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MULCH SURFACE COLOR AFFECTS ACCUMULATION OF EPICUTICULAR WAX ON DEVELOPING LEAVES

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Abstract—Leaf waxes are important to plant growth because they impede water loss and may influence entry of light. Leaf wax quantity and composition were studied in *Capsicum annuum* plants in trickle-irrigated field plots covered with white, black or red plastic mulches. The quantity of reflected blue light (BL) was greatest over white and about the same over black *versus* red surfaces; and reflected far-red to red ratios (FR/R) were about the same over white *versus* black and higher over red. The greatest quantity of total epicuticular wax developed on leaves of plants grown over white mulch (443 $\mu g/cm^2$) while plants grown over black and red mulches had 229 and 227 $\mu g/cm^2$, respectively. When individual lipid classes were expressed as percentages of the totals, esters constituted the highest percentages over white and black surfaces while secondary free fatty alcohols dominated over red. The higher ester content of epicuticular wax on plants grown over black (*versus* red) was almost exactly equalled by increased contents of free fatty alcohols and free fatty acids in the epicuticular wax of plants grown over red mulch. We conclude that the total wax concentration on leaves of field-grown plants was influenced by quantity of BL, and the percentage of individual components was influenced by the FR/R ratio.

INTRODUCTION

Plastic mulches over trickle irrigation systems are widely used to conserve water and to control weeds (with less dependence on herbicides) in production of high value food crops. Black mulches are commonly used to warm the soil in spring, and white-surfaced mulches are often used later in the season to avoid overheating the root zone. Recently, it was discovered that the quantity and spectral distribution of light reflected from different colored soil surfaces or from different colored mulches could act through photomorphogenic pigments such as phytochrome to regulate plant growth and development.[1–4] Basically, different colored mulches can be selected to reflect different quantities of blue (BL)† and photosynthetic light as well as different photon ratios of far-red (FR) relative to red (R). The FR/R ratio can be preselected to mimic effects of different plant population densities and regulate partitioning of photosynthate among new leaf, stem, root and fruit growth.[2,5–8]

Leaf area, stem length, root mass and shoot/root biomass ratios of seedlings were influenced to the same degree by reflected light whether the reflecting surface was a natural soil or an artificially colored material, if the surfaces reflected the same quantity and spectral ratio of light.[9–11] In all of these studies, seedlings grown over white surfaces received greater quantities of reflected photosynthetic light and BL but lower FR/R ratios than those grown over the red surfaces. Even though they received more total photosynthetic light, plants grown in summer sunlight over white surfaces were usually shorter, had thicker leaves, lower shoot/root weight ratios and frequently had less total weight when compared with plants grown over red surfaces.[4] Clearly, the light environment during leaf development can induce morphological and physiological differences that affect photosynthate production and partitioning.[6–14] Some studies have shown that the light that actually gets into a leaf is influenced by the quantity and/or composition of its epicuticular waxes, which may serve as barriers to some wavelengths or they may provide a lens effect to focus light within the tissue.[15–17]

Under controlled environments, variables such as photoperiod, light intensity and the FR/R ratio influence epicuticular components.[18,19] For example, epicuticular wax composition was found to be dependent upon intracellular metabolism where the quantity of isoleucine and valine available for deamination and elongation to long-chain fatty acyl units (which are ultimately extruded to the leaf surface to form branched-chain fatty acids) is determined.[19–21] Altered intracellular metabolism decreased the concentration of isoleucine and/or valine in the cytosol and branched-chain lipids were not present in the epicuticular wax.[20,21]

Long chain fatty acyl units are precursors of: (1) fatty alcohols, (2) alkanes, (3) esters and (4) fatty acids.[22] The fatty alcohols are formed by reduction of the fatty acyl group, alkanes are formed by fatty acyl decarboxylation and esters are formed by joining of a fatty acid carboxyl group with a fatty alcohol hydroxyl group.[22] Thus, although fatty

