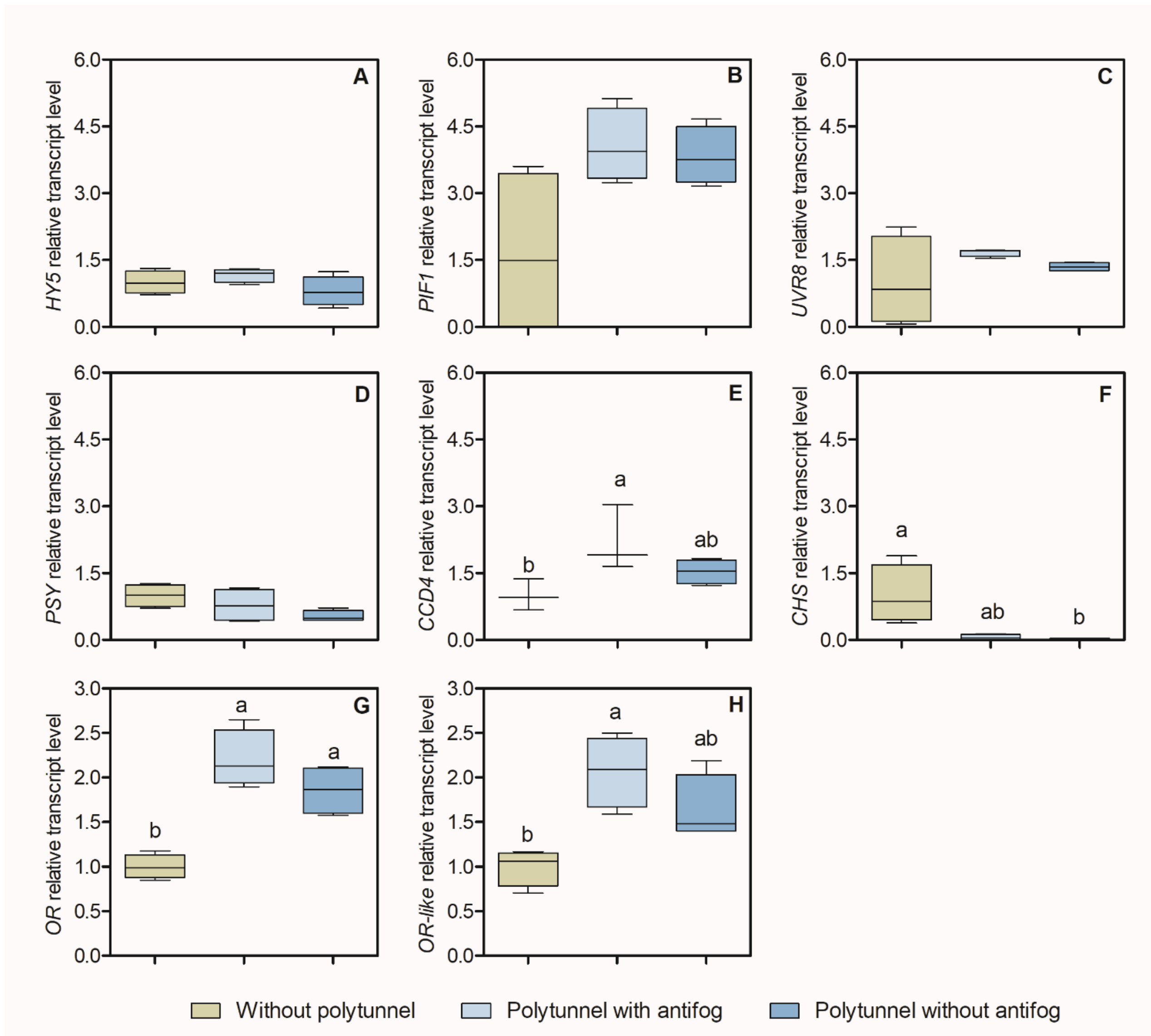


Supplementary Figure S8 Lower flavonoid content in lettuce grown under polytunnels. (A,B) The second, and (C,D) third experimental repetition in May and September are shown. (A,C) Individual, and (B,D) total content of flavonoid glycosides (µg mg^{-1} DM) in lettuce grown without and under polytunnels with and without antifogging additives. The data are expressed as mean ± SE

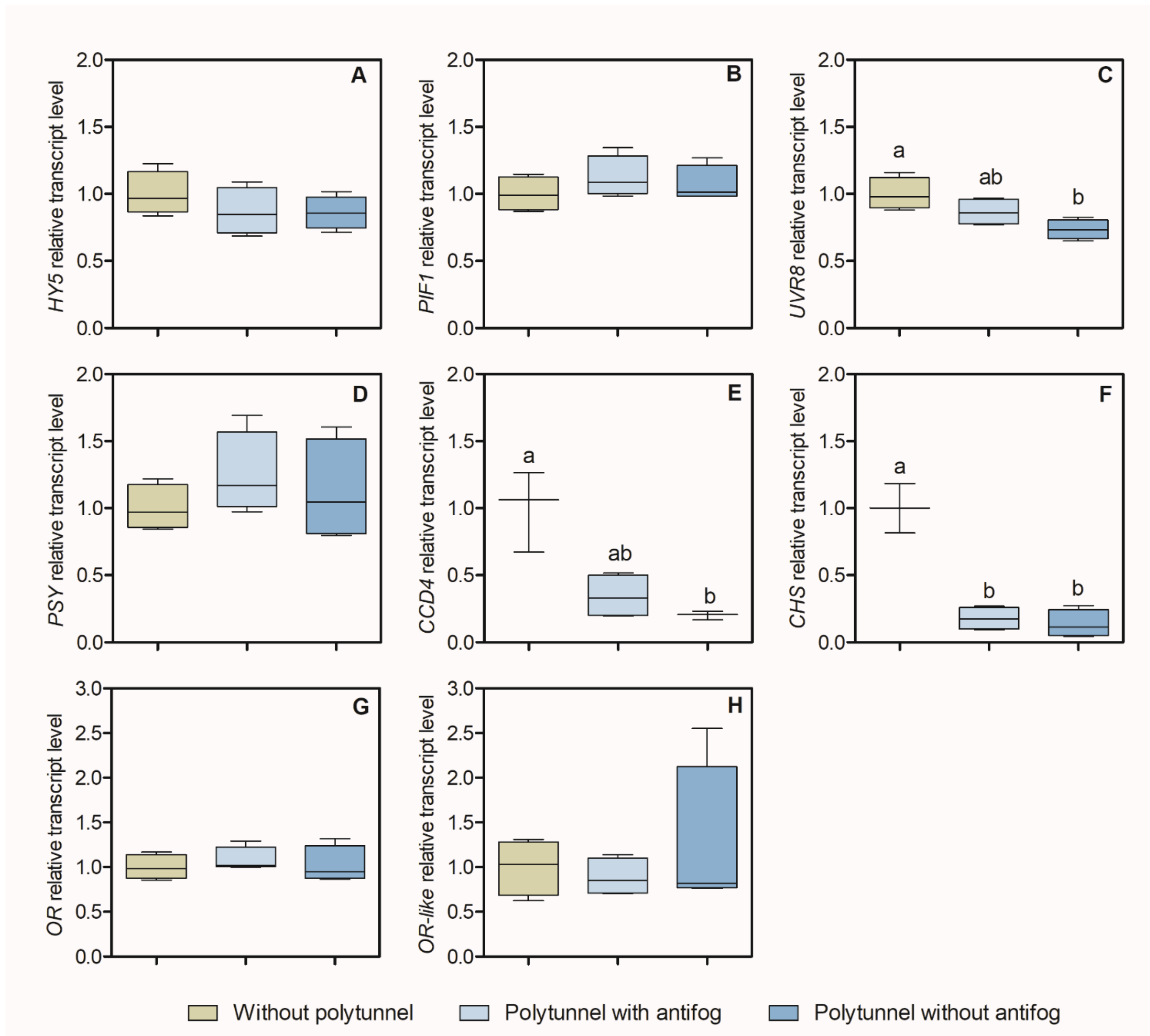
(n = 4). Significant differences ($p \leq 0.05$) of individual and total compounds are indicated by different letters. Gc, glucuronide; MG, malonyl glucoside.



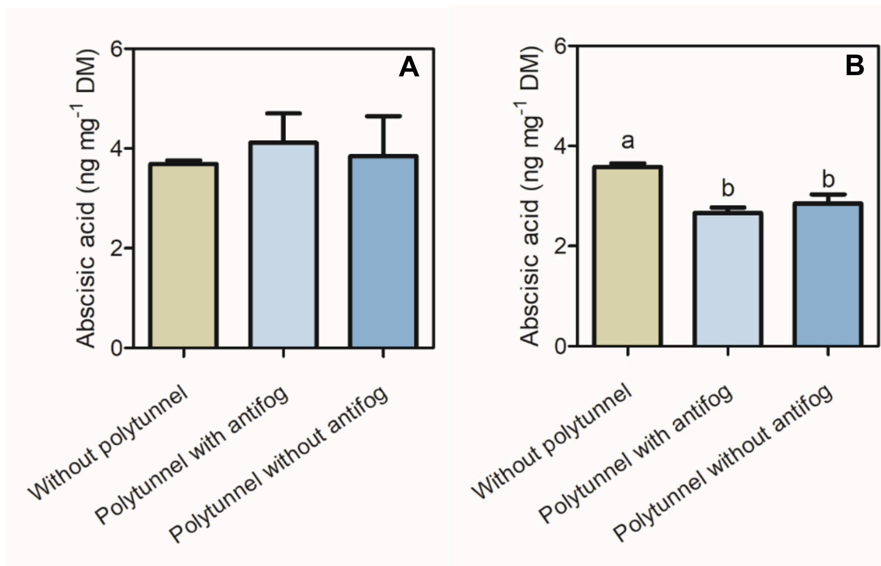
Supplementary Figure S9 Total carotenoid content affected by polytunnel cultivation. Carotenoid content (ng mg^{-1} DM) in lettuce grown without or under polytunnels with and without antifogging additives. (A,D) Individual carotenoids β-/ε-branch, (B,E) downstream upper pathway metabolite phytoene, and (C,F) total carotenoids. Second (A-C) and third (D-F) experimental repetition in May and September are shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) of individual and total compounds are indicated by different letters; no letters indicate absence of significance.



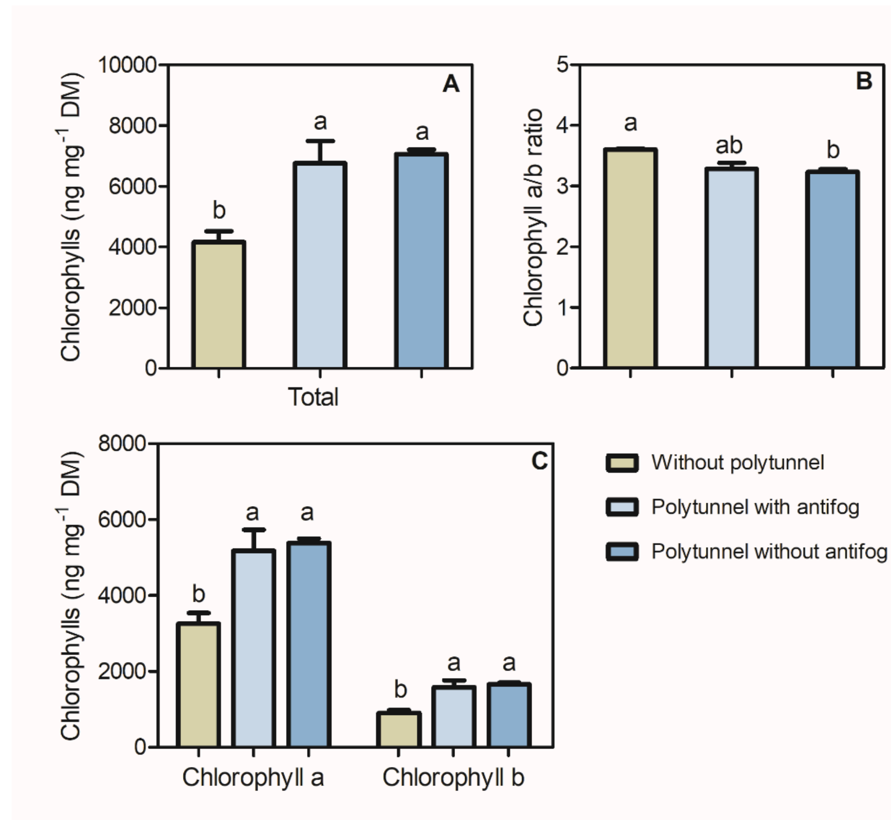
Supplementary Figure S10 Gene transcripts for key enzymes of the core carotenoid and flavonoid biosynthesis pathways. Transcript levels of (A) *HY5*, (B) *PIF1*, (C) *UVR8*, (D) *PSY*, (E) *CCD4*, (F) *CHS*, (G) *OR*, and (H) *OR-like* in lettuce grown without or under polytunnels with and without antifogging additives. The second experimental repetition in May is shown. The data are expressed as Box-Whisker-Plots (n = 4), Whiskers show maximal and minimal values. Data was normalized to lettuce grown without polytunnels. Different letters indicate significant differences ($p \leq 0.05$) of transcripts under different cultivation conditions; no letters indicate absence of significance. *HY5*, Elongated hypocotyl5; *PIF1*, Phytochrome interacting factor1; *UVR8*, Ultraviolet resistance locus8; *PSY*, Phytoene synthase; *CCD4*, Carotenoid cleavage dioxygenase4; *CHS*, Chalcone synthase; *OR*, Orange protein; *OR-like*, Orange-like protein.



