Role of Smoke Stimulatory and Inhibitory Biomolecules in Phytochrome-Regulated Seed Germination of *Lactuca sativa*[1][OPEN]

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The biologically active molecules karrikinolide (KAR_1) and trimethylbutenolide (TMB) present in wildfire smoke play a key role in regulating seed germination of many plant species. To elucidate the physiological mechanism by which smoke-water (SW), KAR_1, and TMB regulate seed germination in photosensitive 'Grand Rapids' lettuce (*Lactuca sativa*), we investigated levels of the dormancy-inducing hormone abscisic acid (ABA), three auxin catabolites, and cytokinins (26 isoprenoid and four aromatic) in response to these compounds. Activity of the hydrolytic enzymes α-amylase and lipase along with stored food reserves (lipids, carbohydrate, starch, and protein) were also assessed. The smoke compounds precisely regulated ABA and hydrolytic enzymes under all light conditions. ABA levels under red (R) light were not significantly different in seeds treated with TMB or water. However, TMB-treated seeds showed significantly inhibited germination (33%) compared with water controls (100%). KAR_1 significantly enhanced total isoprenoid cytokinins under dark conditions in comparison with other treatments; however, there was no significant effect under R light. Enhanced levels of indole-3-aspartic acid (an indicator of high indole-3-acetic acid accumulation, which inhibits lettuce seed germination) and absence of trans-zeatin and trans-zeatin riboside (the most active cytokinins) in TMB-treated seeds might be responsible for reduced germination under R light. Our results demonstrate that SW and KAR_1 significantly promote lettuce seed germination by reducing levels of ABA and enhancing the activity of hydrolytic enzymes, which aids in mobilizing stored reserves. However, TMB inhibits germination by enhancing ABA levels and reducing the activity of hydrolytic enzymes.

Seeds can interact and delineate whether the environmental conditions and cues such as air or oxygen, temperature, water, light, or darkness are suitable for germination (Finch-Savage and Leubner-Metzger, 2006; Oracz and Stawska, 2016). These environmental signals may have promotive or inhibitory roles in germination. The chemical germination cues from plant-derived smoke are of particular interest due to its prominent effects on seed germination of a wide variety of plants. Wildfire smoke contains certain potent bioactive compounds (butenolides) that play a major role in regulating the germination of many plant species, predominantly grasses and shrub species from fire-prone ecosystems (De Lange and Boucher, 1990; Adkins and Peters, 2001; Dixon et al., 2009) but also many non-fire-dependent plants such as rice (*Oryza sativa*), wild oats (*Avena sativa*), and lettuce (*Lactuca sativa*; Kulkarni et al., 2006; Light et al., 2009). Karrikinolide (KAR_1; 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one), a butenolide derived from smoke, exhibits germination promotive activity (Flematti et al., 2004; Van Staden et al., 2004). Conversely, trimethylbutenolide [TMB; 3,4,5-trimethylfuran-2(5*H*)-one] shows germination inhibitory activity (Light et al., 2010). These molecules have great ecological significance, as seeds with KAR_1 regulation germinate when there are fewer competitors and more resources available. The advantage of TMB regulation is that seeds do not germinate until sufficient water is available (Light, 2006). The inhibitory compound TMB is leached with sufficient rainfall and

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provides a mechanism for preventing germination until the conditions are suitable (De Lange and Boucher, 1993). In cv Grand Rapids lettuce, germination is induced by light, and in the dark at a suitable temperature, little or no germination is observed (Borthwick et al., 1952). Red (R) light treatment induces, whereas, far-red (FR) light suppresses, lettuce seed germination. Thus, the regulation of germination in lettuce is strongly influenced by the phytochrome system. However, exogenous application of smoke-water (SW) and smoke promotive biomolecule (KAR_1) to lettuce seeds in the dark replaces the light requirement, resulting in germination. On the contrary, the smoke inhibitory biomolecule (TMB) completely suppresses the germination of lettuce seeds (Van Staden et al., 2004; Light, 2006; Light et al., 2010). SW and KAR_1 have been shown to partially overcome the effect of FR light (Van Staden et al., 1995; Soós et al., 2012). The mechanism by which the plant-derived smoke and its bioactive components regulate seed germination in light-sensitive lettuce seeds is a topic of curiosity among plant physiologists. It was thought that smoke affects membrane permeability or receptor sensitivity rather than influencing the phytochrome system of light-sensitive lettuce seeds (Van Staden et al., 1995). However, the clear mechanism by which these smoke-derived compounds relay the signal to promote or inhibit seed germination in cv Grand Rapids lettuce seeds has not yet been elucidated.

Light signals received by phytochromes are converted to internal cues, which in turn regulate physiological processes in seeds. GA and abscisic acid (ABA) are the internal signals that play central roles in the regulation of seed germination; GA induces, whereas ABA inhibits, seed germination. Recent studies have begun to reveal a strong interaction between light, GA, and ABA signaling pathways in seeds at the molecular level (Soós et al., 2012).

Pfr is the bioactive form of phytochrome induced by R light that promotes seed germination. Pfr is converted to Pr by FR light and suppresses lettuce seed germination. In the dark, Pr is dominant, restricting seed germination. The reversal of germination inhibition is achieved only in light or R light, as all the Pr is converted to Pfr. Plant hormones are essential in all physiological and developmental processes occurring during phytochrome-regulated seed germination (Seo et al., 2009). The endogenous levels of ABA are down-regulated by Pfr in lettuce seeds (Toyomasu et al., 1993, 1994). Levels of the dormancy-inducing hormone, ABA, increase during the onset of dormancy during seed development (Finkelstein et al., 2008), preventing germination by inhibiting the stimulation of endosperm metabolism (Müller et al., 2006). On the contrary, R light treatment up-regulates the endogenous cytokinin levels and FR light reverses this effect (Van Staden, 1973). The inhibition of germination by ABA is only reversed with cytokinins (Van Staden and Wareing, 1972; Van Staden, 1973). However, the connection between light and cytokinin-mediated signaling is still unclear (Seo et al., 2009). There are many reports of phytohormones (such as ABA, auxins, and cytokinins) playing a role in nutrient mobilization during seed germination (Finkelstein and Rock, 2002; Fahad et al., 2015). In the presence of light, the stored food reserves are enzymatically broken down to simpler components and translocated to the embryo, the process known as mobilization, where they provide an energy source for growth. It appears that SW and KAR_1 either substitute R light through interconversion of Pr to Pfr or are somehow involved in phytochrome-mediated signaling of hormones such as ABA or cytokinins (Van Staden et al., 1995). The germination inhibitor TMB might have the reverse role, substituting for the FR light effect. The physiological mode of action of SW- and KAR_1-stimulated germination and TMB-induced suppression of germination is not yet fully understood. A better understanding of the classical role of these smoke stimulatory and inhibitory potent bioactive molecules is necessary to utilize their full potential for biological, ecological, and physiological implications. In this study, the antagonistic relationship between KAR_1 and TMB, in terms of their physiological mode of action, was investigated in phytochrome-regulated seed germination of cv Grand Rapids lettuce.

RESULTS

Influence of SW, KAR_1, and TMB on Lettuce Seed Germination

The effects of SW, KAR_1, and TMB on the germination of cv Grand Rapids lettuce seeds after 24 h were compared for dark and R and FR light (Fig. 1). At 25°C in the dark, germination in water control seeds was 12%. However, when seeds were treated with KAR_1 and SW, the germination increased to 94% and 92%, respectively. TMB treatment almost completely inhibited seed germination (1%) in the dark (Fig. 1A). In R light (1 h of exposure after 3 h of dark incubation), seeds treated with KAR_1 and water control showed 100% germination and SW treatment resulted in 99% germination. Conversely, treatment with TMB significantly inhibited germination (33%) in R light (Fig. 1B). In FR light (1 h of exposure after 3 h of dark incubation), no germination was recorded in TMB-treated seeds. However, SW- and KAR_1-treated seeds significantly reversed the effect of FR light and exhibited 28% and 35% seed germination, respectively (Fig. 1C). The water control showed 6% germination. TMB and KAR_1 (along with SW) significantly reversed the effects of R and FR light, respectively.