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†*Abbreviations:* BL, blue light; FFAc, free fatty acids; FFAlc, free fatty alcohols; 1°FFAlc, primary free fatty alcohols; 2°FFAlc, secondary free fatty alcohols: FR, far-red light; GLC, gas–liquid chromatography. PPF, photosynthetic photon flux; PM, plasma membrane; R, red light; TLC, thin-layer chromatography.

acids and fatty alcohols are presumed to be formed intracellularly in the epidermis and extruded through the plasma membrane (PM), alkanes and esters would have to be formed at, near or on the exofacial surface of the PM. Results of those controlled-environment studies suggest that significant changes in the quantity and composition of epicuticular waxes on plants grown over different colored mulches may result from photocontrol of synthesis at or in the PM. Thus, quantity and composition of epicuticular waxes on leaves that developed in field plots over white, black and red plastic mulches were evaluated.

MATERIALS AND METHODS

Bell pepper (*Capsicum annuum* L. cv. 'Keystone') seedlings were started in 5 cm pots of potting soil and grown to a height of about 5 cm in a greenhouse before transplanting to field plots for evaluation of relationships among reflected light, plant growth and accumulation of leaf waxes. The field plots were located at the USDA-ARS Coastal Plains Soil, Water, and Plant Research Center near Florence, SC. The soil type was Norfolk loamy sand (Typic Paleudults). Plot preparation was according to recommendations of the Clemson University Extension Service. The beds were formed (80 cm wide and 10 cm high), fumigated and covered 2 weeks before transplanting. Trickle irrigation tubing, methyl bromide fumigant and black polyethylene mulch (1.2 m wide and 0.03 mm thick) were applied at the same time with a once-over commercial mulch layer. There were four 18 m long rows, each divided into three 6 m long subplots (mulch colors). The rows (replicates) were 1.8 m apart.

Mulch surface colors were established by application of white and red exterior enamels to the surface of the black polyethylene mulches. Black was obtained by leaving the mulch unpainted. Use of paint to provide different surface colors provided an economical and repeatable approach for these small-plot studies. The three colors were randomized within each of the four rows. Ten centimeter diameter holes were cut at 45 cm intervals in the plastic covered beds. The transplants were hand set in these holes on 5 May. In-row tensiometers were used to determine when to irrigate the plots.

Reflected light from each mulch color was determined at solar noon ± 30 min on a cloudless day, using a LiCor-1800 Spectroradiometer (LiCor Inc., Lincoln, Nebraska) with a remote, hemispherical, cosine-corrected light collector on a 1.5 m fiber optic probe. Upwardly reflected light was measured 20 cm above the mulch surfaces to compare quantities and spectral differences received by developing leaves over the various colors. Measurements were taken at 5 nm intervals from 400 to 750 nm. Incoming sunlight was also measured, and the reflected light was expressed as a percentage of incoming sunlight at each measured wavelength. Spectral irradiances at 735 and 645 nm were used to calculate the FR/R ratios. These values were used because they approach the peaks for phytochrome action spectra in green plants; 645 nm was used instead of 660 nm because chlorophyll competition for light at 660 nm (the approximate phytochrome absorbance maximum *in vitro*) shifts the phytochrome action peak to about 645 nm in green plants.[23] The FR/R ratios shown in this report are relative to the ratio in incoming sunlight, which was arbitrarily assigned a value of 1.00. The rationale for this approach is that plants are adapted to sunlight, and an FR/R ratio that deviates from that in incoming sunlight might signal an adaptive response to the altered light environment.[24]

Stem lengths of 10 consecutive plants were measured from each of the four replicates per color, and leaf discs were sampled for specific leaf weights and epicuticular waxes on 22 June. Two leaf discs were cut (with an 18 mm diameter cork borer) from one recently expanded leaf from each of three plants from each of the four replicates of each color. The leaf discs were freeze-dried and weighed.