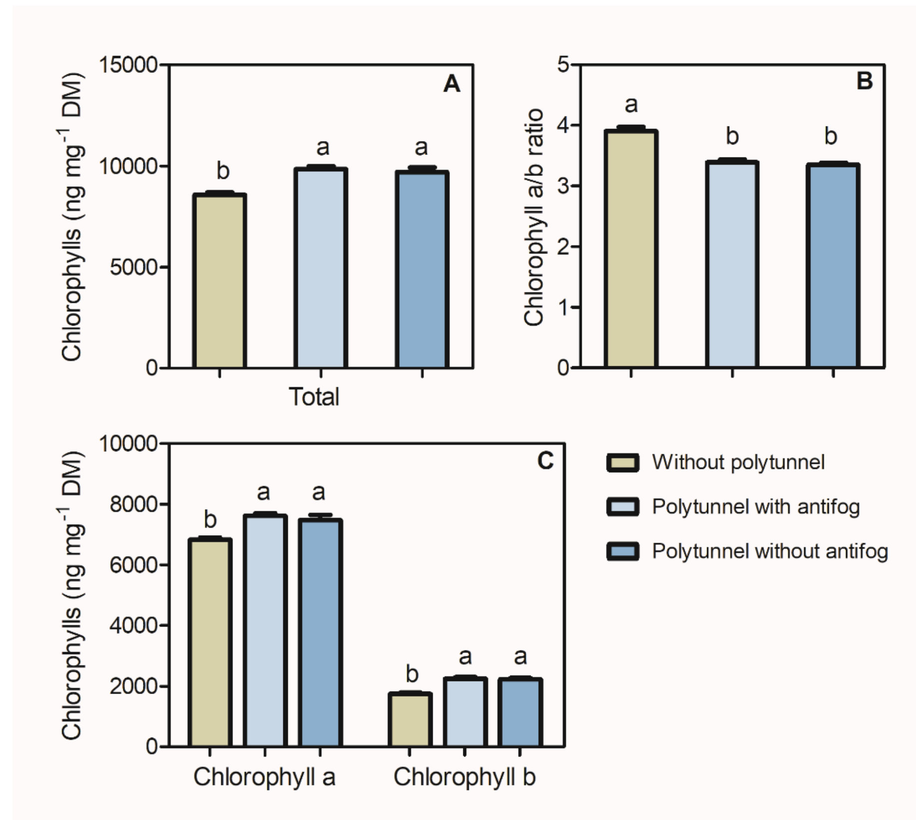
Supplementary Figure S11 Selected gene transcript for key enzymes of the carotenoid and flavonoid biosynthesis pathways. Transcript levels of (A) *HY5*, (B) *PIF1*, (C) *UVR8*, (D) *PSY*, (E) *CCD4*, (F) *CHS*, (G) *OR*, and (H) *OR-like* in lettuce grown without or under polytunnels with and without antifogging additives. The third experimental repetition in September is shown. The data are expressed as Box-Whisker-Plots (n = 4), Whiskers show maximal and minimal values. Data was normalized to lettuce grown without polytunnels. Different letters indicate significant differences ($p \leq 0.05$) of transcripts under different cultivation conditions; no letters indicate absence of significance. *HY5*, Elongated hypocotyl5; *PIF1*, Phytochrome interacting factor1; *UVR8*, Ultraviolet resistance locus8; *PSY*, Phytoene synthase; *CCD4*, Carotenoid cleavage dioxygenase4; *CHS*, Chalcone synthase; *OR*, Orange protein; *OR-like*, Orange-like protein.



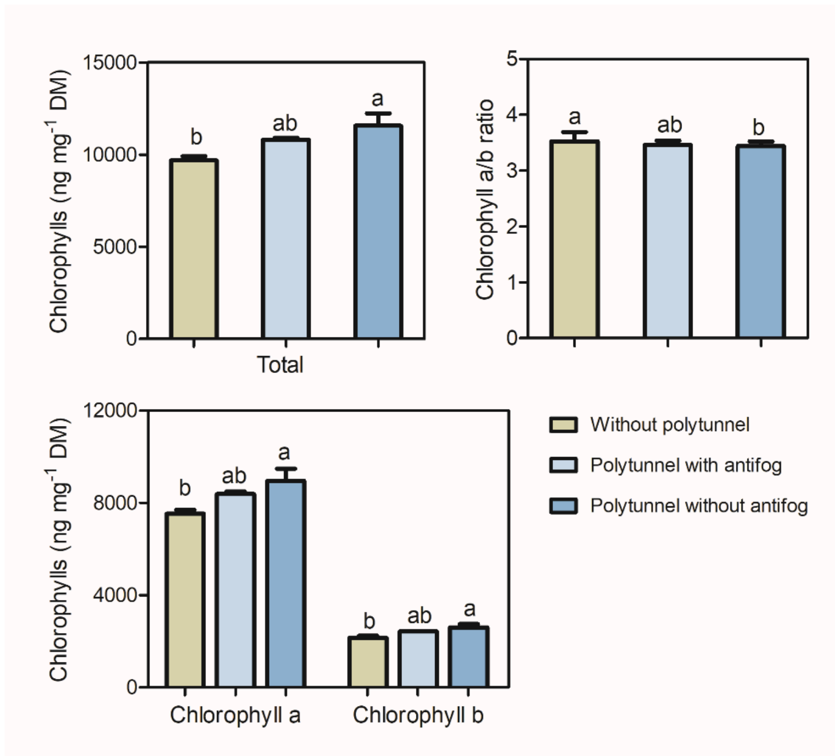
Supplementary Figure S12 Phytohormone ABA content in lettuce. Abscisic acid content (ng mg^{-1} DM) in lettuce grown without or under polytunnels with and without antifogging additives. The second (A, May) and third (B, September) experimental repetitions are shown. The data are expressed as mean ± SE (n = 4). Different letters indicate significant differences ($p \leq 0.05$), no letters indicate absence of significance.



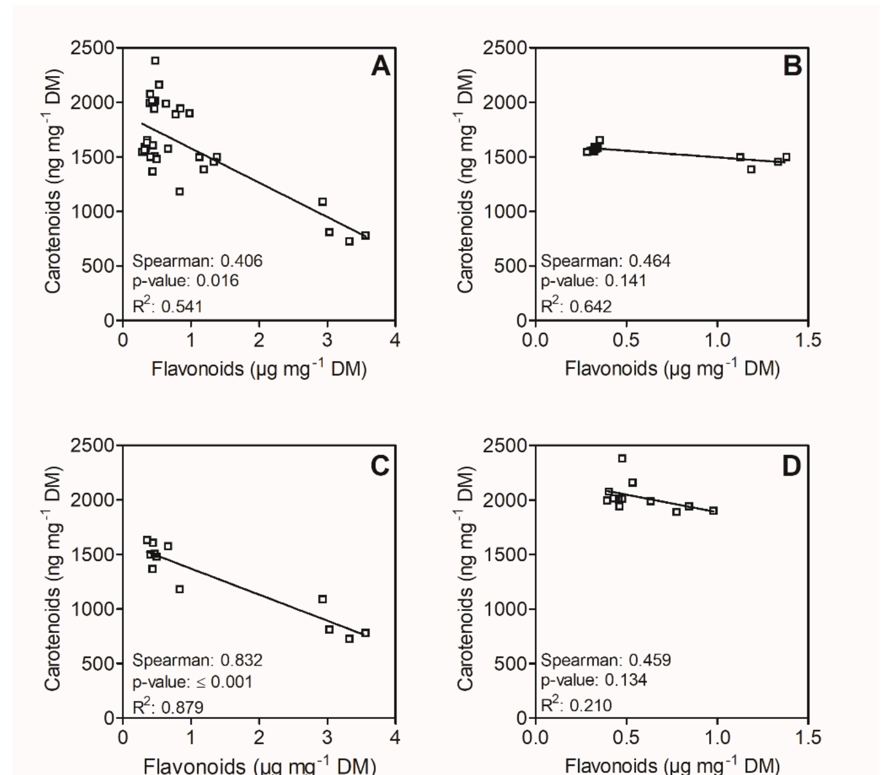
Supplementary Figure S13 Chlorophyll a and b contents. (A) Total, and (C) individual chlorophyll content (ng mg^{-1} DM), and (B) chlorophyll a/b ratio in lettuce grown without and under polytunnels with and without antifogging additives. The first experimental repetition in April is shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) are indicated by different letters.



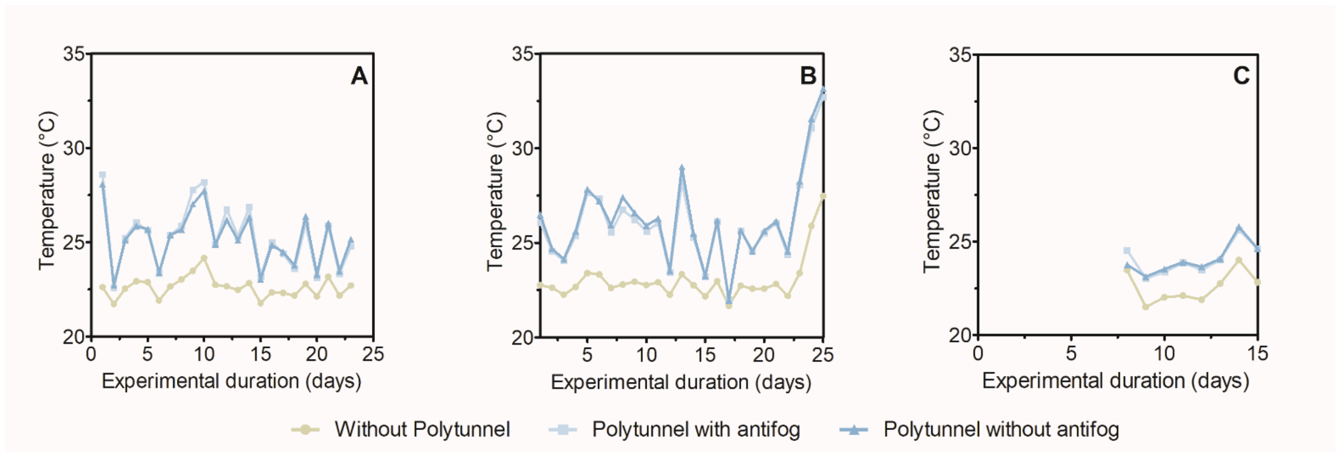
Supplementary Figure S14 Chlorophyll a and b contents. (A) Total, and (C) individual chlorophyll content (ng mg^{-1} DM), and (B) chlorophyll a/b ratio in lettuce grown without and under polytunnels with and without antifogging additives. The second experimental repetition in May is shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) are indicated by different letters.



Supplementary Figure S15 Chlorophyll a and b contents. (A) Total, and (C) individual chlorophyll content (ng mg^{-1} DM), and (B) chlorophyll a/b ratio in lettuce grown without and under polytunnels with and without antifogging additives. The third experimental repetition in September is shown. The data are expressed as mean ± SE (n = 4). Significant differences ($p \leq 0.05$) are indicated by different letters.



Supplementary Figure S16 Correlation analysis of carotenoids and flavonoids. The data represents lettuce grown with protected cultivation, with and without polytunnels (total of n = 12 per repetition). (A) Experimental repetitions 1-3 together, (B) repetition 1 (April), (C) repetition 2 (May), and (D) repetition 3 (September). Because assumption of normal distribution was violated, Spearmans correlation coefficients were determined.



Supplementary Figure S17 Daily average temperatures. Daily temperatures averaged in (A) the first (April), (B) second (May), and (C) third experimental repetition (September) for lettuce grown without or under polytunnels with and without antifogging additives. Due to a malfunction of the temperature sensor, the temperature in the third experiment was recorded starting from the middle of the experiment.

DISCUSSION

1 Use of antifogging additives, a food safety, quality or environmental issue?

Polymer additives are ubiquitous in plastics. More than 5,500 of structurally diverse chemicals are used in plastics[21], and the migration and leaching of many of these additives is potentially problematic.

This was the first time that antifogging additives for greenhouse films have been characterized based on their fatty acid profiles by GC-MS, outside of a polymer matrix. The fatty acid approach was chosen because there are a large number of structurally different commercial antifogging additives, which are UVCBs. It allowed the successful determination of three structurally distinct sorbitan-based, glycerol-based and stearyldiethanolamine-based additives (Publication 1, Fig. 2). This approach was then used for simulation experiments to investigate the fate of intentionally added antifogging additives on lettuce leaves (cv. Attractie) and in soil (Publication 1, Fig. 3). With respect to plastic additives that are used in greenhouse films (and other products), there are three main questions that arise.

(1) What chemical compounds are added to the plastics?
(2) Which and how many compounds are migrating and leaching?
(3) What is the fate of the compounds that are leached out?

1.1 The chemical diversity of compounds added into plastics

A recent study identified approximately 10,547 compounds intentionally added to plastics for various applications (55 % of them were additives).[21] However, the authors noted that there is no centralized and transparent database of compounds added to plastics and their uses. In another study, hexane extracts of 120 plastic food contact materials (FCM) were analyzed in a GC-MS screening approach and over thousands of substances were detected of which only 90 compounds were identified.[85] Since the chemical identity of plastic additives is somewhat ambiguous and antifogging additives are UVCBs, we first characterized the antifogging additives based on their fatty acid profiles after saponification in reference material. In order to be able to identify and assign the source additives, this was deliberately not done after extraction from the polymers. The detected fatty acid composition alone suggests more than the main component stated by the manufacturer (Publication 1, Fig. 2). Instead, the characterized additives contained two (Sabostat A 300, Sabofog MS P) to three (Atmer 1440) major fatty acids along with other minor compound fatty acids (< 5 %).

In general, plastic additives can be analyzed by non-specific (gravimetric), targeted and untargeted approaches using either LC-MS or GC-MS.[86] Leaching studies, focus on soft extraction methods that mimic realistic "in-use" exposure conditions, while extraction studies include hard extraction techniques to capture all compounds in the material.[86] In order to study the simulated leaching of antifogging additives, a prior characterization of their chemical composition is useful. However, untargeted analysis can be an adjunct for leaching studies of plastics with unknown additive composition.