Influence of SW, KAR_1, and TMB on Endogenous Phytohormones

The endogenous levels of ABA; 26 natural isoprenoid cytokinins comprising the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (plastid)-derived cytokinins *tZ*-type cytokinins (*tZ*, trans-zeatin; *tZR*, trans-zeatin

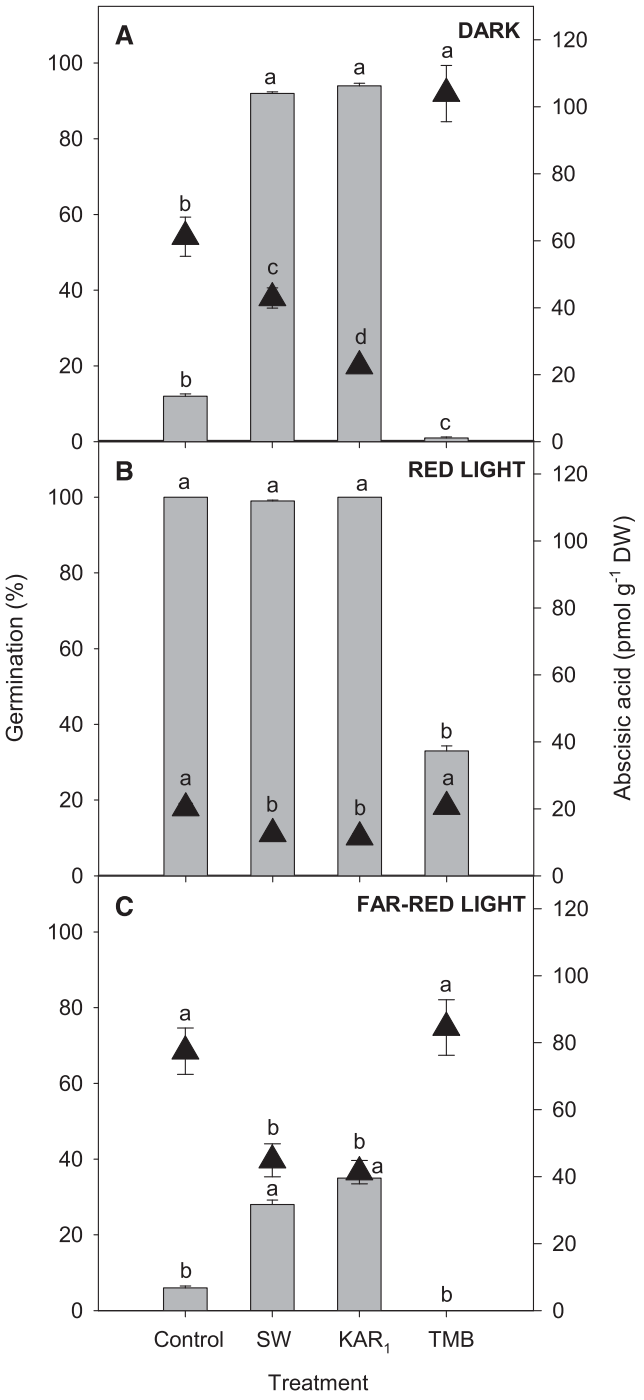

Figure 1. Effects of SW, KAR_1, and TMB on germination ($n = 4$) and ABA ($n = 3$) levels in cv Grand Rapids lettuce seeds under different light conditions for 24 h at 25°C. After 3 h of incubation in the dark, seeds were exposed to R or FR light treatment for 1 h and were replaced in the dark. Bars (germination ± SE) and symbols (ABA ± SE) for each light condition with different letters are significantly different according to Bonferroni correction ($P < 0.05$). DW, Dry weight.

riboside; *t*ZOG, trans-zeatin-*O*-glucoside; *t*ZROG, trans-zeatin riboside-*O*-glucoside; *t*Z7G, trans-zeatin-7-glucoside; *t*Z9G, trans-zeatin-9-glucoside; and *t*ZR5′MP, trans-zeatin riboside 5′-monophosphate), DHZ-type cytokinins (DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; DHZOG, dihydrozeatin-*O*-glucoside; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZ7G, dihydrozeatin-7-glucoside; DHZ9G, dihydrozeatin-9-glucoside; and DHZR5′MP, dihydrozeatin riboside 5′-monophosphate), N^6-(2-isopentenyl)adenine-type (iP) cytokinins [iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenosine; iP7G, N^6-(2-isopentenyl)adenine-7-glucoside; iP9G, N^6-(2-isopentenyl)adenine-9-glucoside; and iPR5′MP, N^6-(2-isopentenyl)adenosine-5′-monophosphate], the mevalonate (MVA) pathway (cytosol)-derived cytokinin *c*Z-type cytokinins (*c*Z, cis-zeatin; *c*ZR, cis-zeatin riboside; *c*ZOG, cis-zeatin-*O*-glucoside; *c*ZROG, cis-zeatin riboside-*O*-glucoside; *c*Z7G, cis-zeatin-7-glucoside; *c*Z9G, cis-zeatin-9-glucoside; and *c*ZR5′MP, cis-zeatin riboside 5′-monophosphate); four aromatic cytokinins (*m*T, *meta*-topolin; *m*TR, *meta*-topolin riboside; *m*T7G, *meta*-topolin-7-glucoside; and *m*T9G, *meta*-topolin-9-glucoside); and three auxin conjugates (IAAsp, IAA-3-Asp; IAAGlu, IAA-3-Glu; and oxIAA, 2-oxindole-3-acetic acid) were determined in cv Grand Rapids using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The identity of ABA, auxins, and all cytokinins was verified by comparing the mass spectra and chromatographic retention times with those of authentic standards.

Endogenous ABA levels were significantly higher in seeds treated with TMB and water control in dark, R light, and FR light, whereas they were significantly lower when seeds were treated with SW or KAR_1 (Fig. 1). In the dark, the maximum endogenous ABA levels were detected in TMB-treated seeds (95.49 ± 8.4 pmol g^{-1}) followed by water control (61.21 ± 5.84 pmol g^{-1}), SW (42.91 ± 3.02 pmol g^{-1}), and KAR_1 (22.57 ± 1.69 pmol g^{-1}; Fig. 1A). In R light, the endogenous levels of ABA in TMB-treated seeds (20.21 ± 1.4 pmol g^{-1}) and water control (19.29 ± 1.83 pmol g^{-1}) were higher than in seeds treated with SW (14.7 ± 2.3 pmol g^{-1}) and KAR_1 (16.0 ± 4.5 pmol g^{-1}; Fig. 1B). In FR light, seeds treated with TMB (84.50 ± 8.31 pmol g^{-1}) showed the highest ABA levels followed by water control (77.45 ± 6.9 pmol g^{-1}), SW (44.86 ± 4.93 pmol g^{-1}), and KAR_1 (41.34 ± 3.5 pmol g^{-1}; Fig. 1C). The endogenous ABA levels in seeds treated with TMB were 4.23-, 1.26-, and 2.04-fold higher than those of KAR_1-treated seeds in dark, R light, and FR light, respectively. These results indicated an overall negative correlation ($R^2 = -0.84$) between germination and ABA production in cv Grand Rapids seeds.