Epicuticular waxes were extracted in boiling chloroform ($CHCl_3$) for 1 min. Appropriate internal standards were added. The solvent was evaporated to <5 mL. Aliquots were subjected to thin-layer chromatography (TLC) for separation of the lipid classes.[19,20] Alkanes were eluted in $CHCl_3$ and concentrated into 3 mL septum closed vials under N_2. Free fatty acids (FFAc) were eluted from the TLC, solvent evaporated and the FFAc were esterified in 10 mL anhydrous methanol-BF_3 with heat (65°C) for 1 h in screw-capped test tubes. The fatty acid methyl esters (FFAc-Me) were separated into *n*-pentane after the addition of deionized water. Free fatty alcohols (FFAlc) were esterified in concentrated formic acid plus 2 drops concentrated H_2SO_4 at 65°C for 1 h. Separation was into *n*-pentane as discussed above. Esters were eluted from the TLC in $CHCl_3$ and concentrated into 3 mL vials under N_2.

Individual constituents of each of the lipid classes were separated *via* gas–liquid chromatography (GLC) and quantitated by ratios to known amounts of internal standards. Total lipid classes per replication per mulch color were calculated and subjected to analysis of variance on a randomized complete block design, and means were separated by the Waller–Duncan least significant difference method.

Table 1. Reflected light from different colored mulches expressed relative to incoming sunlight in the same wavebands

Light characteristics	Mulch surface color		
	White	Black	Red
Upwardly reflected light* (relative to incoming light)			
PPF (400–700 nm) (%)	43	5	14
Blue (BL, 400–500 nm) (%)	41	5	6
Red (R, 645 ± 5 nm) (%)	44	5	35
Far-red (FR, 735 ± 5 nm) (%)	44	5	40
FR/R (ratio)	1.00	—†	1.14

*PPF, photosynthetic photon flux; FR/R, photon ratios are calculated relative to ratio in incoming sunlight, which was assigned a value of 1.00.

†Reflection from black was about 5% across the spectrum. At such low reflectance a very minor difference in reflection in R or FR could result in an apparently large but meaningless ratio.

RESULTS AND DISCUSSION

Reflected light

The percentages of light reflected (relative to incoming light at the same wavelengths) differed over the various colors of mulch (Table 1). Reflected photosynthetic photon flux (PPF) and BL were greatest over white and PPF was least over black surfaces. The red and black surfaces reflected about the same amount of BL, whereas red and white surfaces reflected similar amounts of R. The highest FR/R ratio was reflected from red mulch.

Because of earlier studies on the Beltsville Spectrograph,[23] as well as investigation of plant responses to population density under field conditions,[7,11,24] we hypothesized that even small differences in the FR/R ratio over the variously colored mulch surfaces could have a significant impact on plant development. If true, a higher FR/R ratio (as was found over the red surfaces) should signal the plants to develop characteristics that would favor survival among relatively more competition from other plants, whereas a greater amount of BL and an FR/R ratio as in incoming sunlight (as found over the white surfaces) should signal the plants to develop characteristics that favor survival in very bright sunlight, away from competing plants.

Plant responses

Stem length, leaf thickness and quantity of epicuticular wax differed among plants grown over the various colors of mulch (Table 2). It should be noted that all of the plants in the present study received the same quantity and spectral

Table 2. Plant size, specific leaf weight and total waxes on leaves of pepper plants grown in field plots near Florence, SC, from 5 May until 22 June over different colored mulches

Characteristic	Mulch surface color		
	White	Black	Red
Plant height (mm)	364 b*	401 a	425 a
Leaf dry weight/area (mg/cm^2)	4.64 a	4.06 b	3.98 b
Total leaf waxes expressed as:			
μg/cm^2	443 a	229 b	227 b
μg/mg dry weight	95.5 a	56.5 b	57.0 b

*Values in the same line followed by the same letter are not significantly different at the 5% level.

distribution of incoming sunlight in the field, but they were grown over different colors of soil covers, which reflected different quantities and spectral distributions of light. Stems were shortest, specific leaf weights were greatest and quantities of epicuticular wax were greatest on both leaf area and leaf weight bases when the plants were grown over the white surfaces. The stem length and specific leaf weight responses over different colored mulches were consistent with earlier observations with cotton.[25] The present results with pepper plants that were grown in colored mulch-covered field plots are also consistent with earlier controlled-environment studies in which a low FR/R ratio resulted in thicker leaves than developed on plants that received a higher FR/R ratio.[5,26] Clearly, spectral distribution and quantity of light reflected from the soil surface can influence plant morphology and the quantity of total waxes. Additional analyses were done to determine whether the different components of epicuticular waxes would also respond differently to reflection from different colored mulches under field conditions.