1.2 Migration and leaching of antifogging additives from agricultural films

Although migration and leaching are undesirable for the majority of plastic additives[17], this is in fact the mode of action of antifogging additives. In detail, a concentration gradient of additives to the surface is achieved and maintained by the dissolution of a portion of the additives into the aqueous surface phase (**Fig. 2**). However, leaching studies of antifogging additives from greenhouse films were not the focus of this thesis, as this was simulated. Nevertheless, environmental conditions can affect their migration in greenhouse films. For example, a constant decrease in the concentration of two antifogging additives (glycerol- and stearyldiethanolamine-based) in LDPE films was observed over time in a hot (~ 40 °C) greenhouse environmental simulation.[87] Another issue to consider is aging and weathering, which can affect additive leaching and release.[19] The additive release does not automatically constitute a hazard, but requires examination. Bridson, Gaugler [86] summarized methods already applied, e.g. for FCM or medical devices, and evaluate their applicability to plastic pollution in the environment. As there are no such regulations for agricultural plastics, the release of additives is not covered. Therefore, the methods presented may be of interest for this application.

In this thesis, the leaching of antifogging additives from greenhouse films was simulated by spraying them on lettuce leaves. Their major fatty acids were determined, indicating that the additives adhered to the leaves (24 h after treatment). Moreover, washing the treated leaves with water or hexane after harvest did not effectively remove the additives from the leaves (Publication 1, Fig. 4).

1.3 Do antifogging additives from agricultural films pose a human or environmental risk?

The risk of polymer additives is a function of the degree of exposure and the chemical hazard. To date, the risk of additives has been considered low because the release to the environment post-manufacture was assumed to be negligible.[19]

However, antifogging additives leaching from greenhouse films could contaminate vegetables grown underneath and their non-removability may be an issue. The antifogging additives characterized in this thesis, Sabofog MS P ("sorbitan monostearate") and Atmer 1440 ("glycerol monooleate"), are both approved for use in FCM in the EU with migration limits of 60 mg kg^{-1} food and 10 mg dm^{-2}, respectively.[22] Moreover, the main components of these additives, such as sorbitan stearate or palmitate and mono- and diglycerides of fatty acids, are permitted to be used as emulsifying food additives.[88] Even if the sorbitan and glycerol esters indicate a non-toxic potential of the additives, industrial raw materials are not purified and may contain unknown and toxicologically relevant substances.

Furthermore, antifogging additives leaching from greenhouse films could be released into the environment. The antifogging additives Sabofog MS P and Atmer 1440 are both reported to be readily biodegradable.[89,90] In contrast, Sabostat A 300 ("stearyldiethanolamine monostearate") may be bioaccumulative.[91] Antifogging additives are generally used in low quantities (0.1 to 4 % and Publication 2, 0.35 % in LDPE/EVA films).[92-94] However, Barrick, Champeau [19] pointed out that in chemical terms, weight percent is a high concentration, and the magnitude of the additives is greater than that of other anthropogenic compounds adsorbed to plastics in the environment. In addition, some plastic additives (bisphenol A, phthalates, and some antioxidants) have been identified as PBT (persistent, bioaccumulative, toxic) compounds.[19] Since the chemical identity of additives is often uncertain, their release, transformation and degradation in the environment can only be estimated to a limited extent.

Data from five European countries show that only 50 to 84 % of end-of-life agricultural plastics are collected and plastics may deposited in the environment.[14] Antifogging additives may still be present in the bulk of the polymer[87] and therefore in this end-of-life plastic. Microplastics generated from such materials can act as carriers for plastic additives, and Barrick, Champeau [19] point to plastic additives as a previously overlooked source of microplastic ecotoxicity. Additives in microplastics have been shown to be more toxic to fauna than the plastic particles themselves.[95] In addition, surfactant additives may promote the transport of microplastic particles,[95,96] which could be particularly important for non-biodegradable additives such as Sabostat A 300. In recent years, the adsorption of environmental pollutants such as heavy metals, halogens or (persistent) organic chemicals on microplastics has received more research attention.[97] Although the mechanisms of adsorption/desorption in different environments (water, air, soil) are poorly understood,[97] it can be speculated that surface-adherent additives (especially surfactant additives) may influence these processes.

In this thesis, plant physiological parameters and metabolites were determined after simulated leaching of the additives on lettuce leaves. This had negligible effects in the short experimental period of 24 h (Publication 1, Fig. 5); however, long-term exposure is of interest. Finally, the FAO assessment of agricultural plastics concluded that greenhouse films pose a moderate risk to the environment compared to, for example, polymer-coated fertilizers.[14]

1.4 Future perspectives

As protected cultivation is considered important for food security and adaptation to climate change, future research efforts should address issues related to additives used in plastics. In this thesis, negligible risks to humans and the environment were suggested by the antifogging additives based on the evaluated parameters. However, bioaccumulation in soil and thus in the environment is a concern, especially with respect to the non-removability of the antifogging additives or a possible uptake into plants. Moreover, it may be of great interest to evaluate the effects on plant growth and metabolism on soils contaminated with antifogging additives. Future research could shed light not only on known hazardous additive compounds, but also on those with a high potential for leaching due to their mode of action.

In addition to antifogging additives, the post-manufacturing release of plastic additives needs to be further elucidated. This may include the characterization and the establishment of a comprehensive database to provide an overview of additives used in polymers, the evaluation of leaching potentials, especially in greenhouse environments, or their potential environmental degradation.

2 Cover materials alter cultivation conditions and lettuce quality

The use of protective covers and the choice of cover materials affect the growing conditions of crops. This has implications for crop yield and nutritionally valuable compounds such as plant secondary metabolites. In addition to genetic factors, climatic conditions are expected to have a larger influence on these metabolites than nutrient and water management.[98] In this thesis, the lettuce cultivars Attractie (Publication 2) and Veronique (Publication 2, 3) were grown under polytunnels covered with a three-layer LDPE/EVA film with or without incorporated antifogging additives. Lettuce was also grown without the polytunnel in order to evaluate its effect in general. Experiments were conducted in 2019, 2020 and 2021 in spring (Feb., April, May) and autumn seasons (Sept., Oct.).

2.1 Impact of cover materials on polytunnel and greenhouse microclimate

Protected cultivation using a polytunnel generally increased temperature and relative humidity by 1.11-fold and 1.56- to 1.91-fold, respectively. However, even the choice of cover material may affect the temperature underneath. The use of antifogging additives is associated with the prevention of heat absorption by water droplets, which may lead to an increase in greenhouse temperatures.[23] However, similar temperatures were observed under the polytunnels regardless of the antifogging additives used in this thesis. **Table A** (Appendix) summarizes selected literature dealing with the cultivation of vegetables under different cover materials (also compared to the open field) and their effects on climatic conditions and metabolites. Even in these studies, cover materials have little effect on temperature. Only a double-layer PE and an IR-absorbing PE film led to higher temperatures (1.30- and 1.26-fold compared to outside) than a single-layer PE (1.13-fold compared to outside).

Besides temperature, the covers and cover materials did not significantly modify air humidity (expressed as vapor-pressure deficit or relative humidity). In this thesis, humidity did not differ under polytunnels with and without antifogging additives. However, a greater difference between outdoor and protected cultivation was found compared to other studies. This may be due to the reduced polytunnel dimensions in our model. High humidity can have adverse effects on plants due to reduced transpiration rates, such as heat damage or reduced ion transfer rates from the root to the shoot.[28] In contrast, it was discussed that plant growth is best at higher humidity levels as long as transpiration is sufficient to support physiological functions. Lettuce grown under polytunnels had higher transpiration rates (Publication 2, Fig. S4).

Furthermore, high humidity can promote the spread of plant pathogens.[28] In particular, antifogging additives are used to prevent such contamination by reducing dripping on the plants. However, these effects may not be captured in the short lettuce growing periods (~ 1 month) in this thesis.

Light regime is another factor influenced by cover materials. According to the studies in **Tab. A** (Appendix), differences between the cover materials are evident for photosynthetic photon flux density (PPFD), but also for specific wavelengths. Here, UV transparent and blocking materials are the focus of research. Conventional PE greenhouse films transmit approximately 80 % of the visible light.[27] In general, the PPFD has been reduced up to 0.37-fold by using covers (PE with shading nets). In this thesis, the LDPE/EVA polytunnel covers reduced the PPFD as well as the UVA and UVB transmission (Publication 3, Fig. 2). Furthermore, the use of antifogging additives results in higher PPFD but not UV transmission compared to films without antifogging additives. PPFD under polytunnels with and without

antifogging additives differed by 1.28-fold in Oct. 2019 and 1.20-fold in Feb. 2020 (Publication 2, Tab. 1). The daily light integral (DLI), defined as the total daily amount of photons reaching the plant (PPFD x photoperiod), is a parameter used for recommendations of the light requirements of a vegetable. For example, leafy vegetables require a constant DLI throughout the growing season, while tomatoes have varying DLI requirements depending on the growing or fruiting stage.[29] An optimum DLI of 12 to 14 mol $m^{-2} d^{-1}$ is suggested for lettuce cultivation.[99] The DLI outside the greenhouse was close to this optimal range in the experiments (**Tab. B**, Appendix), however, the PPFD reaching the lettuce was low and thus the DLI (Publication 2 and 3, both Tab. 1). Although differences in light regimes due to cover materials were found, they are highly dependent on the time of cultivation. This was shown in the literature (**Tab. A**, Appendix), but also in this thesis (Publication 2 and 3, both Tab. 1).
By providing a substrate for photosynthesis, carbon dioxide concentrations are known to affect plant growth. One study (**Tab. A**, Appendix) reported minor differences (between 0.99- and 1.05-fold) in carbon dioxide concentrations between greenhouses covered with three different materials. Becker and Kläring [100] observed higher yields and flavonoid glycoside concentrations (~ 1.5-fold) in two red lettuce cultivars grown at elevated carbon dioxide concentration (1000 ppm compared to 200 ppm). Carbon dioxide concentrations between 468 ppm and 488 ppm have been determined under the polytunnels and without polytunnels (**Fig. A1**, Appendix). With respect to Becker and Kläring [100], these minor differences are assumed to have a negligible impact.
Taken together, temperature and light regime are important factors that can be influenced by protected cultivation. However, light seems to be the most important factor in terms of cover materials, as the temperature varies only slightly. Both have the potential to affect the yield and quality of vegetables in protected cultivation.

2.2 Yield and quality of polytunnel grown lettuce

To evaluate the influence of polytunnel cover material on biomass production, the lettuce yield was determined as post-harvest fresh weight (g per plant). Fresh weight did not differ between lettuce grown under polytunnels with and without antifogging additives (except Feb. 2020, Publication 2, Fig. 1). However, the use of polytunnels increased the fresh weight up to 1.55-fold in Oct. and Feb., but decreased it up to 0.71-fold in April and May. As shown in the literature, cover materials affect vegetable yields (**Tab. A**, Appendix), depending on the PPFD underneath the films, but also on its UV transparency. Generally, it can be found that the more PAR and less UV radiation the film transmits, the higher the yield. Moreover, it has been shown

that lettuce fresh weight is higher at higher DLI.[101,102] Since higher DLI but lower fresh weight was determined in the April, May than in the Oct. and Feb. experiments described in this thesis, prevailing temperatures under polytunnels may be a limiting factor.

The term vegetable quality has several meanings depending on the product and consumer perspective. According to the International Organization of Standardization (ISO), quality is the "sum of all characteristics, properties and attributes of a product or commodity which is aimed to fulfilling the established or presumed customer requirements" (ISO 8402, 1989). This includes intrinsic (shape, flavor and ingredients) and extrinsic (packaging, price and brand) quality characterisits.[103] The EU regulates market value with official quality grades and standards. For lettuce, these include color, shape, freshness, and freedom from physiological disorders and defects.[104] However, this does not include other parameters discussed in the scientific literature, such as ecological value, or nutritional and health value. This thesis will focus on selected plant secondary metabolites including carotenoids, flavonoids, chlorophylls and caffeic acid derivatives as examples reflecting the nutritional quality of vegetables in protected cultivation and will use the term quality in this specific meaning.

While total carotenoid and chlorophyll levels in lettuce varied by time of cultivation, total flavonoids and caffeic acid derivatives grown under polytunnels were less variable (Publications 2, 3). The total carotenoid content ranges from 762.98 to 2085.43 ng mg^{-1} DW (39.24 to 119.39 ng mg^{-1} FW; **Tab. C**, Appendix). Kim, Shang [47] screened total carotenoids in different types of lettuce (crisphead, romaine, and oak leaf) and determined values ranging from 54.4 to 129.8 ng mg^{-1} FW. Mou [48] determined the content of two major carotenoids, lutein and β-carotene, in different butterhead cultivars. These varied between 154 to 682 ng mg^{-1} DW (lutein) and 191 to 802 ng mg^{-1} DW (β-carotene), which are in agreement with the carotenoid contents in this thesis. The values differed according to the time of cultivation in summer (April to July) or autumn (July to Oct.). Furthermore, total flavonoids in lettuce varied from 0.320 to 3.210 µg mg^{-1} DW (0.015 to 0.191 µg mg^{-1} FW, **Tab. C**, Appendix). In green lettuce cultivars, Llorach, Martínez-Sánchez [70] found total flavonoids between 0.011 and 0.239 µg mg^{-1} FW, while they were higher in red lettuce cultivars. Thus, lettuce is a good source of carotenoids and flavonoids, and values are comparable to other leafy vegetables like spinach or kale (**Tab. 1**).[46] Nevertheless, they differ due to cultivation conditions and time.

