Role of smoke stimulatory and inhibitory biomolecules in phytochrome-regulated seed germination of Lactuca sativa

Shubhpriya Gupta\textsuperscript{a}, Lenka Plačková\textsuperscript{b}, Manoj G Kulkarni\textsuperscript{a}, Karel Doležal\textsuperscript{b,c} and Johannes Van Staden\textsuperscript{a,*}

\textsuperscript{a}Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa

\textsuperscript{b}Laboratory of Growth Regulators, The Czech Academy of Sciences, Institute of Experimental Botany & Palacký University, Faculty of Science, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic

\textsuperscript{c}Department of Chemical Biology and Genetics, Centre of Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Holice 78371, Czech Republic

One-sentence summary: Karrikinolide and trimethylbutenolide from wildfire smoke regulate germination in lettuce seed by controlling phytochrome-mediated abscisic acid signalling.

Authors’ contributions: SG, JVS and MK conceived the research idea. SG and MGK performed the growth and physiological experiments. LP and KD performed UHPLC/MS-MS analysis. All authors analysed the data. SG designed the experiments and wrote the manuscript. JVS supervised the research and agrees to serve as the author responsible for contact and ensures communication. All authors reviewed and approved the manuscript.

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ABSTRACT

The biologically active molecules karrikinolide (KAR\textsubscript{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\textsubscript{1} and TMB regulate seed germination in photosensitive ‘Grand Rapids’ lettuce (\textit{Lactuca sativa} L.), we investigated levels of the dormancy-inducing hormone abscisic acid (ABA), 3 auxin catabolites and cytokinins (26 isoprenoid and 4 aromatic) in response to these compounds. Activity of the hydrolytic enzymes \(\alpha\)-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 to water controls (100\%). KAR\textsubscript{1} significantly enhanced total isoprenoid cytokinins under dark conditions in comparison to other treatments; however, there was no significant effect under R-light. Enhanced levels of indole-3-aspartate (IAAsp) (an indicator of high indole-3-acetic acid [IAA] accumulation, which inhibits lettuce seed germination) and absence of \textit{trans}-zeatin and \textit{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\textsubscript{1} significantly promote lettuce seed germination by reducing levels of ABA and enhancing 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.

**Keywords:** ABA regulation, natural cytokinins, Grand Rapids, phytochrome, reserve mobilization, seed germination, smoke biomolecules
INTRODUCTION

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 promotory 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 wide variety of plants. Wildfire smoke contains certain potent bioactive compounds (butenolides) which 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, wild oats and lettuce (Kulkarni et al., 2006; Light et al., 2009). Karrikinolide (KAR\textsubscript{1}; 3-methyl-2\textsubscript{H}-furo[2,3-c]pyran-2-one), a butenolide derived from smoke, exhibits germination promotory activity (Flematti et al., 2004; Van Staden et al., 2004). Conversely, trimethylbutenolide (TMB; 3,4,5-trimethylfuran-2(5H)-one) shows germination inhibitory activity (Light et al., 2010). These molecules have great ecological significance, as seeds with KAR\textsubscript{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 provides a mechanism for preventing germination until the conditions are suitable (De Lange and Boucher, 1993). In Lactuca sativa L. cv Grand Rapids, 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 promotory biomolecule (KAR\textsubscript{1}) to lettuce seeds in the dark replaces the light requirement, resulting in germination. On the contrary, the smoke inhibitory biomolecule (TMB) completely supresses the germination of lettuce seeds (Van Staden et al., 2004; Light, 2006; Light et al., 2010). SW and KAR\textsubscript{1} are 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 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. Gibberellin (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 signalling 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; Toyomasu et al., 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 signalling 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 substitutes R-light through inter-conversion of Pr to Pfr or is somehow involved in phytochrome-mediated signalling 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 the present 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 Grand Rapids lettuce.