The cytokinin pool consists of free bases *t*Z, *c*Z, DHZ, iP, and *m*T and their corresponding riboside, *O*-glucoside, 7-glucoside, 9-glucoside, and ribotide (5′-monophosphate) conjugates (Tables 1 and 2). MEP pathway-derived isoprenoid cytokinins such as *t*Z9G, DHZ7G, DHZ9G, DHZR5′MP, iP7G, and iP9G, MVA pathway-derived cytokinins such as *c*Z7G and *c*Z9G, and aromatic cytokinins such as *m*TR, *m*T7G, and *m*T9G were totally absent in all the treatments (Tables 1 and 2). The most prominent cytokinins were MVA pathway-derived less

Table 1. *Endogenous isoprenoid cytokinin content (pmol g^{-1}) in cv Grand Rapids lettuce seeds incubated for 24 h at 25°C* (n = 3)

After 3 h of incubation in the dark, the seeds were exposed to R or FR light treatment for 1 h and were replaced in the dark. Mean values for each cytokinin type and light condition in a column with different letter(s) are significantly different according to Bonferroni correction ($P < 0.05$). Dashes represent values below detection levels. Treatments were as follows: SW (1:2,500, v/v), KAR_1 (10^{-7} M), and TMB (10^{-7} M).

Cytokinin	Treatment	Dark	R Light	FR Light	Dark	R Light	FR Light	Dark	R Light	FR Light	Dark	R Light	FR Light
Ribotide			*t*ZR5′MP			*c*ZR5′MP			DHZR5′MP			iPR5′MP	
	Control	–	–	–	1.6 ± 0.1 a	4.6 ± 0.3 a	1.8 ± 0.1 a	–	–	–	–	2.5 ± 0.1 a	–
	SW	–	–	–	–	2.3 ± 0.1 a	–	–	–	–	–	2.7 ± 0.1 a	–
	KAR_1	–	1.6 ± 0.2 a	–	1.9 ± 0.1 a	3.5 ± 0.1 a	1.5 ± 0.1 a	–	–	–	1.5 ± 0.0 a	1.9 ± 0.1 a,b	–
	TMB	–	–	–	–	–	–	–	–	–	–	1.1 ± 0.1 b	–
Riboside			*t*ZR			*c*ZR			DHZR			iPR	
	Control	–	0.43 ± 0.1 a	–	12.3 ± 0.3 a	14.3 ± 1.1 a	14.7 ± 1.8 a	1.5 ± 0.1 a	1.4 ± 0.1 a	1.7 ± 0.1 a	0.4 ± 0.1 a	0.5 ± 0.1 a	0.4 ± 0.1 a
	SW	–	0.28 ± 0.1 b	–	5.3 ± 0.2 b	3.8 ± 0.2 b	7.5 ± 0.5 b	0.5 ± 0.1 b	0.4 ± 0.1 b	0.5 ± 0.1 b	0.2 ± 0.1 a	0.3 ± 0.0 a	0.2 ± 0.1 b
	KAR_1	–	0.33 ± 0.1 b	–	5.9 ± 0.3 b	4.4 ± 0.3 b	7.7 ± 0.2 b	0.6 ± 0.1 b	0.4 ± 0.1 b	0.4 ± 0.1 b	0.2 ± 0.1 a	0.2 ± 0.1 a	0.1 ± 0.1 b
	TMB	–	–	–	6.1 ± 0.4 b	7.1 ± 1.6 b	5.7 ± 0.4 b	0.5 ± 0.1 b	0.6 ± 0.1 b	0.3 ± 0.2 b	–	0.1 ± 0.0 a	0.1 ± 0.0 b
Base			*t*Z			*c*Z			DHZ			iP	
	Control	–	0.17 ± 0.1 a	–	1.8 ± 0.1 b	1.1 ± 0.0 c	1.1 ± 0.0 c	–	–	–	1.5 ± 0.1 a	1.2 ± 0.1 a	1.0 ± 0.1 a
	SW	–	0.21 ± 0.1 a	–	4.3 ± 0.3 a	3.7 ± 0.3 b	4.6 ± 0.0 b	2.4 ± 0.2 a	2.6 ± 0.6 a	2.8 ± 0.4 a	1.4 ± 0.1 a	1.1 ± 0.1 a	1.0 ± 0.1 a
	KAR_1	0.19 ± 0.1 a	0.21 ± 0.1 a	–	5.8 ± 0.2 a	3.2 ± 0.2 b	7.3 ± 0.3 a	2.2 ± 0.2 a	1.5 ± 0.1 a	2.2 ± 0.1 a	1.7 ± 0.1 a	0.8 ± 0.1 b	1.1 ± 0.1 a
	TMB	–	–	–	4.0 ± 0.1 a	5.8 ± 0.5 a	5.9 ± 0.4 b	2.3 ± 0.2 a	2.7 ± 0.1 a	1.6 ± 0.1 a	1.1 ± 0.1 a	1.1 ± 0.1 a	1.2 ± 0.1 a
Glucoside			*t*ZOG			*c*ZOG			DHZOG				
	Control	86.7 ± 7.8 a	46.5 ± 3.5 a	52.4 ± 3.7 a	87.1 ± 9.0 a	46.6 ± 3.5 a	51.7 ± 4.0 a	1.1 ± 0.1 a	0.9 ± 0.1 a	0.8 ± 0.1 a	–	–	–
	SW	61.4 ± 1.3 a,b	49.9 ± 0.1 a	49.7 ± 2.6 a	60.3 ± 0.7 a,b	50.1 ± 0.0 a	48.9 ± 2.5 a	1.0 ± 0.1 a	1.0 ± 0.1 a	0.8 ± 0.1 a	–	–	–
	KAR_1	61.1 ± 1.7 b	51.3 ± 3.6 a	49.5 ± 3.4 a	58.9 ± 1.8 b	50.4 ± 3.6 a	49.1 ± 3.2 a	1.0 ± 0.1 a	0.9 ± 0.1 a	0.8 ± 0.1 a	–	–	–
	TMB	61.2 ± 0.2 a,b	55.8 ± 2.4 a	49.1 ± 2.3 a	64.1 ± 6.1 a,b	55.2 ± 1.5 a	48.6 ± 2.0 a	0.9 ± 0.1 a	0.9 ± 0.1 a	0.8 ± 0.1 a	–	–	–

(*Table continues on following page.*)

Table 1. (*Continued from previous page.*)

Cytokinin	Treatment	Dark	R Light	FR Light	Dark	R Light	FR Light	Dark	R Light	FR Light	Dark	R Light	FR Light
Riboside-*O*-glucoside			*t*ZROG			*c*ZROG			DHZROG				
	Control	1.5 ± 0.1 a	1.3 ± 0.1 a	1.3 ± 0.0 a	515 ± 13 a	766 ± 68 a	444 ± 34 a	7.7 ± 0.3 b	11.5 ± 0.2 a	6.4 ± 0.4 a	–	–	–
	SW	1.5 ± 0.1 a	1.5 ± 0.1 a	1.2 ± 0.1 a	516 ± 18 a	792 ± 46 a	571 ± 43 a	9.5 ± 0.5 a,b	10.6 ± 0.3 a	6.9 ± 0.1 a	–	–	–
	KAR_1	1.8 ± 0.1 a	1.0 ± 0.1 a	1.2 ± 0.1 a	705 ± 51 a	753 ± 60 a	464 ± 8 a	11.0 ± 0.6 a	11.7 ± 0.0 a	6.4 ± 0.5 a	–	–	–
	TMB	1.5 ± 0.1 a	1.2 ± 0.1 a	1.2 ± 0.1 a	501 ± 18 a	765 ± 93 a	428 ± 10 a	8.3 ± 0.6 a,b	9.5 ± 0.0 a	7.8 ± 0.6 a	–	–	–
Riboside-7-glucoside			*t*Z7G			*c*Z7G			DHZ7G			iP7G	
	Control	10.2 ± 0.4 a	7.8 ± 0.4 a	9.5 ± 0.5 a,b	–	–	–	–	–	–	–	–	–
	SW	9.4 ± 0.5 a	9.2 ± 0.6 a	9.7 ± 0.3 a	–	–	–	–	–	–	–	–	–
	KAR_1	9.4 ± 1.0 a	8.2 ± 0.5 a	8.1 ± 0.4 b,c	–	–	–	–	–	–	–	–	–
	TMB	9.0 ± 0.7 a	9.2 ± 0.5 a	7.8 ± 0.5 c	–	–	–	–	–	–	–	–	–
Riboside-9-glucoside			*t*Z9G			*c*Z9G			DHZ9G			iP9G	
	Control	–	–	–	–	–	–	–	–	–	–	–	–
	SW	–	–	–	–	–	–	–	–	–	–	–	–
	KAR_1	–	–	–	–	–	–	–	–	–	–	–	–
	TMB	–	–	–	–	–	–	–	–	–	–	–	–