2.3 Do cover materials affect phenolic compounds?

Flavonoids and phenolic acids are highly dependent on the UV light regime. As shown in **Tab. A** (Appendix), UV transmitting materials or open field resulted in higher levels of total phenolic compounds as well as flavonoids in lettuce cultivars and rocked salad, up to 3.86-fold compared to UV blocking materials. Flavonoids in lettuce are not affected by the use of antifogging additives in polytunnel covers, probably because of similar UV light environment. Although it has been shown that low PPFD (225 to 400 µmol m^{-2} s^{-1}) results in low flavonoids in lettuce[78], small differences in PPFD due to the use of antifogging additives in polytunnels are probably not sufficient to cause differences in lettuce flavonoids. However, caffeic acid derivatives (individual and total) were affected in lettuce Veronique (Publication 2, Tab. S4), although these were different in the two experiments and no pattern was apparent. In contrast, no effects were observed for lettuce phenolic acids exposed to 225 and 400 µmol m^{-2} s^{-1}, which the authors suggested could be due to the small difference in PPFD.[78]

2.4 Do cover materials affect photosynthetic pigments?

The evidence of a cover material effect is less conclusive for carotenoids and chlorophyll. Studies show both an effect and no effect on vegetable carotenoid and chlorophyll content using different cover materials (**Tab. A**, Appendix). For those showing an effect, lower PPFD tended to result in accumulation of carotenoids and chlorophylls. This was also observed for lutein and β-carotene in spinach grown at different PPFDs, although with minor differences (1.28- and 1.19-fold comparing 125 and 620 µmol m^{-2} s^{-1}).[62] Furthermore, lower chlorophyll contents were determined in kale at higher DLI (constant photoperiod but increasing PPFD) and similar trends in lettuce and spinach.[101] Palmer and van Iersel [105] showed higher chlorophyll contents in lettuce and mizuna at lower PPFD, longer photoperiod at constant DLI. While chlorophylls did not differ, total and individual carotenoids were higher in lettuce grown under polytunnels without compared to with antifogging additives in the 2019 and 2020 experiments (Publication 2), which is probably related to PPFD. However, the effect of antifogging additives was not observed in 2021 experiments (Publication 3). This could be due to the supplemental lighting in 2019 and 2020 not being implemented in 2021. DLI and temperature in the 2019 and 2020 experiments were comparable to those in Sept. 2021. However, the supplemental light may have altered the spectral quality. With the addition of blue or red light in the spectrum, microgreen and baby leaf lettuce accumulated different amounts of carotenoids compared to white light only.[106]

Table A (Appendix) demonstrates that an effect of cover material on carotenoids was more likely to be detected in leafy vegetables, while little effect was detected in fruiting vegetables. It can be hypothesized that this is due to the location of the carotenoids in chloroplasts and their related involvement in photosynthesis in leafy vegetables. Differences in the occurrence of carotenoids in photosynthetic and non-photosynthetic tissues of plants are known.[55,60] However, the effects were shown to be cultivar-specific, as carotenoid levels in cultivar Attractie (Publication 2, Fig. 5) were independent of polytunnel cultivation and cover material. Cultivar-specific differences in response to cover material were also found for carotenoids and phenolic compounds in lettuce and tomato plants in previous studies (**Tab. A**, Appendix).

2.5 Future perspectives

Not only valuable compounds such as plant secondary metabolites contribute to the quality of vegetables, but also anti-nutritional compounds such as nitrate. Nitrate itself is not harmful, but it can form nitrosamines in the human body, which are known to promote gastrointestinal cancer, for example.[106] Lettuce, like other leafy vegetables, has a high potential to accumulate nitrate. This depends, among other things, on the DLI and the spectral quality of the light.[28,106] For example, winter-grown lettuce accumulates higher levels, and the EU regulates nitrate limits based on season, open field and protected cultivation.[107] Surprisingly, only one study evaluates nitrate content in lamb's lettuce depending on cover material. At a certain fertilization level, nitrate content is more than tripled in PE diffused compared to PE standard films (**Tab. A**, Appendix).

Cover materials used in protected cultivation have the ability to modify climatic conditions, especially the light regime. Thus, they are able to alter the content of health-promoting compounds such as plant secondary metabolites, although this depends on time of cultivation, vegetable species and cultivar. In order to select cover materials to produce high quality vegetables, future research is needed to evaluate other vegetables commonly grown under protective cover such as cucumber, lamb's lettuce, pepper or radish. In particular, the effect of antifogging covers could be of interest for fruiting vegetables due to the longer cultivation time. In addition, the effect of the cover material on anti-nutritional compounds such as nitrate should be determined since its accumulation is highly dependent on the light regime. Furthermore, future research may clarify whether the observed effects differ in greenhouse grown vegetables, as the studies within this thesis were conducted with polytunnels.

3 Protected cultivation affects carotenoids and flavonoids in a light and temperature dependent manner

It has been demonstrated that the cover material can have an effect on the levels of plant secondary metabolites in the vegetables grown underneath. In this thesis, carotenoids in lettuce were affected by the use of antifogging covers, and the effects are assumed to be dependent on the light regime. Not only the light regime, but also the temperature generally differs in protected cultivation with polytunnels compared to without. Thus, as noted previously, light and temperature are the most important factors to consider when evaluating effects on plant secondary metabolites in lettuce. However, it is not possible to distinguish between (solar) light- and temperature-dependent changes in metabolites due to their strong relation (Publication 3, Fig. S7), known as greenhouse effect.[27] In all experiments conducted similar trends and changes in carotenoid and flavonoid contents were shown. While the carotenoid content is generally higher in polytunnel grown lettuce, the flavonoid content is reversed. It is noteworthy that carotenoids differ at the individual level according to the experiment, but flavonoids do not.

3.1 Transcriptional regulation of flavonoids in polytunnel grown lettuce

The flavonoids detected in lettuce were mainly quercetin derivatives and a luteolin derivative (**Fig. 6**; Publication 2, Tab. S1), which is in accordance to the literature.[70] Flavonoids function in plants as UV light shielding compounds and antioxidants, among other things. However, there is an ongoing debate as to whether the protective properties are due to the UV light absorbing properties of flavonoids or rather to their ROS-scavenging ability.[108] For example, the synthesis of dihydroxy B-ring flavonoids (quercetin over kaempferol) in *Trifolium repens* L. is favored by additional UVB light, probably due to their efficient antioxidant properties.[109] Since only dihydroxy B-ring flavonoids were present in lettuce, it is not surprising that similar changes occurred for all flavonoids due to polytunnel cultivation. In contrast, the caffeic acid derivative composition of lettuce varied, with decreased major and increased minor caffeic acid derivatives such as chlorogenic and *iso*-chlorogenic acids, respectively (Publication 2, Fig. 3, Tab. S4).

Consistent with flavonoid contents, transcript levels of *CHS*, a gene encoding a key enzyme in the flavonoid biosynthetic pathway, are also reduced in lettuce grown under polytunnels. This indicates transcriptional control. Flavonoid contents as well as *CHS* expression are higher in plants grown at lower temperatures.[110,111] This is consistent with lower temperature and higher flavonoid contents using no polytunnels in this thesis. In addition, flavonoid accumulation is

favored at higher PPFD[78], which in turn is in agreement with higher flavonoid contents in lettuce grown without polytunnels. Nevertheless, lettuce grown under UV transmitting and UV blocking covers with almost equal PAR transmittance are reduced in flavonoids.[112] This shows a strong UV light dependent accumulation of flavonoids and the reduced transcripts of the *UVR8* photoreceptor in lettuce grown under the polytunnels provide further evidence (Publication 3, Fig. 5). Of note, UVR8 has long been thought to be exclusively a UVB light photoreceptor, but it has recently been reported that short-wave UVA radiation (315-350 nm) may also be involved in UVR8 signaling.[113] UVA radiation is the major component of solar UV radiation. Furthermore, long-wave UVA radiation (350-400 nm) is also sensed by the blue light photoreceptor CRY. Although flavonoid biosynthesis is transcriptionally regulated in polytunnel grown lettuce, signal transduction from UVR8 to CHS remains ambiguous because *HY5*, the integral transcription factor downstream of all photoreceptors, is not affected at all. Therefore, the involvement of other transcription factors remains to be elucidated.
Moreover, the phytohormone ABA was shown to promote flavonol biosynthesis. *Vitis vinifera* L. grown under UVB light filtering covers had low leaf flavonol content, but this was increased by exogenously applied ABA.[114] Since lettuce flavonoids correlate with ABA in the majority of experiments, a regulatory effect is possible (Publication 3, Fig. 6, S12 and **Fig. A2**, Appendix), although the ABA-flavonol relationship is less understood.[115]

3.2 Post-transcriptional regulation of carotenoids in polytunnel grown lettuce

In this thesis, lutein and β-carotene, along with violaxanthin and neoxanthin, are the major carotenoids detected in lettuce (Publication 3, Tab. S2). In chloroplasts, these are the predominant carotenoids.[116] In addition, the lettuce specific carotenoid lactucaxanthin was detected (**Fig. 5**). Unlike flavonoids, carotenoids in lettuce, albeit increased in total amount under polytunnels, differed at the individual level across experiments. While all carotenoids were higher under polytunnels in 2019, this was not the case in the other experiments (Publication 2, Fig. 5 and 3, Fig. 4, S9). Only lutein was higher in lettuce grown under polytunnels in all experiments. Higher levels of neoxanthin and violaxanthin were also found, while β-carotene was largely unaffected. Carotenoids in plants are discussed as a fine-tuning mechanism, probably to cope with environmental fluctuations.[116] In this context, it is less surprising that individual carotenoids were differently adapted to the naturally fluctuating greenhouse conditions, while chlorophylls were not (Publication 2, Fig. 4, and 3, Fig. S13-15). Carotenoids have multiple functions in plants. In photosynthetic tissue, their light-harvesting properties as photosynthetic antenna pigments, membrane stabilization, and involvement in

photoprotection by NPQ are important to consider.[116] They are bound in light-harvesting complexes (violaxanthin, neoxanthin, lactucaxanthin, and lutein) and in the core of photosystems (β-carotene) to act as light-harvesting pigments and ROS scavengers, respectively.[116,117] Higher chlorophyll contents together with a lower chlorophyll a/b ratio in lettuce under polytunnels indicate an adaptation to the lower PPFD to ensure effective photosynthesis. The involvement of the light-harvesting carotenoids and not β-carotene further supports this assumption. Lutein in particular has an important function in the transfer of excitation energy to chlorophyll molecules.[118,119]

The transcription of PSY, the key enzyme in the carotenoid biosynthetic pathway, is highly light dependent.[64,116] However, it has to be pointed out that the *PSY* transcripts do not correspond to the levels of carotenoids, since they are either unaffected or reduced in lettuce under polytunnels (Publication 3, Fig. 5). The same applies to the transcript levels of a carotenoid cleavage enzyme (*CCD4*) and storage proteins (*OR* and *OR-like*) (Publication 3, Fig. 5). Thus, transcriptional control does not explain the high carotenoid content in lettuce grown under polytunnels. However, a higher metabolic flux determined with the phytoene desaturase (PDS) inhibitor norflurazon suggests an involvement of post-transcriptional mechanisms (Publication 3, Fig. 7). Higher temperature led to decreased expression of *ZmPSY*, while carotenoids accumulated[120] and Stanley and Yuan [64] concluded that regulation of *PSY* expression is unlikely to be responsible for the temperature adaptation of carotenoids. The results of this thesis support this hypothesis.

High temperatures are associated with the prevention of PSII damage repair by ROS introduction.[64] Carotenoids also serve to stabilize the membranes and influence membrane fluidity to optimize the photosynthetic electron transport chain in the thylakoid membrane. It has been shown that there is a pool of free carotenoids in the membrane bilayer that acts at this site. Zeaxanthin is oriented perpendicular to the membrane, which increases its rigidity.[121] Moreover, overexpression of the *Arabidopsis* β-hydroxylase (*CHYB*) leads to an increase in zeaxanthin, which has been associated with its tolerance to heat and light.[122] In this thesis, zeaxanthin tends to be higher in lettuce under polytunnels, albeit not significant. This is especially true for the experiment in May 2021, where the highest temperatures have been measured (Publication 3, Fig. S9). Lutein can also act as a membrane stabilizer with perpendicular membrane orientation, contributing to its rigidity.[121] Although temperature- and light-induced changes are almost indistinguishable in protected cultivation, elevated temperature may contribute to carotenoid accumulation in lettuce.

This is further indicated by the fatty acid content of lettuce grown under polytunnels (Publication 2, Tab. 2). Palmitic acid was higher in lettuce grown under polytunnels, which is in agreement with the observations of Falcone, Ogas [123] for *Arabidopsis* grown at elevated temperature. In contrast, a decrease in trienoic fatty acids as in their experiments was not detected in lettuce. The saturation of membrane fatty acids may also contribute to its rigidity in response to higher temperatures. An adaptation of thylakoid membrane architecture by decreasing membrane fluidity has been discussed in response to high light and temperature conditions.[124]

However, it remains to be elucidated how the temperature response is regulated, and the experiments conducted in this thesis suggest post-transcriptional regulation. This may occur at PSY protein level or depends on PSY protein solubility in relation to its enzyme activity at the thylakoid membrane. For example, the OR protein family is known to interact with AtPSY in chloroplasts, leading to enhanced PSY activity.[125] In this context, transgenic *Arabidopsis* and sweet potato plants overexpressing *IdOR* showed enhanced heat tolerance.[126]

3.3 The inverse relation between flavonoids and carotenoids

Carotenoids and flavonoids in lettuce grown under polytunnels showed a negative correlation (Publication 3, Fig. S6, **Fig. A3**, Appendix). This led us to first hypothesize that the metabolic pathways are co-regulated at the transcriptional level *via* the HY5/PIF1 signaling network. Since the two metabolites have been shown to be regulated at different levels, the relationship must occur elsewhere. A stronger correlation was observed in experiments with the highest DLI and the highest temperatures, indicating the involvement of environmental factors. Thus, this inverse relationship could occur due to different relative importance of the metabolites for lettuce photosynthesis. High light/UV radiation leads to an adaptation of protective flavonoids to scavenge ROS in chloroplasts, whereas low light/high temperature leads to an adaptation of light-harvesting and membrane-modulating carotenoids to ensure efficient light utilization.