Leaf waxes

Concentrations of epicuticular wax components present on the leaf discs were influenced by the mulch color (Table 3). Because the total quantity of fatty acyl units available for extrusion through the PM would be strongly influenced by intracellular metabolism, these data are explicable on the basis of the quantity of long-chain fatty acyl precursors available for metabolism inside and outside the PM. White mulch reflected the greatest quantities of BL and photosynthetic light (Table 1) and the leaves developed over white mulch had greater quantities of total epicuticular wax (Table 2). This corroborates previous reports that light intensity contributes,[19,20] but the quantity of BL might be the component that is sensed by the plant as an indicator of "intensity," as suggested by controlled-environment studies.[27,28]

Alkane concentrations varied among plants grown over the different mulch colors (Table 3). Because the conversion of fatty acyl → alkanes occurs on or at the PM exofacial surface,[22] these differences might be influenced by a phytochrome-controlled enzyme reaction near, at or on the exofacial PM surface. Similar considerations apply to the esters (Tables 3 and 4). Secondary free fatty alcohol (2°FFAlc) percentages also varied among plants grown over different colored mulches, but these constituents could be synthesized intracellularly and extruded through the PM. Therefore, 2°FFAlc percentages

Table 3. Concentrations of alkanes, esters, secondary free fatty alcohols (2°FFAlc), primary free fatty alcohols (1°FFAlc) and free fatty acids (FFAc)*

Lipid class	Mulch surface color			
	White	Black (B)	Red (R)	Δ† (B-R)
A. On area basis (μg/cm^2)				
Alkanes	1.4 b‡	2.5 a	0.1 c	2.4↓
Esters	180.4 a	80.9 b	63.3 c	17.6↓
2°FFAlc	100.5 a	59.8 c	70.5 b	10.7↑
1°FFAlc	107.0 a	49.6 b	50.5 b	0.9↑
FFAc	54.0 a	36.6 b	42.8 b	6.2↑
B. On dry weight basis (μg/mg of dry leaf)				
Alkanes	0.30 b	0.62 a	0.03 c	0.59↓
Esters	38.86 a	19.94 b	15.90 c	4.04↓
2°FFAlc	21.65 a	14.73 c	17.70 b	2.97↑
1°FFAlc	23.05 a	12.22 b	12.68 b	0.46↑
FFAc	11.63 a	9.02 b	10.75 ab	1.73↑

*Values are expressed as weight of each component per cm^2 of leaf surface and per mg of dry leaf tissue.

†Δ = Epicuticular wax component on plants grown over black mulch minus the epicuticular wax component on plants grown over red mulch.

‡Values on a line followed by the same letter are not significantly different at the 5% level as determined by least significant differences.

might be explicable as an intracellular phytochrome response. Because fatty acyl units are the precursors of FFAc, alkanes, FFAlc and esters, the FFAc content is probably a measure of fatty acyl units not utilized in the biosynthesis of the other constituents, but FFAc content was responsive to mulch color (Table 3). Thus, quantity and composition of leaf epicuticular wax were influenced by spectral composition of the light reflected from mulches located on the soil surface below the developing plants. The data suggest a regulatory system for the production of epicuticular waxes that appears to be much more responsive to phytochrome control in field-grown plants than previously recognized.