Table 2. *Endogenous aromatic cytokinin and auxin content (pmol g^{-1}) in cv Grand Rapids lettuce seeds incubated for 24 h at 25°C* (n = 3)

After 3 h of incubation in the dark, seeds were exposed to R or FR light treatment for 1 h and were replaced in the dark. Mean values for each aromatic cytokinin and auxin in a column with different letter(s) are significantly different according to Bonferroni correction ($P < 0.05$). Dashes in the columns represents values below detection levels. Treatments were as follows: SW (1:2,500, v/v), KAR_1 (10^{-7} M), and TMB (10^{-7} M).

Treatment	Dark	R Light	FR Light	Dark	R Light	FR Light
		Cytokinin			Auxin	
		*m*T			IAAsp	
Control	4.7 ± 0.3 a	9.3 ± 0.9 a	2.8 ± 0.2 b	47.6 ± 1.8 a	68.3 ± 3.3 b	59.6 ± 5.8 a
SW	3.8 ± 0.3 a	2.5 ± 0.1 b	1.8 ± 0.2 b	58.3 ± 5.6 a	79.6 ± 2.2 a,b	58.1 ± 2.6 a
KAR_1	2.4 ± 0.2 a	1.9 ± 0.1 b	4.5 ± 0.1 a	60.7 ± 6.2 a	60.9 ± 3.3 b	64.4 ± 0.7 a
TMB	4.0 ± 0.5 a	6.3 ± 0.3 a,b	5.6 ± 0.3 a	46.3 ± 0.1 a	115.6 ± 15.3 a	50.6 ± 3.5 a
		*m*TR			IAAGlu	
Control	–	–	–	–	–	–
SW	–	–	–	–	–	–
KAR_1	–	–	–	–	–	–
TMB	–	–	–	–	–	–
		*m*T7G			oxIAA	
Control	–	–	–	–	–	–
SW	–	–	–	–	–	–
KAR_1	–	–	–	–	–	–
TMB	–	–	–	–	–	–
		*m*T9G				
Control	–	–	–	–	–	–
SW	–	–	–	–	–	–
KAR_1	–	–	–	–	–	–
TMB	–	–	–	–	–	–

biologically active *c*Z-type cytokinins, particularly *c*ZROG, which is a deactivation-reversible form and can revert back to base *c*Z.

Precursor *t*ZR5′MP was only present in seeds treated with KAR_1 in R light. *c*ZR5′MP was present in KAR_1-treated seeds and water control in the dark. In R light, *c*ZR5′MP level increased and was present in seeds treated with KAR_1, SW, and water control. In FR light, it was only present in water control and KAR_1-treated seeds. In dark, iPR5′MP was detected in KAR_1-treated seeds and was absent in FR light. In R light, it was present in all the treatments, with lowest levels observed in TMB-treated seeds. However, DHZ5′MP was not present in any of the treatments (Table 1).

The transport form *t*ZR was detected only in R light in seeds treated with KAR_1, SW, and water. The levels of riboside *c*ZR and DHZR were significantly higher in the water control compared with SW-, KAR_1-, and TMB-treated seeds in dark, R light, and FR light. There was no significant difference in the levels of *c*ZR and DHZR in SW-, KAR_1-, and TMB-treated seeds. Riboside iPR was not detected in TMB-treated seeds in dark, and there was no significant difference in levels of iPR in dark and R light. However, in FR light, the iPR levels were highest in the water control.

Among the active bases, *c*Z was most prevalent followed by DHZ, iP, and *t*Z. Under dark conditions, *t*Z was only detected in lettuce seeds treated with KAR_1. In R light, *t*Z was detected in SW- and KAR_1-treated and control seeds. However, no significant difference was observed. The levels of *c*Z were significantly increased in treatments compared with the control. DHZ was absent in the water control in dark, R light, and FR light, and no significant difference was observed in the rest of the treatments. iP showed no significant difference in all the treatments except for KAR_1-treated seeds in R light, where it decreased significantly (Table 1).

The levels of the storage/inactive (reversible forms) cytokinins *t*ZOG and *c*ZOG were higher in the dark compared with R and FR light in all the treatments (Table 1). The levels of *t*ZOG and *c*ZOG were significantly higher in the water control compared with KAR_1-treated seeds, with no significant differences in seeds treated with SW and TMB in the dark. However, no significant difference was observed among the treatments under R and FR light. DHZOG was present in all the treatments, but these results were not significantly different. *t*ZROG was present in all the treatments; however, the differences were nonsignificant. *c*ZROG increased significantly under all light conditions. The overall levels of *c*ZROG and DHZROG increased in R light (Table 1). Among the irreversible deactivation forms (7-glucosides and 9-glucosides), *c*Z7G, DHZ7G, iP7G, *t*Z9G, *c*Z9G, DHZ9G, and iP9G were absent in all the treatments. However, *t*Z7G was present in all treatments and showed no significant difference in dark and R light. In FR light, the SW-treated seeds showed the highest levels of *t*Z7G.

The MVA pathway-derived cytokinins were significantly higher in comparison with MEP pathway-derived cytokinins (Supplemental Fig. S1). The levels of MEP pathway-derived cytokinins were significantly lower after treatments in comparison with the control under dark. The levels of MVA pathway-derived cytokinins were significantly greater in KAR_1-treated seeds in comparison with other treatments under dark conditions.

A much clearer picture was revealed when the total cytokinins were compared. An increase in total isoprenoid cytokinins was recorded in R light in comparison with dark and FR light in all the treatments, with the exception of KAR_1-treated seeds under dark conditions (Supplemental Fig. S2). In the dark, the levels of total cytokinins were significantly higher in KAR_1-treated seeds in comparison with the other treatments. In R light, the levels of total cytokinins were nonsignificant in all the treatments (Supplemental Fig. S2).

The only aromatic cytokinin detected was *m*T (Table 2). The levels of *m*T were lower in SW- and KAR_1-treated seeds compared with TMB-treated seeds and water control under all light conditions, with the exception of KAR_1-treated seeds in FR light. The auxin catabolites oxIAA and IAGlu were not present in lettuce seeds in all the treatments. There were no significant differences in levels of IAAsp except for TMB treatment under red light, which was significantly higher than SW- and KAR_1-treated seeds and the water control (Table 2).