3.4 Future perspectives

In conclusion, flavonoids and carotenoids in lettuce were influenced by protected cultivation with polytunnels in relation to light and temperature regime. While flavonoids are under transcriptional control, carotenoids are assumed to be regulated post-transcriptionally. Future research should aim to elucidate the regulatory transcriptional cascade of the flavonoid pathway, as the transcription factor *HY5*, which is an integrating point downstream of all photoreceptors, was not affected by polytunnel coverage. Targeted analysis of known involved

transcription factors and photoreceptors, such as *COP1* and *CRY*, or RNA-Seq analysis may help to further understand the regulatory network. To shed light on carotenoid regulation and accumulation, future work could focus on analyzing the protein levels of PSY and its post-transcriptional regulator OR by Western blot analysis. In addition, the determination of the enzyme activity of PSY and its chloroplastic localization are of interest.

Since light and temperature are interrelated in polytunnel cultivation, it is not possible to distinguish whether the accumulation of these secondary plant metabolites is due to one or the other. Nevertheless, the result is an adaptation to ensure optimal photosynthesis under the given conditions. Experiments in controlled environments such as phytochambers using "greenhouse conditions" (including daily light fluctuations) may help to distinguish between temperature and light related adaptations in lettuce. This knowledge could be used to develop cover materials to target temperature and/or light regimes.

4 Choice of cover materials and incorporated additives as potential for sustainable lettuce cultivation and quality improvement

In this thesis, it has been shown that protected cultivation has a significant effect on plant secondary metabolites in lettuce and thus on lettuce quality. However, the incorporation of antifogging additives into the cover material had a negligible effect on most metabolites, with the exception of carotenoids. It was recognized that the effect of cover material depends mainly on the light regime with changes in plant metabolites up to 3.86-fold (for lettuce flavonoids; **Tab. A**, Appendix). On the other hand, the effect of covers compared to no covers has light and temperature dependent dimensions.

4.1 Using light strategically to grow high quality lettuce

In order for cultivation systems to be sustainable and future-feasible, an efficient use of resources is necessary. For example, this can be determined as light use efficiency (LUE), which is defined as shoot dry weight per incident photon flux density.[127] Jin, Formiga Lopez [127] evaluated the LUE of different systems and found that greenhouse cultivation was more efficient than open field, although the highest LUE was for vertical farming. In protected cultivation, LUE varies seasonally due to the availability of sunlight. During the summer months, PPFD may be close to photosynthetic saturation, while in the winter, light availability may be a bottleneck. Thus, there is an opportunity to improve LUE through material selection.

Materials could be optimized according to the growing season or the selection of vegetables with different light requirements. However, LUE does not evaluate the accumulation of health-promoting vegetable compounds.
Although the use of antifogging additives in this thesis slightly increased DLI and PPFD under polytunnels, this change was probably too small to have an effect on phenolic compounds. However, the use of the (partially UV transparent) polytunnels did. Phenolic compounds were reduced in lettuce grown under polytunnels (flavonoids up to 0.23-fold, caffeic acid derivatives up to 0.69-fold). In terms of its health-promoting properties, this is a major limitation of lettuce quality due to the use of covers, as also shown in other studies (**Tab. A**, Appendix). Some options exist to improve lettuce quality in protected cultivation by increasing phenolic compounds. For example, the absence of UV blocking stabilizers in films would increase UV transmittance and thus enhance flavonoids as shown in **Tab. A**, (Appendix). However, UV stabilizers are used to extend the service life of a film by preventing its degradation.[12] In terms of sustainable strategies, this would increase plastic waste and may be counterproductive. Light supplementation (PAR or UV radiation), which could be provided by energy-efficient LED systems, may be another way to enhance phenolic compounds without reducing film lifespan. Increasing the PPFD (from 225 to 410 $\mu mol\ m^{-2}\ s^{-1}$) two weeks before harvest increased flavonoids in lettuce.[78] In addition, UVB light treatment 10 days prior to harvest of lettuce grown under UV blocking film resulted in increased flavonoids and less yield reduction than is typically seen with UV light supplementation.[39] Of course, it remains to be elucidated how this supplementation might affect other health-promoting compounds such as carotenoids. A study by Assumpção, Assis [79] showed that daily UVB light treatment of lettuce for one hour two weeks before harvest tended to increase flavonoids as well as chlorophylls and carotenoids.

4.2 Other factors to improve lettuce quality

In this thesis, polytunnels altered lettuce flavonoids and carotenoids up to 0.23-fold and 1.23-fold, respectively, while antifogging additives altered carotenoids up to 1.09-fold. However, in addition to environmental factors, other agronomic or crop-related factors may influence the levels of these metabolites. For example, different vegetable cultivars naturally vary in their levels of secondary metabolites. Lettuce cultivars vary in lutein (up to 27-fold) and β-carotene (up to 30-fold) content.[48] Furthermore, differences in flavonoids (up to 127-fold) and phenolic acids (up to 12-fold) were found among green and red lettuce cultivars.[70] The nutrient status of the vegetable, which can be altered by the degree of fertilization, also affects the level of plant secondary metabolites. Nitrogen supply between 0.75 and 12 mM for green and red lettuce

showed increased chlorophyll and carotenoid levels (up to 4.30-fold and 2.50-fold), while flavonoids and phenolic acids decreased (up to 0.11-fold and 0.06-fold).[128]

Taken together, other factors may have a greater influence on the profile of health-promoting plant secondary metabolites than the cover material used. Nevertheless, particularly the combination of these factors offers the potential to produce high quality vegetables.

4.3 Developing sustainable cover materials

Sustainable cover material selection must take into account the service life of the films, the waste generated, as well as the migration of additives into the environment. To improve the sustainability of films with antifogging additives, there is potential in extending the antifog effect (extending service life) and preventing migration and leaching of additives from the films. The PermAFog project, within which this thesis was conducted, aims to develop cover materials with "permanent" antifog properties. These newly developed materials were shown to have no negative effect on lettuce quality in terms of flavonoids and chlorophylls compared to conventional films (**Fig. A4**, Appendix). Such developments for other types of additives can make a positive contribution to more sustainable protected agriculture.

4.4 Future perspectives

A sustainable improvement for future food production in terms of vegetable quality, including the selection of cover materials, will require consideration of multiple factors ranging from plant-environment interactions to environmental safety issues and efficient use of resources. However, systematic research on the impact of cover materials and their strategic selection is lacking. This thesis contributes by focusing on the LDPE/EVA cover material with incorporation of a specific type of additives, the antifogging additives. Due to the variety of possible materials and combinations with different additives, future research could follow two parallel approaches. Firstly, from a material point of view, with the improvement of materials in terms of migration issues, but also the impact of different materials on the climatic conditions and the resulting changes in health-promoting plant secondary metabolites. Different polymers for films (without additives) could be evaluated for their general effect on light or temperature regimes in protected cultivation and corresponding adaptations of secondary metabolites. The next step could be to test the influence of commonly used additives on the quality of the vegetables. The second approach could be taken from the plant's point of view. Understanding the regulatory mechanisms and adaptation strategies of plants and their metabolites to climatic

conditions that reflect realistic greenhouse scenarios could further advance the strategic development of cover materials.

Finally, when producing highly nutritious vegetables, not only pre-harvest conditions need to be considered. Post-harvest conditions, such as storage, can also affect the quality of vegetables and several polymer materials and additives are used as food packaging to protect them.[18] The choice of packaging material may also influence the health-promoting plant secondary metabolites. The application of antifogging additives in PP packaging material and the influence on selected metabolites in green and red lettuce were evaluated in this thesis. Antifogging additives were found to have no effect on storage stability of the metabolites in green and little effect in red lettuce (**Fig. A5**, **A6**, Appendix). In addition to higher carotenoids and chlorophylls after one day of storage, the antioxidant capacity of hydrophilic extracts tended to be higher, along with higher levels of water-soluble anthocyanins in red lettuce stored in antifog bags for ten days. Consistent with this observation, Lee and Chandra [129] showed more stable chlorophylls and anthocyanins in red lettuce stored in non-perforated, antifog PP bags for 16 days compared to perforated and non-perforated PP materials.

This highlights that sustainable quality improvement of vegetables such as lettuce does not end at harvest, but requires consideration along the entire production chain.

SUMMARY AND CONCLUSION

Protected cultivation in greenhouses or polytunnels offers the potential for sustainable production of high-yield, high-quality vegetables. The FAO also considered this as a way to meet the challenges of climate change and food security in the future.[14] Thus, protected cultivation can contribute to achieving SDG2 "Zero Hunger". Nevertheless, it was pointed out that there is a lack of systematic research on the influence of cover materials used in protected cultivation.[10] This thesis summarizes the research on LDPE/EVA films with incorporated antifogging additives as polytunnel covers and examines them from two perspectives (**Fig. 7**).

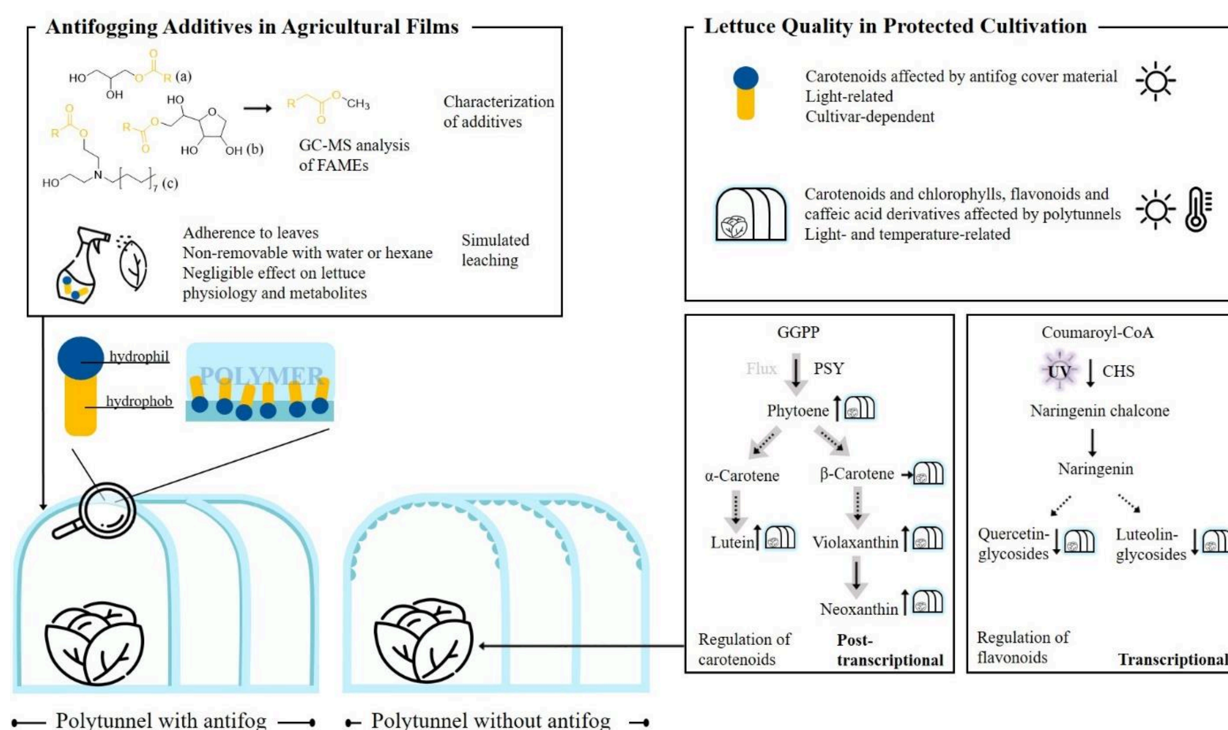


Figure 7: Key effects of antifogging additives used in cover materials and protected cultivation of lettuce under polytunnels. (a) Glycerol-, (b) sorbitan- and (c) stearyldiethanolamine- based antifogging additives. Hydrophobic fatty acid moieties of antifogging additives are highlighted. Detailed pathways of carotenoids and flavonoids are shown in **Fig. 5** and **6**. CHS, chalcone synthase; CoA, coenzyme A; FAMES, fatty acid methyl esters; GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase.

First, the direct impact of antifogging additives used in agricultural films and leaching from them was demonstrated. For this purpose, a GC-MS method has been developed to determine the fatty acid moieties of commonly used antifogging additives. Three structurally different additives have been characterized using this method, and all of them contain more than the main fatty acid specified by the manufacturer. In simulated leaching experiments on lettuce

leaves (*Lactuca sativa* var. *capitata* L. cv. Attractie), leaf adhesion of antifogging additives was observed that could not be removed with either water or hexane. Depending on the nature of the additive, environmental and vegetable contamination issues arise. Nevertheless, these foliar adherent antifogging additives appear to have a negligible effect on plant physiology and nutritionally valuable metabolites after short-term exposure.

The second approach in this thesis focused on the indirect effects of antifogging additives in LDPE/EVA polytunnel covers on the quality of lettuce (cv. Veronique and Attractie) grown underneath. It has been shown that both protected cultivation and the incorporation of antifogging additives modify the climatic conditions. The use of these additives mainly affected the light regime, but not the temperature, relative humidity or carbon dioxide concentration. This resulted in a cultivar-specific adaptation of carotenoids in lettuce under antifog polytunnels, but not of flavonoids, caffeic acid derivatives or chlorophylls. It has been suggested that this is related to their involvement in photosynthesis as a fine-tuning mechanism to ensure efficient photosynthesis under prevailing environmental conditions. The differences at the level of individual carotenoids in the experiments further support this hypothesis.

It was concluded that lettuce cultivation under polytunnels has a light and temperature dependent dimension compared to cultivation without polytunnels, both of which are closely related. Higher levels of carotenoids in lettuce grown under polytunnels may be associated to their light-harvesting and membrane stabilization function. Both hypotheses are supported by the chlorophyll a/b ratio and fatty acid saturation. Moreover, carotenoids are assumed to be regulated post-transcriptionally, as indicated by the lack of correlation between carotenoid content and *PSY* transcripts and the increased carotenoid metabolic flux.