In earlier controlled-environment studies, sicklepod (*Cassia obtusifolia* L.) responses to photoperiod differed for plant height and weight, total epicuticular wax development and epicuticular wax constituent formation.[18] Tobacco (*Nicotiana tabacum* L.) epicuticular wax alkane, FFAc and FFAlc contents were strongly influenced by photoperiod and spectral distribution,[19,20] both of which act through the phytochrome system. The biosynthetic mechanisms, inhibitors and carbon pathways have been established in barley (*Hordeum*

Table 4. Individual lipid classes expressed as percentages of totals from surfaces of leaves that developed on plants grown over white, black and red mulches

Lipid class	Mulch surface color		
	White	Black	Red
Alkanes	0.3	1.1	<0.1
Esters	**40.7**	**35.3**	27.8
2°FFAlc	22.7	26.1	**31.0**
1°FFAlc	24.1	21.6	22.2
FFAc	12.2	15.9	18.8

vulgare L.).[29–31] After elongation of fatty acyl units to C_{22}–C_{34}, the fatty acyl units are converted to: (1) alkanes *via* decarboxylation,[22] (2) FFAc,[31] (3) 1°FFAlc or 2°FFAlc, *via* reduction[31] and (4) esters *via* esterification of FFAlc + FFAc.[29–31] Photosynthetic photon flux density and quantity of BL over the white mulch were greater than over the red or black mulch (Table 1), and quantity of epicuticular wax was significantly greater over the white mulch than over the black or red mulches (Table 2). The total quantities of epicuticular wax on leaves grown over the black and red mulches were not statistically different (Table 2) but the composition of the waxes suggested an FR/R influence (Tables 3 and 4).

Alkane concentrations ($\mu g/cm^2$) were decreased in plants grown over the red relative to black mulch and this decrease in alkane concentration (Table 3) was almost identical to the decrease in total wax ($\Delta\mu g/cm^2$) on leaves of plants grown over the red mulch. Although this change in alkane concentration was approximately 1% or less of the total epicuticular wax content (Table 4), it resulted in a >20-fold difference in alkane concentration ($\mu g/cm^2$) on the leaves of plants grown over black *versus* red mulches. In essence, alkane production was virtually eliminated in plants grown over the red mulch (Table 3). Thus, it appears that the higher FR/R ratio reflected from the red mulch influenced alkane biosynthesis. Because (1) alkanes are produced by a decarboxylation reaction,[22] (2) alkanes are transported very slowly through PM[32] and (3) a phytochrome response requires the presence of the cytosol, this change in alkane concentration must occur in or at the surface of the PM so that the alkanes were released outside the PM.

Although all classes were in highest concentrations in leaves grown over the white surfaces (Table 3), esters were the most abundant of the classes in waxes on leaves grown over both white and black surfaces and second most abundant over red surfaces (Tables 3 and 4). It should be noted that white surfaces reflected the most BL, white and black reflected about the same FR/R ratio and red reflected the highest FR/R ratio (Table 1).

Ester concentration decreased while 2°FFAlc increased in the epicuticular wax on leaves grown over red mulch in comparison to leaves grown over black mulch (Table 3A). This decrease of 17.6 $\mu g/cm^2$ was equivalent to a 21.7% decrease in ester concentration in plants grown over the red mulch. The decrease in ester concentration on leaves of plants grown over the red mulch was almost exactly balanced by increased concentrations (17.8 $\mu g/cm^2$) of 2°FFAlc, 1°FFAlc and FFAc (Table 3A). Long chain esters are carried through the PM with difficulty[32]; but triglyceride components are readily transported through the PM[32] and a phytochrome response would require the presence of cytosol. Thus, these changes might be explicable by phytochrome influence on the esterase responsible for the formation of esters from 2°FFAlc, 1°FFAlc and FFAc.

In summary, our results suggest that the enzymatic biosyntheses of alkanes and esters are influenced by phytochrome. Whether these influences are within the cytosol or PM has yet to be determined, as is the role of BL receptors. The results also suggest along with those of Bradburne *et al.*[25] that mulch color can affect the chemical composition and quality of sun-grown leaf crops.

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