Effects of SW, KAR_1, and TMB on Hydrolytic Enzymes and Mobilization of Reserve Food

The effects of SW, KAR_1, and TMB on the activity of the hydrolytic enzymes lipase and α-amylase were evaluated under dark, R light, and FR light. In comparison with the control, KAR_1 treatment significantly increased the α-amylase activity in dark and FR light; however, SW treatment significantly enhanced α-amylase activity in dark and FR light. TMB treatment significantly inhibited α-amylase activity compared with the water control in all light treatments (Fig. 2, A–C). α-Amylase activity in KAR_1-treated seeds was 1.7-, 3.6-, and 2.5-fold higher than that in TMB-treated seeds in dark, R light, and FR light, respectively. The starch and carbohydrate content was maximum in KAR_1-treated seeds and lowest in TMB-treated seeds in all light conditions, suggesting that TMB inhibited carbohydrate and starch mobilization (Fig. 2, D–F). The starch content was very low in all treatments; however, there was a significant difference in the levels of starch. Protein content was highest in KAR_1-treated seeds under all light treatments. TMB-treated seeds showed the lowest protein content in R light. However, in dark and FR light, the protein content in these seeds was significantly greater than in the water control (Fig. 2, D–F). The lipids are major food reserves of lettuce seeds. In the dark, the maximum lipid content was observed in SW treatment followed by KAR_1, water control, and TMB treatment (Fig. 3A). In R and FR light, the lipids were maximum in KAR_1-treated seeds. Lipase activity was maximum in KAR_1-treated seeds followed by SW-treated seeds, water control, and TMB-treated seeds in all the light treatments (Fig. 3, B and C). The lipase activity in KAR_1-treated seeds was 1.8-, 2.9-, and 2.5-fold higher compared with TMB-treated seeds in dark, R light, and FR light, respectively. The results from this study indicate that there was a greater mobilization and utilization of stored reserves due to enhanced activity of hydrolytic enzymes in KAR_1- and SW-treated seeds.

DISCUSSION

Lettuce seeds treated with water showed 12% germination in the dark. However, 1 h of R and FR light exposure resulted in 95% and 5% seed germination, respectively. The SW- and KAR_1-treated seeds showed more than 90% and 97% germination, respectively.

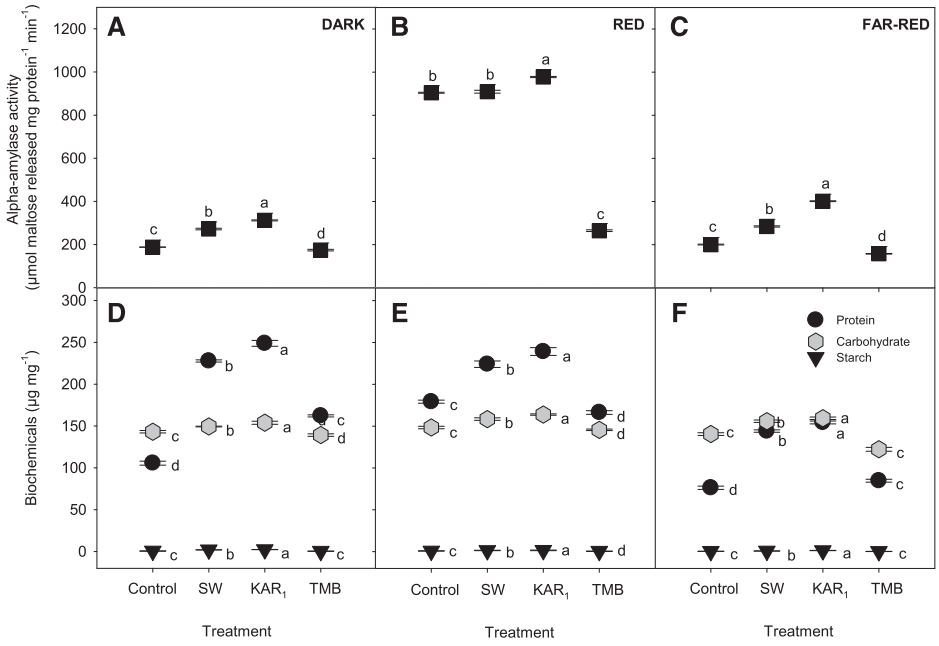

Figure 2. Influence of SW, KAR_1, and TMB on α-amylase activity (A–C) and starch, sugar, and protein content (D–F) in cv Grand Rapids lettuce seeds under different light conditions for 24 h at 25°C ($n = 3$). After 3 h of incubation in the dark, seeds were exposed to R or FR light treatment for 1 h and were replaced in the dark. Symbols (value ± SE) for each light condition with different letters are significantly different according to Bonferroni correction ($P < 0.05$).

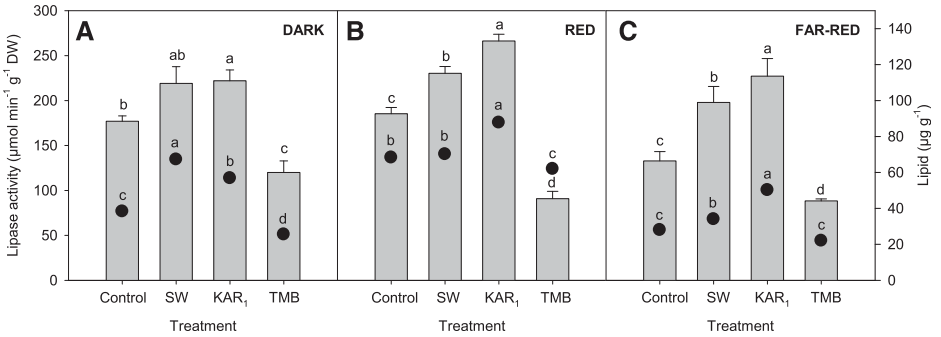

Figure 3. Influence of SW, KAR_1, and TMB on lipase activity and lipid content in cv Grand Rapids lettuce seeds under different light conditions for 24 h at 25°C ($n = 3$). After 3 h of incubation in the dark, seeds were exposed to R or FR light treatment for 1 h and were replaced in the dark. Bars (lipase ± SE) and symbols (lipids ± SE) for each light condition with different letters are significantly different according to Bonferroni correction ($P < 0.05$). DW, Dry weight.

On the other hand, TMB completely inhibited germination in the dark, and even after 1 h of R light exposure, it significantly inhibited germination (33%). SW- and KAR_1-treated seeds significantly overcame the inhibitory effect of FR light and resulted in 28% and 35% germination, respectively, compared with no germination in TMB-treated seeds. This might have occurred due to substantial decreases in ABA content of SW- and KAR_1-treated seeds. Plant-derived smoke and KAR_1 partially inhibits the effect of FR light (Van Staden et al., 1995; Soós et al., 2012). The dynamic balance between the Pfr and Pr forms of phytochromes, induced in R and FR light, respectively, has a unique role in regulating cv Grand Rapids lettuce seed dormancy and germination (Black et al., 1974). The Pfr form of phytochrome deactivates ABA synthesis genes, while the Pr form activates these genes (Seo et al., 2006). ABA is a dormancy-inducing hormone that inhibits seed germination by inhibiting the transition of the embryo to plant and radicle elongation (Fountain and Bewley, 1976; Müller et al., 2006; Finkelstein et al., 2008). It also inhibits storage oil mobilization and hydrolyzing enzymes. The levels of the dormancy-inducing hormone ABA in seeds treated with SW, KAR_1, and TMB were quantified in this study. UHPLC-MS/MS analysis revealed that levels of ABA were highest in TMB-treated seeds followed by water control, SW, and KAR_1 in the dark, R light, and FR light (Fig. 1). Correspondingly, a negative correlation was found between percentage seed germination and ABA content in the dark ($R^2 = -0.87$), R light ($R^2 = -0.49$), and FR light ($R^2 = -0.99$) in all the treatments. These findings correspond with the study of Soós et al. (2012), who reported that KAR_1 suppressed, while TMB up-regulated, ABA-related transcripts in lettuce seeds. Similarly, in smoke-treated seeds of *Nicotiana attenuata*, a decrease in ABA level was observed (Schwachtje and Baldwin, 2004). The regulation of ABA metabolism in lettuce seeds is controlled by phytochrome, which is also supported by this study. Smoke compounds tested in this study have been shown to modulate ABA levels very precisely, as KAR_1 decreased and TMB increased ABA levels in dark, R light, and FR light treatments. This indicates that KAR_1 and TMB may mimic the effect of R and FR light, respectively, and have an additive effect in modulating ABA levels. A basic helix-loop-helix transcription factor, PIL5, acts as a key negative regulator in phytochrome-mediated seed germination and preferentially interacts with the Pfr forms of phyA and phyB (Oh et al., 2004). When activated by light, phytochromes bind to PIL5 and accelerate its degradation, releasing its repression of seed germination and allowing seeds to germinate (Shen et al., 2005; Oh et al., 2006). PIL5 represses seed germination by directly binding to the promoters of two GA repressor (DELLA) genes in Arabidopsis (*Arabidopsis thaliana*), RGL2 and possibly RGL1, and activating their expression (Tyler et al., 2004). It might be possible that the smoke compounds KAR_1 and TMB directly aid in the conversion of Pr to Pfr and vice versa, or they may also interact with PIL5 or DELLA genes involved in seed germination. It will be of great interest to study these molecular mechanisms to investigate the influence of KAR_1 and TMB on expression of the PIL5 or DELLA genes.