In contrast, the flavonoid content of lettuce under polytunnels was reduced and similar at the individual flavonoid level, which was assumed to be related to their ROS scavenging potential. Furthermore, they have been shown to be transcriptionally regulated (*CHS*), mainly in response to UV light (*UVR8*).

Taken together, the use of LDPE/EVA polytunnels with and without antifogging additives affected health-promoting plant secondary metabolites in lettuce. However, although antifogging additives were demonstrated to adhere to lettuce leaves after simulated leaching, this had negligible effects on plant physiology and these metabolites in this thesis. Furthermore, it was confirmed that newly developed cover materials with “permanent” antifog properties are suitable for lettuce cultivation. To achieve high quality vegetables, not only pre-harvest factors but also post-harvest storage must be taken into account. This was demonstrated by storing two types of lettuce in antifogging PP food bags for ten days.

This research has added to the knowledge of the effects of antifog LDPE/EVA cover materials; however, there are some limitations as discussed in more detail in the publications (Publication 1, *"3.5 Limitations and analytical challenge"*; Publication 2, *"Experimental setup and general restrictions of the study"*; Publication 3, *"4.4 Limitations and potential"*). Briefly, it may not have been possible to capture all the effects that could be caused by antifogging additives sprayed directly on lettuce leaves (24 h treatment) and used in polytunnels (about one month of cultivation) within the experimental durations. In addition, the fatty acid-based GC-MS analysis cannot detect intact molecules of antifogging additives. Therefore, the study of foliar uptake of intact molecules or their degradation is not possible. For the benefit of determining differently structured molecules, this loss of information must be tolerated. Finally, the size of the polytunnel used for the experiments provides an opportunity to minimize bias due to fluctuating light/shade conditions in the greenhouse. However, the microclimate created may differ from larger dimensions, as shown for relative humidity, which in turn could alter the plant response. Nevertheless, the research presented provides insight into the effects of antifogging additives incorporated into polytunnel cover material on microclimate and lettuce quality. In addition, research gaps were identified and future research could build on these model-like experiments to examine greenhouse scenarios.

Future challenges for sustainable vegetable production were identified as ensuring the planetary health by producing more healthy food while using resources sparingly and responsibly and without using additional land. Protected cultivation offers the opportunity to produce more on less agricultural land, by using resources more efficiently. The selection and development of cover materials in combination with other strategies such as targeted supplemental light, fertilization, and cultivar selection, among other factors, can contribute to the production of lettuce with high nutritional value. This can help meet consumer demand for healthy vegetables in plant-based diets as recommended for sustainable human nutrition.

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APPENDIX

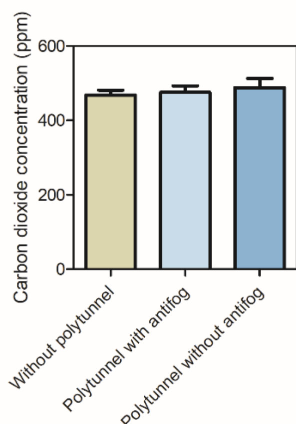

Figure A 1: Determination of carbon dioxide concentrations (ppm) under polytunnels with and without antifogging additives or without polytunnel. Data are represented as mean of daily averaged CO_2 ± SD (n = 57). Measurement was conducted from Nov. 2020 to Jan. 2021.

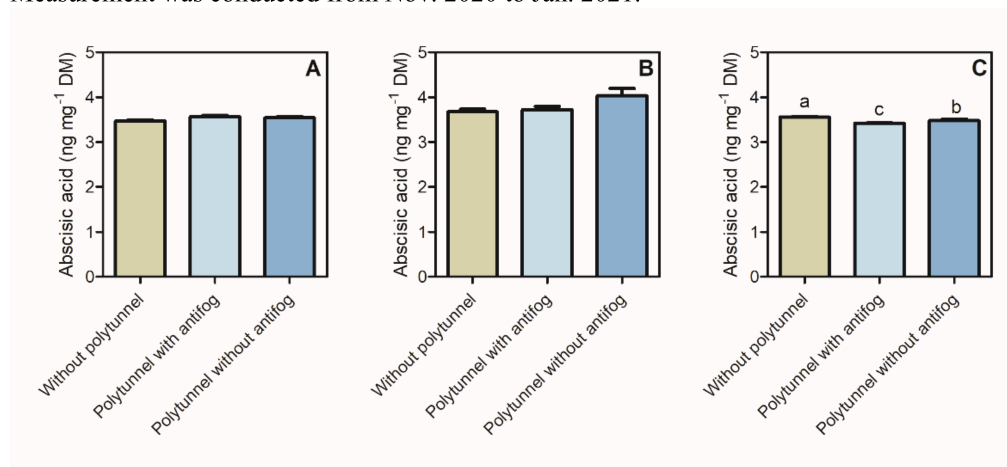

Figure A 2: Abscisic acid content (ng mg^{-1} DM) in lettuce (Veronique, A, C; Attractie B) grown without or under polytunnels with and without antifogging additives. Data from experiments performed in 2019 (A, B) and 2020 €. The data are expressed as mean ± SE (n = 12 to 16 in 2019 and n = 28 in 2020). Different letters indicate significant differences ($p \leq 0.05$), no letters indicate absence of significance.

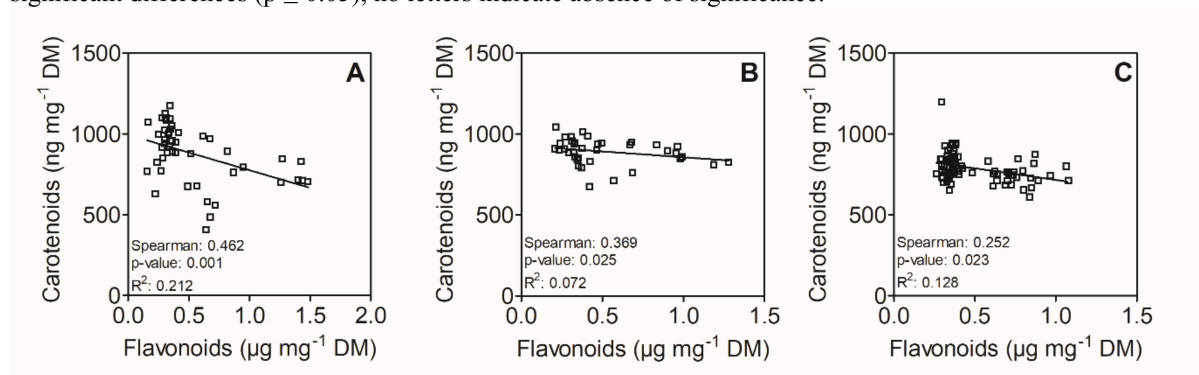

Figure A 3: Correlation analysis (Spearman's) of carotenoids and flavonoids in lettuce grown without or under polytunnels with and without antifogging additive. Data from experiments in 2019 (Veronique, A; Attractie, B) and 2020 (Veronique, C).

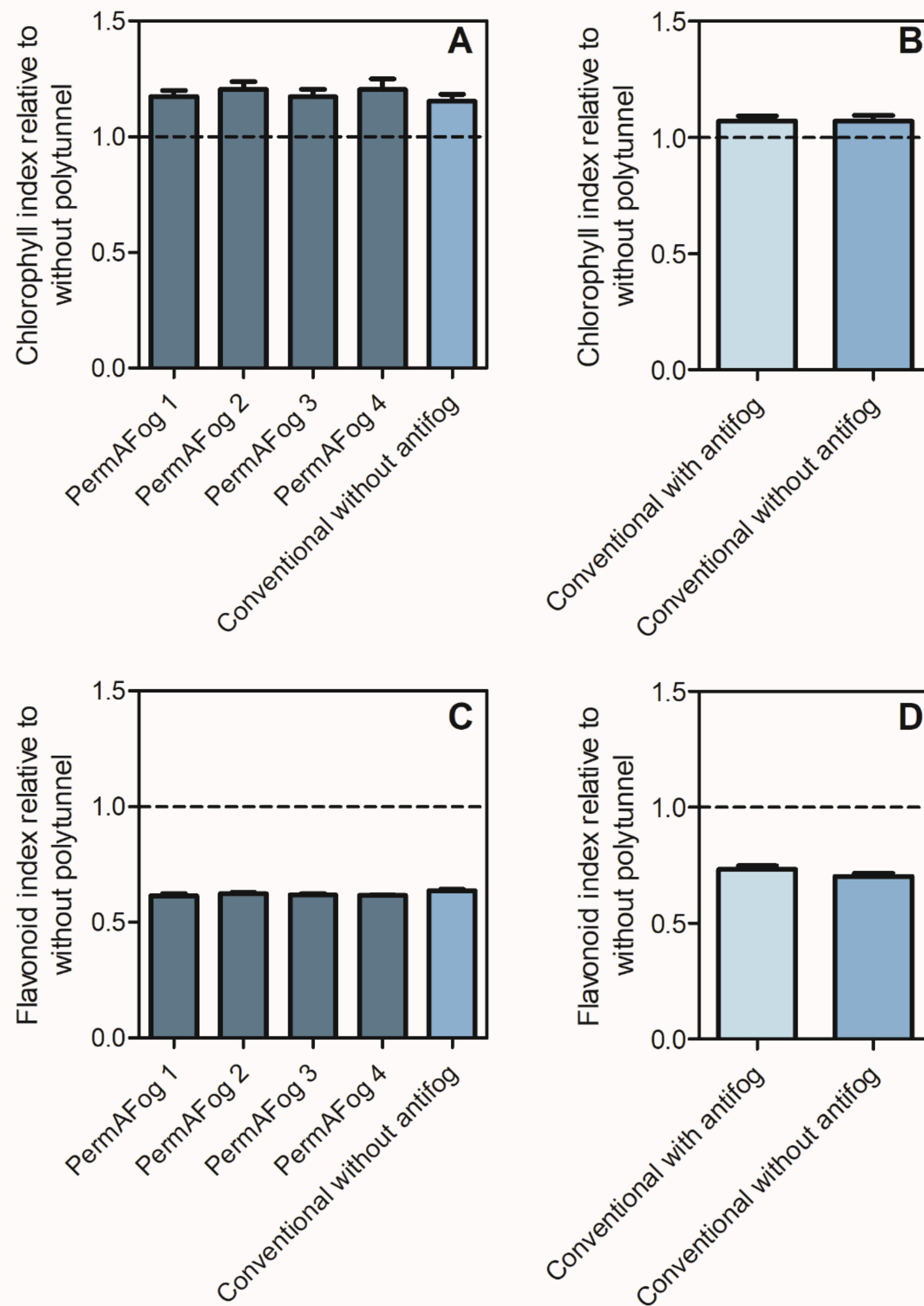

Figure A 4: Chlorophyll and flavonoid index in lettuce (cv. Veronique) grown under polytunnels covered with novel developed "permanent-antifog" greenhouse films (PermAFog 1-4; A, C) or with conventional films with and without antifogging additives (B, D). Lettuce was grown under polytunnels in Oct. 2022 (A, C) or Oct. 2019 (B, D). Data are shown as mean ± SE (n = 3 to 5) relative to lettuce grown without polytunnels. Indices were determined adaxial with non-invasive measurement (DUALEX) as described previously. [130]

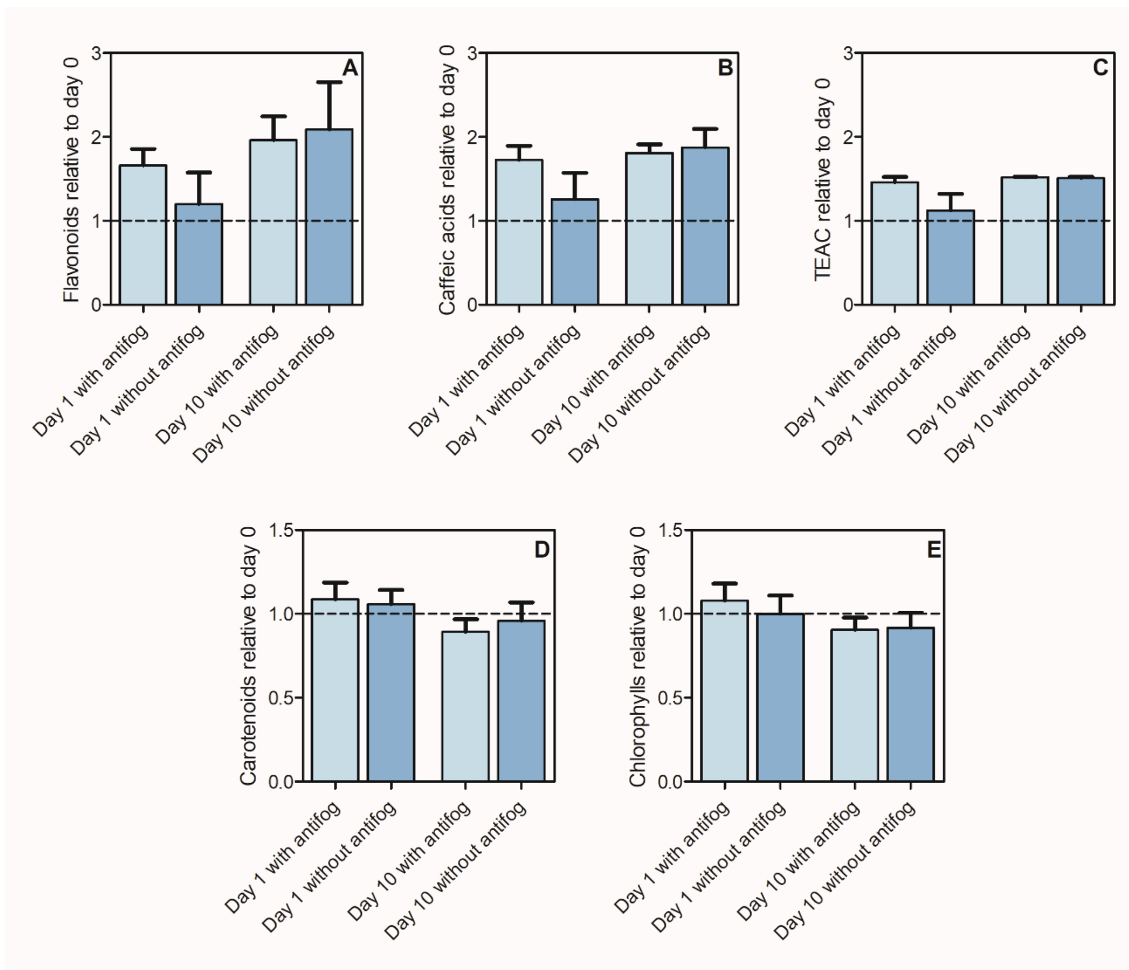

Figure A 5: Storage of green lettuce (cv. Attractie) in PP food bags with or without incorporated additive (Atmer 1440) for 10 days in a refrigerator at 7.5 °C and 64 % relative humidity. Phenolic compounds (flavonoids, A; caffeic acid derivatives, B), trolox equivalent antioxidant capacity (TEAC, C), carotenoids (D) and chlorophylls € were analyzed. Data are presented as mean ± SE (n = 4) based on dry weight values, relative to lettuce fresh from the field (day 0). TEAC assay was performed with hydrophilic extracts (60 % MeOH) as described previously.[131] Absence of asterisks indicate absence of significance.