**RESULTS**

**Influence of SW, KAR$_1$ and TMB on Lettuce Seed Germination**

The effects of smoke-water (SW), karrikinolide (KAR$_1$) and trimethylbutenolide (TMB) on
the germination of Grand Rapids lettuce seeds after 24 h were compared for dark, red (R) and
far-red (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 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 of TMB significantly inhibited germination (33%) in R-
light (Fig. 1B). In FR-light (1 h 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 abscisic acid (ABA); twenty-six natural isoprenoid cytokinins
comprising 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (plastid) derived cytokinins
viz. $tZ$-type cytokinins ($tZ$: trans-Zeatin, $tZ$R: trans-Zeatin riboside, $tZ$OG: trans-Zeatin-O-
glucoside, $tZ$ROG: trans-Zeatin riboside-O-glucoside, $tZ$7G: trans-Zeatin-7-glucoside, $tZ$9G:
trans-Zeatin-9-glucoside, $tZ$R5'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, DHZR5'MP: dihydrozeatin riboside 5'-
monophosphate), $N^6$-(2-isopentenyl)adenine-type (iP) cytokinins (iP: $N^6$-(2-
isopentenyl)adenine, iP$_R$: 5'-isopentenyladenosine, iP$_{7G}$: 5'-isopentenyladenine-7-
glucoside, iP9G: \( \text{N}^6-(2\text{-isopentenyl})\text{adenine-9-glucoside}, \) iP5'MP: \( \text{N}^6-(2\text{-isopentenyl})\text{adenosine-5'-monophosphate} \) and mevalonate (MVA) pathway (cytosol) derived cytokinin viz. cZ-type cytokinins (cZ: cis-Zeatin, cZR: cis-Zeatin riboside, cZOG: cis-Zeatin-O-glucoside, cZORG: cis-Zeatin riboside-O-glucoside, cZ7G: cis-Zeatin-7-glucoside, cZ9G: cis-Zeatin-9-glucoside, cZR5'MP: cis-Zeatin riboside 5'-monophosphate); four aromatic cytokinins (mT: meta-topolin, mTR: meta-topolin riboside, mT7G: meta-topolin-7-glucoside, mT9G: meta-topolin-9-glucoside); and three auxin conjugates (IAAsp: IAA-3-aspartate, IAAGlu: IAA-3-glutamate, oxIAA: 2-oxindole-3-acetic acid) were determined in Grand Rapids using 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 KAR1 (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 KAR1-treated seeds (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 KAR1 (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 KAR1 (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 KAR1-treated seeds in dark, R and FR-light respectively. These results indicated an overall negative correlation (\(R^2 = -0.84\)) between germination and ABA production in *Lactuca sativa* L. Grand Rapids seeds.

The cytokinin pool consists of free bases \(tZ, cZ, DHZ, iP\) and \(mT\) and their corresponding ribosides, \(O\)-glucosides, 7-glucosides, 9-glucosides and ribotides (5’monophosphates) conjugates (Table 1 and Table 2). MEP pathway-derived isoprenoid cytokinins viz. \(tZ9G, DHZ7G, DHZ9G, DHZR5'MP, iP7G\) and \(iP9G,\) and MVA pathway-derived cytokinins, \(cZ7G, cZ9G\) and aromatic cytokinins viz. \(mTR, mT7G\) and \(mT9G\) were totally absent in all the treatments (Table 1 and Table 2). The most prominent cytokinins were MVA pathway-derived less biologically active \(cZ\) type cytokinins, particularly \(cZROG\) which is a deactivation reversible form and can revert back to base \(cZ.\)
Precursor \( tZR5'\)MP was only present in seeds treated with KAR\(_{1}\) in R-light. \( cZR5'\)MP was present in KAR\(_{1}\)-treated seeds and water control in the dark. In R-light, \( cZR5'\)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, iP\( R5'\)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, DHZ\( R5'\)MP was not present in any of the treatments (Table 1).

The transport form \( tZR \) was detected only in R-light in seeds treated with KAR\(_{1}\), SW and water. The levels of riboside \( cZR \) and DHZR were significantly higher in the water control compared to SW, KAR\(_{1}\) and TMB-treated seeds in dark, R- and FR-light. There was no significant difference in the levels of \( cZR \) and DHZR in SW, KAR\(_{1}\) and TMB-treated seeds. Riboside iP was not detected in TMB-treated seeds in dark and there was no significant difference in levels of iP in dark and R-light. However in FR-light the iP levels were highest in the water control.