Although the levels of ABA in R light were not significantly different in seeds treated with water and TMB, a significant difference was observed in seed germination. This may be due to the changes in levels of cytokinins, as previous research has shown that cytokinin production is also a phytochrome-controlled process (Van Staden and Wareing, 1972). R light treatments increased endogenous cytokinin levels and FR light reversed this effect (Van Staden, 1973). Cytokinins overcome the inhibitory effect of ABA and promote germination (Black et al., 1974). The increased levels of cytokinins in R light enhanced cell division and enlarged radicles, allowing germination (Van Staden and Wareing, 1972). GAs are known for releasing seed dormancy; however, higher concentrations of GA are nearly ineffective in releasing dormancy caused by ABA. This inhibition of germination caused by ABA may only be reversed with cytokinins (Khan, 1967, 1968; Bewley and Fountain, 1972). Consequently, the endogenous levels of natural isoprenoid and aromatic cytokinins were quantified in this study.

A detailed assessment of the concentrations of various endogenous cytokinins and their fluctuations after 24 h of germination in lettuce seeds treated with smoke compounds in various light treatments revealed that the content of MVA pathway (cytosol)-derived cytokinins (*c*Z type) was much higher compared with that of MEP pathway-derived cytokinins (*t*Z, DHZ, and iP types; Table 1; Supplemental Fig. S1). The results are in agreement with Wang et al. (2015), who also reported the involvement of the MVA pathway-derived cytokinins for isoprenoid biosynthesis in lettuce seed germination. In Arabidopsis seeds, *c*Z levels were higher after 24 h of imbibition (Gajdosová et al., 2011). Similarly, *c*Z concentrations are higher during seed development in specific chickpea (*Cicer arietinum* and *Cicer anatolicum*) cvs (Lulsdorf et al., 2013). Dwarf hops (*Humulus lupulus*) varieties contain significantly higher amounts of *c*Zs (Patzak et al., 2013), and *c*ZR is a major cytokinin in unfertilized hops (Watanabe et al., 1981). These results reveal that *c*Z-type cytokinins tend to accumulate under particular circumstances such as seed germination.

Ribotides play a central role in the regulation of cytokinin levels, as they are readily converted to both less active ribosides and highly active free base forms (Laloue and Pethe, 1982; Palmer et al., 1984). The ribotides DHZR5′MP and *t*ZR5′MP (except in KAR_1-treated seeds in R light) were absent in all the treatments. Ribotide *c*ZR5′MP was absent in SW-treated seeds in dark and FR light, whereas TMB treatment inhibited it in dark, R light, and FR light. The absence of ribotides by particular smoke compounds or light treatment indicates that they are either not formed or might have been utilized and converted to other forms and play an active role in seed germination. The absence of irreversible deactivation forms (7-glucosides and 9-glucosides), except *t*Z7G type, suggests that in lettuce seed germination, the cytokinins are mostly stored as reversible deactivation forms (*O*-glucosides and riboside-*O*-glucosides). The reversible deactivation forms may be converted to active forms (bases and ribosides) when needed. The levels of the riboside-*O*-glucoside *c*ZROG were significantly highest of the quantified cytokinins. *t*ZOG and *c*ZOG accumulated high levels in lettuce seeds in all the treatments, as they are readily converted to the free base forms and are also less susceptible to degradation by cytokinin oxidase (Spíchal et al., 2004).

In this study we observed that R light treatment triggered or enhanced levels of some of the cytokinins such as precursors *t*ZR5′MP, *c*ZR5′MP, and iPR5′MP, transport form *t*ZR, base *t*Z, and storage reversible inactive forms *c*ZROG and DHZROG. The levels of the active isoprenoid cytokinins, base *t*Z, and transport form *t*ZR were low. KAR_1-treated seeds had *t*Z only in the dark. In R light, *t*Z and *t*ZR were detected in seeds treated with SW and KAR_1 and water control. TMB completely inhibited *t*Z and *t*ZR in R light.

The levels of transport form *c*ZR were higher in the control as compared with the treatments, whereas the levels of base *c*Z were higher in the treatments as compared with the control. This suggests that the treatments enhanced the conversion of transport form *c*ZR to base *c*Z under all light conditions. In contrast, DHZ, DHZR, iP, and iPR did not show any trend or significant difference in various treatments. Bean (*Phaseolus vulgaris*; Mok et al., 1978) and tobacco (*Nicotiana tabacum*) cell-culture assays (Schmitz and Skoog, 1972; Gajdosová et al., 2011) revealed that *c*Z-type cytokinins have little or no activity compared to iP and *t*Z types, which are generally considered as the most active natural cytokinins. *t*Z-type cytokinins have very high cell division-promoting activity (Matsumoto-Kitano et al., 2008). The possible explanations for low levels of the highly active free bases, particularly *t*Z, may be due to their rapid utilization or confined regulation, so that they do not accumulate high levels. This indicates that there were no active cytokinins (*t*Z and *t*ZR) in TMB-treated seeds to overcome the inhibitory effects of ABA in R light, which resulted in a significant decline in germination (33%). The presence of *t*Z and *t*ZR might be responsible for the significant level of germination in the water control seeds, irrespective of having similar ABA levels to TMB-treated seeds after 1 h of R light exposure. On the other hand, an increase in total cytokinins in R light and a decrease in FR light in comparison with dark were observed in all treatments. Similar results were reported for *Rumex obtusifolius* (Van Staden and Wareing, 1972). On comparing the total isoprenoid cytokinins, it was revealed that KAR_1 was able to regulate the total isoprenoid cytokinins in the dark (Supplemental Fig. S2). The results suggest that under R and FR light, the smoke compounds are unable to regulate the total isoprenoid cytokinins and there might be involvement of some unknown product(s) or pathway(s) for lettuce seed germination, regulated by KAR_1 and TMB (Wang et al., 2015).

The levels of aromatic cytokinin *m*T were low in SW- and KAR_1-treated seeds compared with TMB-treated seeds and water control in all the light treatments. Although isoprenoid and aromatic cytokinins have an overlapping spectrum of biological activity, they are not considered as alternative forms of the same signals (Strnad, 1997). They are believed to be involved in the metabolism and development of mature tissues rather than in the stimulation of cell division (Kaminek et al., 1987) and to play a role in retarding senescence (Strnad, 1997).