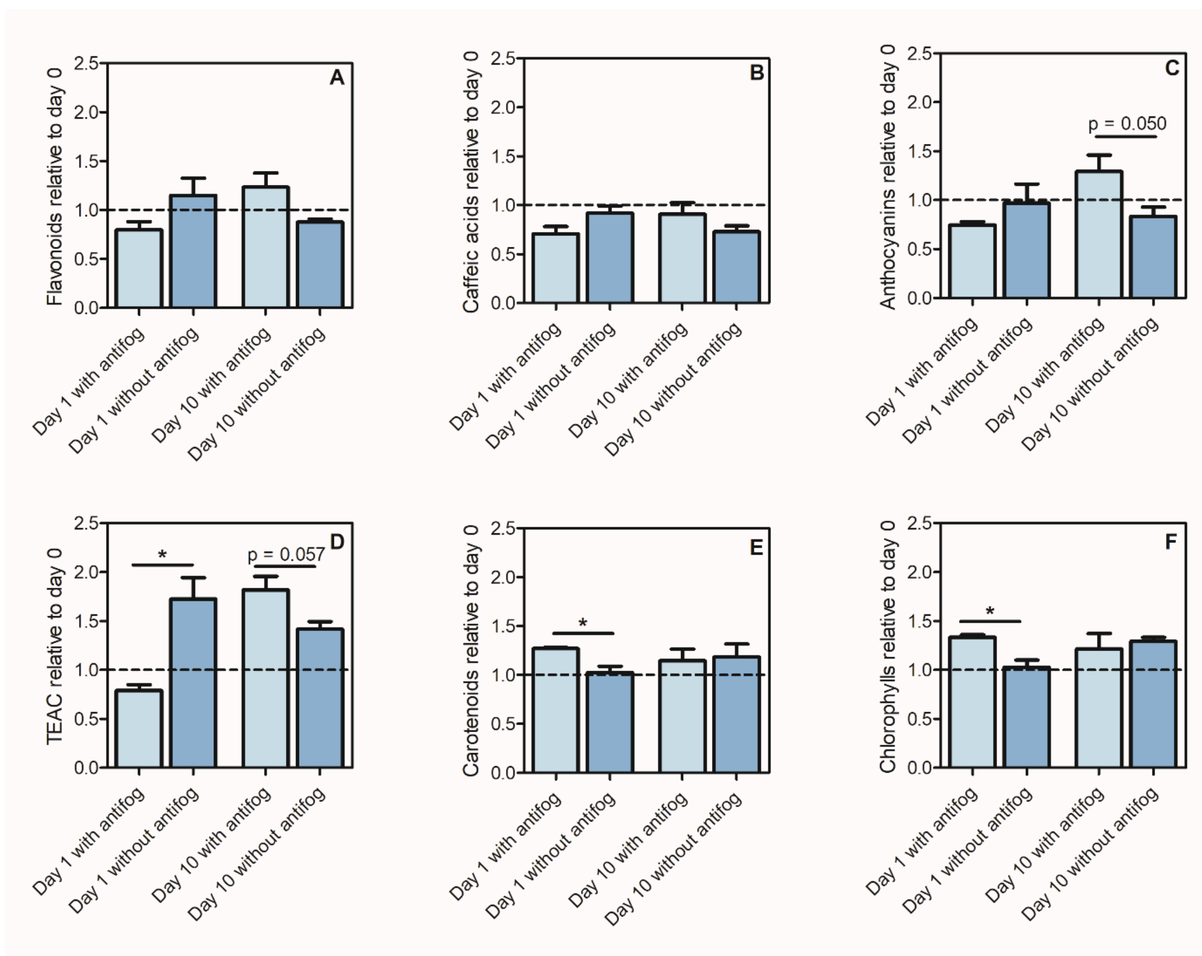

Figure A 6: Storage of red lettuce (cv. Merveille de 4 saison) in PP food bags with or without incorporated additive (Atmer 1440) for 10 days in a refrigerator at 7.5 °C and 64 % relative humidity. Phenolic compounds (flavonoids, A; caffeic acid derivatives, B; anthocyanins, C), trolox equivalent antioxidant capacity (TEAC, D), carotenoids € and chlorophylls (F) were analyzed. Data are presented as mean ± SE (n = 4) based on dry weight values, relative to lettuce fresh from the field (day 0). TEAC assay with hydrophilic extracts (60 % MeOH) and anthocyanin analysis were performed as described previously.[78,131] Asterisks indicate significant difference ($p \leq 0.05$) between lettuce in food bags with and without antifogging additives, no asterisks indicate absence of significance.

Table A: Influence of cover materials on climate conditions, yield and metabolites in vegetables. Relative differences in climatic conditions were calculated over entire cultivation time as reported in the literature, unless otherwise noted. If the data were shown only in figures, the numbers were extracted using WebPlotDigitizer (Ankit Rohatgi, Version 4.3.) for calculation. Significant differences ($p \leq 0.05$) were indicated using lower case letters. Relative differences from the experiments in this thesis were calculated separately according to the experiments presented in Publication 2 (P2) and Publication 3 (P3).

Cover material	Construction, region, season	Plant species	Climate conditions measured			Factors investigated			Reference
			Condition	Relative differences due to cover		Factor	Relative differences due to cover		
PE-UV stabilized	Mini-GH, Turkey, Autumn (June to Nov.)	Eggplant (cv. Valentine F1)	T	PE-UV	1.21	Yield	PE-UV	1.09^{b}	Cemek 2006[31]
PE-IR absorbing				PE-IR	1.26		PE-IR	1.19^{b}	
D-PE (double layer)				D-PE	1.30		D-PE	1.60^{a}	
S-PE (single layer)				S-PE	1.13		S-PE	1.00^{c}	
Outside				Outside	1.00		Outside	n.d.	
			RH	PE-UV	0.88				
				PE-IR	0.93				
				D-PE	1.01				
				S-PE	0.93				
				Outside	1.00				
			PPFD	PE-UV	0.65				
				PE-IR	0.63				
				D-PE	0.56				
				S-PE	0.71				
				Outside	1.00				
PE clear (PE-C)	Tunnel, Italy, Spring (March to June)	Lamb's lettuce (cv. Princess)	n.d.			Yield	PE-C	1.00^{b}	Cozzolino 2020[132]
PE light diffusing (PE-LD)							PE-LD	1.23^{a}	
						Nitrate (50 kg N·ha^{-1} fertilization)	PE-C	1.00^{b}	
							PE-LD	3.61^{a}	
						Ascorbic acid (total)	PE-C	1.00^{b}	
							PE-LD	1.09^{a}	
						AC, lipophilic	PE-C	1.00^{a}	
							PE-LD	0.91^{b}	
						AC, hydrophilic	PE-C	1.00^{a}	

Cover material	Construction, region, season	Plant species	Climate conditions measured			Factors investigated			Reference
			Condition	Relative differences due to cover		Factor	Relative differences due to cover		
							PE-LD	0.66^{b}	
						Chlorophyll (total)	No influence		
						Carotenoids (total)	No influence		
						Phenolics (total)	No influence		
Single-layered glass (GL)	Mini-GH, Canada, Winter (Nov. to Feb.)	Cucumber (cv. Flamingo)	T	GL	1.00	Yield	GL	1.00^{b}	Hao 1999[32]
				D-PE	1.01		D-PE	1.06^{b}	
Double-inflated PE (D-PE)				A	1.02		A	1.64^{a}	
			VDP	GL	1.00	Chlorophyll (fruit skin)	No influence		
				D-PE	0.75				
Twin-wall acrylic (A)				A	0.89				
			CO2	GL	1.00				
				D-PE	1.05				
				A	0.99				
			PPFD	GL	1.00				
				D-PE	0.84				
				A	0.93				
Single-layered glass (GL)	Mini-GH, Canada, Spring (Jan. to July)	Tomato	T	GL	1.00	Yield	No influence		Papadopoulos 1997[33]
				D-PE	1.01				
Double-inflated PE (D-PE)				A	1.01				
			VDP	GL	1.00				
				D-PE	0.94				
Twin-wall acrylic (A)				A	0.98				
UV block film	Tunnel, UK, Spring (April to June)	Red leaf lettuce (cv. Revolution)	UV transmission	Block (~ 5% up to 380 nm)		Yield	Block	1.00^{a}	García-Macías 2007[112]
				Low (~ 25% up to 380 nm)			Low	0.66^{b}	
UV low film				Window (~ 70 %)			Window	0.57^{b}	

Cover material	Construction, region, season	Plant species	Climate conditions measured		Factors investigated		Reference
			Condition	Relative differences due to cover	Factor	Relative differences due to cover	
UV window All with IR reducing and light-diffusing additives					Phenolics (total)	Block 1.00^{c} Low 1.59^{b} Window 1.78^{a}	
					Flavonoids (total)	Block 1.00^{c} Low 3.19^{b} Window 3.86^{a}	
					Caffeic acid	Block 1.00^{c} Low 1.36^{b} Window 1.46^{a}	
					AC (ORAC)	Block 1.00^{c} Low 1.66^{b} Window 1.77^{a}	
UV block UV window	GH, Italy, Spring (April to May)	Rocket salad	UV transmission	UV block (UVB 0%) UV window (UVB 27%)	Flavonoids (total)	Block 1.00^{b} Window 2.49^{a}	Mormile 2019[133]
					Chlorogenic acid	Block 1.00^{a} Window 0.84^{b}	
PE/EVA (PE) Double-layer diffused PE/EVA (D-PE) Diffused twin-wall polycarbonate (PC)	GH, Canada, Autumn (Sept to n.m.)	Tomato (cv. Black cherry, Brandy sweet plum, Cuban yellow, Esterina hybrid F1, Flavorita F1)	Light transmission	PE (78 % VIS) D-PE (79% VIS) PC (79% VIS)	Flavonoids and phenolic acids (individual)	Cultivar-dependent, up to 3.77-fold change (D-PE to PE)	Ahmadi 2018[37]
					Carotenoids (lutein, lycopene)	Cultivar-dependent, up to 3.76-fold change (D-PE and PC to PE)	
					AC, hydrophilic (FRAP, ORAC, DPPH)	No influence	
7-layer low-density PE (7-PE)	GH, Greece, Winter/spring (Dec. to June)	Tomato (cv. Elpida)	n.d.		Yield (total)	7-PE 0.94^{b} S-PE 1.00^{a} ID-PE 0.86^{c}	Petropoulos 2019[36]

Cover material	Construction, region, season	Plant species	Climate conditions measured		Factors investigated		Reference
			Condition	Relative differences due to cover	Factor	Relative differences due to cover	
Single three-layer PE (S-PE) Double inflated three-layer PE (ID-PE)					Macronutrients Tocopherols Sugars Organic acids Fatty acids AC	Influence Influence Influence Influence Influence Influence	
					Carotenoids (total) Lutein Lycopene	Depends on DAT, up to 1.46 (total) and 1.75 (Lu, 7-PE to S-PE) and 1.32 (Ly, ID-PE to S-PE)	
					Chlorophylls (total)	Depends on DAT, up to 1.60 (7-PE to S-PE)	
Standard PE (PE) Standard PE (removal 2 weeks prior to harvest, mov.) Diffuse PE (D-PE) Clear PE (no UV filter, C-PE) UVA/B Block (Block) 55% Shade Cloth + Standard PE (Shade)	Tunnel, USA, Summer (tomato, April to Aug.); Spring and autumn (lettuce, Feb. to May and Sept. to Nov.)	Tomato (cv. BHN 589) Lettuce, (cv. New Red Fire, Two Star)	T (canopy)	Spr / Sum / Aut PE 1.00 / 1.00 / 1.00 Mov. 1.09 / 1.02 / 1.10 D-PE 0.96 / 0.98 / 1.01 C-PE 0.93 / 1.01 / 1.04 Block 0.97 / 1.02 / 1.01 Shade 0.93 / 1.02 / 0.91	Yield (tomato)	PE 1.00^{a} Mov. 0.94^{a} D-PE 1.10^{a} C-PE 1.13^{a} Block 1.01^{a} Shade 0.67^{b}	Gude 2022[134]
			PPFD	PE 1.00^{ab} / 1.00^{b} / 1.00^{ab} Mov. 1.13^{a} / 1.36^{a} / 1.22^{a} D-PE 0.74^{b} / 0.89^{b} / 0.85^{ab} C-PE 0.89^{ab} / 0.93^{b} / 1.00^{ab} Block 0.87^{ab} / 1.03^{b} / 0.95^{ab} Shade 0.37^{c} / 0.34^{c} / 0.48^{b}	Yield (lettuce red, spring)	PE 1.00^{a} Mov. 0.77^{bc} D-PE 0.96^{a} C-PE 0.99^{a} Block 0.88^{ab} Shade 0.69^{c}	
					Yield (lettuce green spring and both, autumn)	no influence	