Among the active bases, \( cZ \) was most prevalent followed by DHZ, iP and \( tZ \). Under dark conditions, \( tZ \) was only detected in lettuce seeds treated with KAR\(_{1}\). In R-light, \( tZ \) was detected in SW- and KAR\(_{1}\)-treated and control seeds. However, no significant difference was observed. The levels of \( cZ \) were significantly increased in treatments compared to the control. DHZ was absent in the water control in dark, R- 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 storage/inactive (reversible forms) cytokinins \( tZOG \) and \( cZOG \) were higher in the dark compared to R- and FR-light in all the treatments (Table 1). The levels of \( tZOG \) and \( cZOG \) were significantly higher in the water control compared to 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. \( tZROG \) was present in all the treatments, however, the differences were non-significant. \( cZROG \) increased significantly under all light conditions. The overall levels of \( cZROG \) and DHZROG increased in R-light (Table 1). Among the irreversible deactivation forms (7-glucosides and 9-glucosides) \( cZ7G \), DHZ7G, iP7G, \( tZ9G \), \( cZ9G \), DHZ9G and iP9G were absent in all the
treatments. However, tZ7G 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 tZ7G.

The MVA pathway-derived cytokinins were significantly higher in comparison to MEP pathway-derived cytokinins (Supplemental data, Supplemental Figure S1). The levels of MEP-derived cytokinins were significantly lower after treatments in comparison to the control under dark. The levels of MVA pathway-derived cytokinins were significantly greater in KAR$_1$-treated seeds in comparison to 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 to dark and FR-light in all the treatments with the exception of KAR$_1$-treated seeds under dark conditions (Supplemental data, Supplemental Figure S2). In the dark, the levels of total cytokinins were significantly higher in KAR$_1$-treated seeds in comparison to the other treatments. In R-light, the levels of total cytokinins were non-significant in all the treatments (Supplemental data, Supplemental Figure S2).

The only aromatic cytokinin detected was mT (Table 2). The levels of mT were lower in SW- and KAR$_1$-treated seeds compared to TMB-treated seeds and water control under all light conditions with the exception of KAR$_1$-treated 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).

Effect 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- and FR-light. In comparison to 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 to the water control in all light treatments (Fig. 2A-C). Alpha-amylase activity in KAR$_1$-treated seeds was 1.7, 3.6 and 2.5-fold higher than the TMB-treated seeds in dark, R- 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.
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\textsubscript{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 the water control (Fig. 2D-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\textsubscript{1}, water control and TMB treatment (Fig. 3A). In R- and FR-light the lipids were maximum in KAR\textsubscript{1}-treated seeds. Lipase activity was maximum in KAR\textsubscript{1}-treated seeds followed by SW-treated seeds, water control and TMB-treated seeds in all the light treatments (Fig. 3B and C). The lipase activity in KAR\textsubscript{1}-treated seeds was 1.8, 2.9 and 2.5-fold higher compared to TMB-treated seeds in dark, R- and FR-light. The results from the present study indicate that there was a greater mobilization and utilization of stored reserves due to enhanced activity of hydrolytic enzymes in KAR\textsubscript{1}- and SW-treated seeds.