The levels of IAAsp, the main naturally occurring irreversible catabolite of IAA, was significantly higher in TMB-treated seeds under R light compared with SW- and KAR_1-treated seeds and the water control (Table 2). IAAsp is rapidly formed following high concentrations of IAA in plant tissue (Delbarre et al., 1994; Sasaki et al., 1994) and is synthesized in places of IAA retention (Paliyath et al., 1989; Nordström and Eliasson, 1991). IAA conjugates have no auxin activity; however, their activity is directly related to the amount of free auxin released by hydrolysis (Bialek et al., 1983). The accumulation of IAAsp in TMB-treated seeds in R light indicates high IAA accumulation, which has been shown to be a potent inhibitor of lettuce seed germination

and is known to reduce root and hypocotyl elongation (Khan and Tolbert, 1966; Sankhla and Sankhla, 1972; Zelená, 2000; Chiwocha et al., 2003). Therefore, it can be envisaged that there might be a high accumulation of IAA, as supported by high IAAsp levels in TMB-treated seeds in R light, as a consequence of which significant inhibition of germination (33%) was observed (Fig. 1).

The major food reserves stored in the lettuce seed (mostly in the cotyledons) are lipids (~33%) and proteins (~3.7%), with smaller amounts of soluble sugars being present, and very little starch (Mayer and Poljakoff-Mayber, 1975). Therefore, lipid, total carbohydrate, protein, and starch were quantified along with the hydrolytic enzymes lipase and α-amylase. In this study, the hydrolytic enzymes lipase and α-amylase and storage reserve, lipids, carbohydrates, and starch were significantly increased in KAR_1-treated seeds in comparison with TMB-treated seeds (Figs. 2 and 3). The increased protein content in TMB-treated seeds compared with the water control may be attributed to an increase in some germination inhibitory proteins in dark and FR light that might have been suppressed in R light. In this respect, further investigations are necessary. KAR_1 and SW activated α-amylase and lipase and initiated mobilization of storage reserves, which resulted in increased germination, whereas TMB reversed the effect by deactivating these enzymes. KAR_1 acted in a similar manner to GA, mediating the release of hydrolytic enzymes that hydrolyze the storage reserves (Hopkins and Hüner, 1995). Blank and Young (1998) extrapolated that the active compounds present in smoke influence enzyme systems that control growth rate. SW enhanced soluble sugar and proteins related to signaling and transport (Rehman et al., 2018). α-Amylase activity was slightly elevated in okra (*Abelmoschus esculentus*) roots with SW and KAR_1 (Papenfus et al., 2015). SW and KAR_1 also stimulate growth in bean and maize (*Zea mays*) seedlings by efficient starch mobilization (Sunmonu et al., 2016). TMB down-regulates genes required for storage reserve mobilization (Soós et al., 2012). Our results here showed a positive correlation (amylase:starch, $R^2 = 0.32$; lipase:lipid, $R^2 = 0.74$) between elevation in hydrolytic enzymes (lipase and α-amylase) and their substrates (lipids and starch). The germinated seeds showed greater mobilization of lipids and starch compared with ungerminated seeds. This is in agreement with the reports of Rentzsch et al. (2012) and Sunmonu et al. (2016). Wang et al. (2015) also found that the abundance of transcripts encoding LIPOXYGENASE2 and ISOCITRATE LYASE enzymes involved in the mobilization of lipids were higher in germinated seeds than in both dry and ungerminated lettuce seeds. The increased α-amylase and lipase activity in the lettuce seeds may be attributed to the relatively higher starch and lipid contents. In this study, increases in mobilization of food reserves with SW and KAR_1 treatments and decreases in mobilization with TMB treatment were observed. This may be attributed to changes in endogenous ABA levels that were regulated by these compounds in photosensitive lettuce seeds. It has been proposed that ABA inhibits seed germination by preventing the mobilization of storage reserves (Garciarrubio et al., 1997; Bethke and Jones, 2001; Finkelstein et al., 2002; Graham, 2008). In other studies, ABA inhibited the expression of genes involved in storage reserve mobilization. This corresponds well with our study, as the seeds having high ABA levels had low storage reserves, thus blocking the supply of energy and nutrients to the developing embryo. Seeds having high ABA levels inhibited the radicle emergence in this study. This is also supported by the study of Gimeno-Gilles et al. (2009), who showed that ABA inhibits cell wall loosening and expansion and consequently inhibits radicle emergence and germination. It is extrapolated that the active compounds present in smoke influence enzyme systems that control the growth rate of many plant species (Blank and Young, 1998).

CONCLUSION

The results presented in this work clearly indicate that the smoke-related compounds KAR_1 and TMB control germination of lettuce seeds by modulating the phytochrome system and/or phytochrome-mediated ABA signaling. This in turn influences the activity of hydrolytic enzymes and mobilization of food reserves. The possible mechanism by which this is achieved may be due to the substitution for R and FR light by KAR_1 and TMB, respectively, via the interconversion of Pr and Pfr. The results also revealed that TMB significantly inhibited MVA pathway-derived cytokinins in the dark and FR light; however, the treatments significantly reduced the levels of MEP derived-cytokinins only in the dark. This suggests that lettuce seeds treated with SW, KAR_1, and TMB affected cytokinin homeostasis and metabolism primarily in the dark. A significant inhibition of germination in TMB-treated seeds in R light treatment compared with the control seeds, irrespective of similar ABA levels, might be due to the accumulation of IAA or the absence of *t*Z and *t*ZR. Further research is needed to identify the influence of smoke compounds on other growth regulators such as GA and ethylene in phytochrome-regulated germination.

MATERIALS AND METHODS

Plant Material

Lettuce (*Lactuca sativa* 'Grand Rapids') seeds were purchased from Stokes Seeds (lot no. 212388). The seeds were checked for light sensitivity. Mature seeds of cv Grand Rapids lettuce do not germinate in the dark, at temperatures that are suitable for germination, and are termed light sensitive. They were stored in the dark at 4°C in an opaque bag and box until used.

Smoke Compounds and Chemicals

SW (Gupta et al., 2019), KAR_1 (Flematti et al., 2004; Van Staden et al., 2004), and TMB (Light et al., 2010) solutions were prepared according to previously described methods. All the chemicals used were of analytical grade.

Cv Grand Rapids Bioassay

For performing the cv Grand Rapids bioassay (Drewes et al., 1995; Light et al., 2010), all precautions were taken to protect the seeds from light. Lettuce seeds were immediately brought to the dark room from the refrigerator, counted under a green safelight (0.5 μmol m^{-2} s^{-1}), and placed on 65-mm polystyrene petri plates containing two sheets of Whatman No. 1 filter paper. The seeds were soaked in 2.2 mL of the different test solutions, SW (1:2,500 [v/v]), KAR_1 (10^{-7} M), and TMB (10^{-7} M), and distilled water was used as a control. The petri plates were then wrapped in aluminum foil and placed in wooden light-proof boxes that were painted black inside, and the lid of the boxes was again wrapped in aluminum foil. The boxes were then placed in an incubator in the dark at 25°C for 3 h. After this period of imbibition in the dark, one set of lettuce seeds was exposed to R light (660 nm) and another to FR light (730 nm) for 1 h and then again placed in the incubator in the dark for 24 h. The seeds were considered as germinated when the radicle was visible. Four replicates with 25 seeds each were used for the germination of lettuce seeds, and four replicates with 200 mg of seeds for each treatment were used for the biochemical determinations.

Biochemical Determinations

Protein Estimation

Total protein was estimated according to the Bradford (1976) method with minor modifications, using BSA as a standard. Seeds (200 mg) were homogenized in an ice-chilled mortar and pestle with 6 mL of ice-cold phosphate-buffered saline (8 g of NaCl [137 mM], 0.2 g of KCl [2.7 mM], 1.44 g of Na_2HPO_4 [10 mM], and 0.24 g of KH_2PO_4 [1.8 mM] in 1 L of distilled water, pH 7.2). The homogenate was centrifuged at 15,000*g* for 15 min at 4°C. Sample (100 μL) was pipetted out into the test tube, and the volume was made up to 1 mL in all test tubes with phosphate-buffered saline. Bradford dye (1 mL) was added to all the test tubes. The contents of the test tubes were mixed by vortexing, and the tubes were allowed to stand for 5 min. Red dye turns blue as the dye binds protein, and absorbance was recorded at 595 nm against the blank.