Cover material	Construction, region, season	Plant species	Climate conditions measured			Factors investigated			Reference
			Condition	Relative differences due to cover		Factor	Relative differences due to cover		
Standard PE (PE)	Tunnel,	Tomato (cv.	PPFD	PE	1.00	Tomato:			Lee 2021[35]
	USA, Summer	BHN 589)	(Summer)	Mov.	1.27	Carotenoids			
Standard PE	(tomato, April			D-PE	0.94	Phenolics (total)			
(removal 2 weeks	to Aug.);	Lettuce, (cv.		C-PE	0.95	AC (ABTS)	no		
prior to harvest,		New Red Fire,		Block	0.97	Macro and micro	influence		
mov.)	Spring and	Two Star)		Shade	0.66	nutrients			
	autumn								
Diffuse PE (D-PE)	(lettuce, Feb.		UVA	PE	1.00	Lettuce (TS):			
	to May and		(Summer)	Mov.	6.14	Chlorophyll	PE	1.00^{b}	
Clear PE (no UV	Sept. to Nov.)			D-PE	0.48	(total)	Mov.	1.26^{ab}	
filter, C-PE)				C-PE	3.70		D-PE	1.14^{a}	
				Block	1.44		C-PE	1.00^{ab}	
UVA/B Block				Shade	0.45		Block	1.14^{a}	
(Block)							Shade	1.30^{a}	
			UVB	PE	1.00	Carotenoids			
55% Shade Cloth +			(Summer)	Mov.	6.37	(total)	PE	1.00^{b}	
Standard PE				D-PE	0.43		Mov.	1.20^{ab}	
(Shade)				C-PE	4.17		D-PE	1.08^{ab}	
				Block	0.40		C-PE	0.95^{b}	
				Shade	0.37		Block	1.08^{ab}	
							Shade	1.21^{a}	
						Luteolin-7-			
						glucoside	PE	1.00^{bc}	
							Mov.	1.27^{ab}	
							D-PE	0.66^{bc}	
							C-PE	1.28^{a}	
							Block	0.95^{ab}	
							Shade	0.46^{c}	
						Lettuce (NRF):			
						Carotenoids	PE	1.00^{b}	
						(total)	Mov.	1.01^{b}	
							D-PE	1.19^{a}	
							C-PE	1.04^{ab}	
							Block	1.05^{ab}	
							Shade	1.16^{a}	

Cover material	Construction, region, season	Plant species	Climate conditions measured		Factors investigated			Reference
			Condition	Relative differences due to cover	Factor	Relative differences due to cover		
					Luteolin-7-glucoside	PE	1.00^{b}	
						Mov.	1.74^{a}	
						D-PE	0.62^{b}	
						C-PE	0.93^{a}	
						Block	0.61^{b}	
						Shade	0.32^{c}	
					Chlorogenic acid	PE	1.00^{a}	
						Mov.	0.94^{ab}	
						D-PE	1.16^{a}	
						C-PE	1.20^{a}	
						Block	1.07^{a}	
						Shade	0.78^{b}	
					AC, hydrophilic (ABTS)	PE	1.00^{ab}	
						Mov.	1.02^{ab}	
						D-PE	0.97^{ab}	
						C-PE	1.23^{a}	
						Block	0.77^{bc}	
						Shade	0.65^{c}	
PE-UV transmittive (Window) PE-UV block (block)	GH, Greece, Spring (March- May)	Lettuce (cv. Redino lollo rosso)	UVA transmission	Window (26 % UVA) Block (4,5 % UVA)	Yield	Window	1.00^{b}	Lycoskoufis 2022[39]
						Block	1.33^{a}	
					Phenolics (total)	Window	1.00^{a}	
						Block	0.55^{b}	
					Flavonoids (total)	Window	1.00^{a}	
						Block	0.53^{b}	
					AC, hydrophilic (DPPH)	Window	1.00^{a}	
						Block	0.47^{b}	
Open field (OF)	Tunnel,	Lettuce (cv. Australe,	T	OF 1.00 D-PE 1.04	Yield (year 1, all cultivars)	No influence		O'Connell 2021[135]

Cover material	Construction, region, season	Plant species	Climate conditions measured			Factors investigated			Reference
			Condition	Relative differences due to cover		Factor	Relative differences due to cover		
Double- inflated PE, 95% ultraviolet-block roof and twin-wall polycarbonate ends (D-PE)	Georgia, Spring (March– May)	Baby Green Oakleaf, Bambi, Breen, Dragoon, Rhazes, Spretnek, Maraine)	RH	OF	1.00	Yield (year 2, Baby green oak leaf, Spretnek)	OF	1.00^{b}	
				D-PE	0.98		D-PE	2.52^{a}	
			DLI	OF	1.00	Anthocyanins (Rhazes, total)	OF	1.00^{a}	
				D-PE	0.63		D-PE	0.34^{b}	
Low-density PE UV transparent (window)	GH, Colombia, n.d.	Lettuce (cv. Casabella, Vera, Lollo Rosso)	Temperature	No impact		Yield	No influence		Quintero-Arias 2021[40]
			RH	No impact		Anthocyanins (total, all cultivars)	Window	1.00^{a}	
Low-density PE UV block (up to 380 nm, block)			PAR	Window ~ 10% higher			Block	0.35^{b}	
Open field (OF)	Tunnel, USA, Summer (June – Sept.)	Lettuce (cv. Two Star, New Red Fire)	T	No impact (air circulation due unclosed system)		Macro and micronutrients	Influence (lettuce and tomato)		Woolley 2019[34]
UV clear PE (PE)			PPFD	OF	1.00	Cichoric acid (lettuce TS)	OF	1.00^{a}	
		Tomato (cv. Celebrity, Mountain Fresh)		PE	0.83		PE	0.08^{b}	
			UVA	OF	1.00	Luteolin-7-glucoside (lettuce TS)	OF	1.00^{a}	
				PE	0.67		PE	0.20^{b}	
			UVB	OF	1.00	Gallic acid (lettuce NRF)	OF	1.00^{a}	
				PE	0.53		PE	0.23^{b}	

Cover material	Construction, region, season	Plant species	Climate conditions measured				Factors investigated				Reference
			Condition	Relative differences due to cover			Factor	Relative differences due to cover			
					P2	P3			P2	P3	
Without tunnel (WT)	Mini tunnel	Lettuce (cv. Veronique)	T	WT	1.00	1.00	Yield	WT	1.00^b	1.00^a	Thesis
Low-density PE/EVA with antifog (AF)	Germany, Spring (Feb.; April; May) Autumn (Sept.; Oct.)			AF	1.11	1.11		AF	1.55^a	0.80^b	
Low-density PE/EVA without antifog (WAF)				WAF	1.10	1.11		WAF	1.47^a	0.71^b	
			RH	WT	1.00	1.00	Carotenoids (total)	WT	1.00^c	1.00	
				AF	1.56	1.86		AF	1.12^b	1.20	
				WAF	1.57	1.91		WAF	1.22^a	1.23	
			DLI	WT	1.00	1.00	Chlorophyll (total)	WT	1.00^b	1.00	
				AF	0.74	0.77		AF	1.11^b	1.22	
				WAF	0.60	0.65		WAF	1.21^a	1.27	
			PPFD	WT	1.00	1.00	Flavonoids (total)	WT	1.00^a	1.00^a	
				AF	0.66	0.78		AF	0.40^b	0.26^b	
				WAF	0.54	0.65		WAF	0.40^b	0.23^b	
			UVA	WT	n.d.	1.00	Caffeic acid derivatives (total)	WT	1.00^a	1.00^a	
				AF		0.70		AF	0.69^b	0.85^b	
				WAF		0.71		WAF	0.71^b	0.86^b	
			UVB	WT	n.d.	1.00					
				AF		0.65					
				WAF		0.68					

Polyethylene, PE; ethylene vinyl acetate, EVA; polycarbonate, PC; greenhouse, GH; temperature, T; relative humidity, RH; vapor pressure deficit, VPD; photosynthetic photon flux density, PPFD; daily light integral, DLI; cultivar, cv.; antioxidant capacity, AC; not determined, n.d.

Table B: Daily light integral (mol m^{-2} d^{-1}) determined outside the greenhouse

Experiment	Daily light integral (DLI) outside
October 2019	12.78
February 2020	8.84
April 2021	12.99
May 2021	29.58
September 2021	15.97

Table C: Plant secondary metabolites in lettuce (cv. Veronique, V; Attractie, A) grown under polytunnels with and without antifogging additives or without polytunnels, calculated on a fresh weight basis.

	Total carotenoids (ng mg^{-1} FW)		
Experiment	Without polytunnel	Polytunnel with antifog	Polytunnel without antifog
October 2019	$52.15 \pm 4.83^{(V)}$ $50.64 \pm 0.94^{(A)}$	$42.05 \pm 0.90^{(V)}$ $41.03 \pm 1.74^{(A)}$	$46.99 \pm 1.04^{(V)}$ $39.59 \pm 2.28^{(A)}$
February 2020	39.24 ± 1.05	45.14 ± 1.71	43.29 ± 1.26
April 2021	87.05 ± 0.77	79.09 ± 0.82	80.00 ± 1.12
May 2021	65.58 ± 2.13	119.39 ± 2.66	117.94 ± 2.00
September 2021	87.07 ± 3.51	89.94 ± 1.11	96.02 ± 2.15

	Total chlorophylls (ng mg^{-1} FW)		
Experiment	Without polytunnel	Polytunnel with antifog	Polytunnel without antifog
October 2019	$466.55 \pm 37.14^{(V)}$ $447.68 \pm 11.67^{(A)}$	$432.16 \pm 12.19^{(V)}$ $398.72 \pm 18.12^{(A)}$	$457.71 \pm 14.18^{(V)}$ $400.15 \pm 24.81^{(A)}$
February 2020	375.71 ± 9.07	392.39 ± 16.75	387.70 ± 11.39
April 2021	510.48 ± 3.20	489.66 ± 3.79	495.39 ± 11.67
May 2021	320.21 ± 9.00	560.27 ± 22.38	550.29 ± 4.84
September 2021	436.45 ± 17.26	471.92 ± 6.94	540.08 ± 12.34

	Total caffeic acid derivatives (ng mg^{-1} FW)		
Experiment	Without polytunnel	Polytunnel with antifog	Polytunnel without antifog
October 2019	$576.00 \pm 50.09^{(V)}$ $320.11 \pm 22.83^{(A)}$	$228.01 \pm 17.27^{(V)}$ $197.30 \pm 20.45^{(A)}$	$188.59 \pm 7.09^{(V)}$ $177.13 \pm 9.95^{(A)}$
February 2020	260.33 ± 10.13	222.94 ± 10.80	224.71 ± 9.28
April 2021	350.19 ± 7.26	272.64 ± 2.38	301.62 ± 12.61
May 2021	435.16 ± 6.49	287.01 ± 5.25	284.49 ± 7.20
September 2021	n.d.	n.d.	n.d.

	Total flavonoids (ng mg^{-1} FW)		
Experiment	Without polytunnel	Polytunnel with antifog	Polytunnel without antifog
October 2019	$70.43 \pm 7.66^{(V)}$ $51.17 \pm 4.46^{(A)}$	$15.27 \pm 1.35^{(V)}$ $16.65 \pm 1.88^{(A)}$	$15.11 \pm 0.55^{(V)}$ $14.78 \pm 1.14^{(A)}$
February 2020	39.72 ± 1.70	19.50 ± 0.85	18.33 ± 0.74
April 2021	74.92 ± 3.30	16.36 ± 0.79	16.59 ± 0.39
May 2021	191.29 ± 6.92	28.33 ± 4.47	23.36 ± 0.52
September 2021	36.92 ± 5.30	19.80 ± 1.54	20.23 ± 0.54

Not determined, n.d.

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Icons in this thesis are made by Freepik from www.flaticon.com.

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PUBLICATIONS, CONFERENCES AND PROFESSIONAL TRAININGS

Articles in peer-reviewed journals

A. Fricke, V. Harbart, M. Schreiner, S. Baldermann, A proof of concept for inland production of the "sea-vegetable" *Ulva compressa* in Brandenburg (Central Europe) using regional saline groundwater, Algal Research, 103226, 2023. DOI: 10.1016/j.algal.2023.103226.

A. Fricke, V. Harbart, M. Schreiner, S. Baldermann, Study on the nutritional composition of the sea vegetable *Ulva compressa* in a brine-based cultivation system, Frontiers in Marine Science, 10:1292947, 2023. DOI: 10.3389/fmars.2023.1292947.

Presentations

Vergleich von destruktiven und nicht-destruktiven Methoden zur Bestimmung des Gehaltes von Chlorophyllen und Flavonolen im Kopfsalat, 2022, Auftakt der Regionalverbandstagung Lebensmittelchemische Gesellschaft (LChG, organized by AG JLC), digital.

Poster presentations

V. Harbart, S. Baldermann, The cover matters - Impact of antifogging agents on food security and quality, 2019, N^2 PhD network event, Berlin, Germany.

V. Harbart, S. Baldermann, Accessing the impact and migration of antifogging additives from plastic films into leafy vegetables, 2021, Lebensmittelchemikertag, digital.

V. Harbart, S. Baldermann, Impact of antifogging food packaging material on phenolic compounds in green and red lettuce cultivars, 2021, XXI European Food Chemistry Conference, digital.

V. Harbart, S. Baldermann, A rapid and simple method for determining antifogging additives foliar applied to plants and in soil, 2022, Analytica Conference, München, Germany.

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Professional trainings

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CURRICULUM VITAE

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung von anderen als den angegebenen Hilfsmitteln und Quellen angefertigt habe.

Die Arbeit wurde an keiner anderen Universität eingereicht.

Berlin, 24.08.2023

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Vanessa Harbart