α-Amylase Activity

α-Amylase activity was determined in cv Grand Rapids seeds using the method described by Sadasivam and Manickam (1996) with minor modifications. Seeds (200 mg) were extracted in 5 mL of ice-cold 10 mM calcium chloride solution. The homogenate was centrifuged at 15,000*g* for 15 min at 4°C in a refrigerated centrifuge. The supernatant was saved and used as the enzyme source. To 5 mL of enzyme extract (supernatant), 3 mM calcium chloride was added and heated for 5 min at 70°C to inactivate β-amylase. Starch solution (1 mL) was mixed with 1 mL of properly diluted enzyme extract (from the previous step) in a test tube and incubated at 27°C for 5 min. The reaction was stopped by the addition of 2 mL of 3,5-Dinitrosalicylic acid reagent, and the solution was then heated in a boiling-water bath for 5 min. Rochelle salt solution (1 mL) was added while the tubes were warm, and then the test tubes were cooled in running tap water. Absorbance was recorded at 560 nm after the volume was made up to 5 mL by adding 1 mL of distilled water. The standard curve was made using 0 to 100 μg of maltose.

Lipase Activity

Lipase activity was assayed using the method of Itaya and Ui (1965) with minor modifications. The cv Grand Rapids seeds (200 mg) were homogenized with 2 mL of borate buffer (0.2 M, pH 7.2) containing 20% (w/v) polyvinylpyrrolidone and centrifuged at 10,000*g* for 20 min. To 100 μL of supernatant, 1 mL of substrate (0.98% [w/v] NaCl, 5 g of gum acacia, and 5 mL of olive oil) was added and incubated for 1 h at 37°C. To stop the reaction, it was then placed in the water bath (90°C) for 2 min. Afterward, 6 mL of chloroform and 2 mL of sodium phosphate buffer (0.66 mM, pH 6.2) were added and allowed to settle for 30 min at room temperature. The lower layer was separated, and three copper triethanolamine reagents (1 M triethanolamine, 1 N acetic acid, and 6.45% [w/v] copper nitrate) were mixed with it and reincubated for the next 30 min. Thereafter, in the lower layer, 100 μL of diethyldithiocarbamate (11 mM) was added and absorbance was taken at 440 nm. The standard curve was prepared using stearic acid and expressed as μmol min^{-1} g^{-1} dry mass of seeds.

Estimation of Starch

Starch content was estimated using the method described by Sadasivam and Manickam (1996) with minor modifications. The cv Grand Rapids seeds (200 mg) were homogenized in hot ethanol (80%, v/v) to remove sugars. The homogenate was centrifuged at 3,000*g* for 15 min, and the residue was retained and repeatedly washed with hot ethanol (80%, v/v) until the washings did not give color with anthrone reagent. The residue was then dried well over a water bath and was extracted at 0°C for 20 min after adding 2 mL of water and 3 mL of 52% (v/v) perchloric acid. The mixture was then centrifuged at 3,000*g* for 15 min, and the supernatant was retained. The residue was again extracted using 3 mL of perchloric acid and centrifuged. The supernatants were pooled and were made up to 10 mL with distilled water. Diluted supernatant (100 μL) was pipetted out and made up to 1 mL with distilled water. Anthrone reagent (4 mL) was added to each test tube and heated in a boiling-water bath for 8 min. Test tubes were rapidly cooled in running tap water, and absorbance was taken at 630 nm as the color changed from green to dark green. The standard curve was made using 0 to 100 μg of Glc.

Estimation of Total Carbohydrate

Total carbohydrate content was estimated using the method described by Sadasivam and Manickam (1996) with minor modifications. Seeds (200 mg) were weighed in test tubes and were hydrolyzed in a boiling-water bath for 3 h with 3 mL of 2.5 N HCl and then cooled to room temperature. The hydrolyzed seeds were neutralized with sodium carbonate until the effervescence ceased. The volume was made up to 5 mL by adding distilled water and was centrifuged at 3,000*g* for 15 min. Supernatant (100 μL) was taken, and 4 mL of anthrone reagent was added after the volume was made up to 1 mL using distilled water. The test tubes were heated in a boiling-water bath for 8 min and were cooled rapidly in running tap water. The absorbance was taken at 630 nm as the color changed from green to dark green. The standard curve was prepared using 0 to 100 μg of Glc.

Estimation of Lipid

Lipid content estimation was performed by the method of Becker et al. (1978) with minor modifications. Seeds (200 mg) were ground in a mortar and pestle with a chloroform and methanol mixture (2:1, v/v). The mixture was poured in flasks and was kept at room temperature in the dark for complete extraction. Later, chloroform and water (1:1, v/v) were added. The solution was shaken, and after phase separation, three layers were observed. The methanol layer was discarded, and the lower organic layer was collected in a preweighed beaker and evaporated in a water bath at 60°C. The weight of the lipid was determined. The results were expressed in terms of weight in milligrams of total lipids per gram of fresh seed.

Estimation of Phytohormones

After appropriate treatment with SW, KAR_1, and TMB in dark, R light, and FR light, the seeds were ground in liquid nitrogen. For cytokinin analysis, technical triplicates of seeds (2 mg per sample) were homogenized and extracted with 1 mL of modified Bieleski buffer (60% methanol, 10% formic acid, and 30% water) with a cocktail of stable isotope-labeled internal standards (0.25 pmol of cytokinin bases, ribosides, and *N*-glucosides and 0.5 pmol of cytokinin *O*-glucosides and nucleotides per sample) for determination of endogenous cytokinins. The extracts were purified using a combination of C18 (1 mL per 30 mg) and MCX (1 mL per 30 mg) cartridges (Dobrev and Kamínek, 2002). Eluates were evaporated to dry, dissolved in 30 μL of 10% (v/v) methanol, and analyzed by the separation methods described by Svačinová et al. (2012).

To determine levels of auxins and ABA, 1 mL of 50 mM sodium phosphate buffer (pH 7) was used as the extraction solution with isotope-labeled internal standards (5 pmol of [$^{13}C_6$]IAA, [$^{13}C_6$]IAAsp, [$^{13}C_6$]oxIAA, [$^{13}C_6$]IAGlu, and [D_6]ABA per sample) added before homogenization and purified using HLB (1 mL per 30 mg) cartridges. Eluates were evaporated to dry, subsequently dissolved in 30 μL of 10% (v/v) methanol, and analyzed by the methods described by Novák et al. (2012). The endogenous levels of phytohormones were determined using a UHPLC device (Acquity UPLC I-Class System; Waters) coupled to a triple quadrupole mass spectrometer with an electrospray interface (Xevo TQ-S; Waters). Quantification was obtained by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. The levels of individual phytohormones were quantified by comparing the ratio of endogenous

hormones and internal standards of known concentration using Masslynx software.

Statistical Analysis

The germination data were arcsine transformed prior to statistical analysis. For the germination assay, total protein estimation, total carbohydrates, starch, and α-amylase activity, significant differences between treatments were determined using one-way ANOVA according to Bonferroni correction ($P < 0.05$; Goedhart, 2014). Correlation was calculated using the MS Excel software program.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Endogenous MEP pathway-derived and MVA pathway-derived isoprenoid cytokinin content.

Supplemental Figure S2. Total endogenous isoprenoid cytokinin content in cv Grand Rapids lettuce seeds incubated for 24 h at 25°C.

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