**DISCUSSION**

Lettuce seeds treated with water showed 12% germination in the dark. However, 1 h of red (R) and far-red (FR) light exposure resulted in 95% and 5% seed germination respectively. The SW- and KAR\textsubscript{1}-treated seeds showed more than 90% and 97% germination respectively. 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\textsubscript{1}-treated seeds significantly overcame the inhibitory effect of FR-light and resulted in 28% and 35% germination respectively, compared to no germination in TMB-treated seeds. This might have occurred due to substantial decreases in ABA content of SW- and KAR\textsubscript{1}-treated seeds. Plant-derived smoke and KAR\textsubscript{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 phytochrome, induced in R- and FR-light respectively, has a unique role in regulating Grand Rapids lettuce seed dormancy and germination (Black et al., 1974). The Pfr form of phytochrome deactivates abscisic acid (ABA) synthesis genes, whilst the Pr form activates these genes (Seo et al., 2006). ABA is a dormancy-inducing hormone and it inhibits seed germination by inhibiting the transition of the embryo to plant and radicle elongation (Fountain and Bewley, 1976; Muller et al., 2006; Finkelstein et al., 2008). It also inhibits storage oil mobilization and hydrolysing enzymes. The levels of dormancy-inducing hormone ABA in seeds treated with SW, KAR\textsubscript{1} and TMB were quantified in the present 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- 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 the current study. Smoke compounds tested in the present study have been shown to modulate ABA levels very precisely as KAR$_1$ decreased and TMB increased ABA levels in dark, R- 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 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). Red light treatments increased endogenous cytokinin levels and FR-light reversed this effect (Van Staden, 1973). Cytokinins also 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). Gibberellins are known for releasing seed dormancy, however, higher concentrations of gibberellin is nearly
ineffective in releasing dormancy caused by ABA. This inhibition of germination caused by ABA may only be reversed with cytokinins (Khan, 1967; Khan, 1968; Bewley and Fountain, 1972). Consequently, the endogenous levels of natural isoprenoid and aromatic cytokinins were quantified in the present study.

The detailed assessment of the concentration of various endogenous cytokinins and their fluctuations after 24 h of germination in lettuce seeds treated with smoke compounds in various light treatment revealed that the content of MVA pathway (cytosol) derived cytokinins (cZ type) was much higher compared to MEP pathway-derived cytokinins (tZ, DHZ and iP type) (Table 1 and Supplemental data, Supplemental Figure 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, cZ levels were higher after 24 h of imbibition (Gajdošová et al., 2011). Similarly, cZ concentrations are higher during seed development in specific chickpea cultivars (Lulsdorf et al., 2013). Dwarf hops varieties contain significantly higher amounts of cZs (Patzak et al., 2013) and cZR is a major cytokinin in unfertilized hops (Watanabe et al., 1981). These results reveal that cZ-type cytokinins tend to accumulate under particular circumstances such as seed germination.

Ribotides play a central role in 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 tZR5’MP (except in KAR1-treated seeds in R-light) were absent in all the treatments. Ribotide cZR5’MP was absent in SW-treated seeds in dark and FR-light, whereas TMB treatment inhibited it in dark, R- 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-glucoside and 9-glucoside), except tZ7G 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 riboside-O-glucosides cZROG were significantly highest of the quantified cytokinins. tZOG and cZOG 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 (Spičhal et al., 2004).
In the present study we observed that R-light treatment triggered or enhanced levels of some of the cytokinins such as precursors tZR5’MP, cZR5’MP and iPR5’MP, transport form tZR, base tZ, storage reversible inactive forms cZROG and DHZROG. The levels of the active isoprenoid cytokinins, base tZ and transport form tZR were low. KAR1-treated seeds had tZ only in the dark. In R-light, tZ and tZR were detected in seeds treated with SW and KAR1 and water control. TMB completely inhibited tZ and tZR in R-light.

The levels of transport form cZR were higher in the control as compared to the treatments, whereas, the levels of base cZ were higher in the treatments as compared to the control. This suggests that the treatments enhanced the conversion of transport form cZR to base cZ under all light conditions. In contrast, DHZ, DHZR, iP and iPR did not show any trend or significant difference in various treatments. Phaseolus (Phaseolus vulgaris) (Mok et al., 1978) and tobacco (Nicotiana tabacum) cell-culture assays (Schmitz et al., 1972; Gajdošová et al., 2011) revealed that cZ type cytokinins have little or no activity compared to iP and tZ types, which are generally considered as the most active natural cytokinins. trans-Zeatin type cytokinin 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 tZ, 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 (tZ and tZR) 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 tZ and tZR 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 decrease in FR-light in comparison to dark was observed in all treatments. Similar results were reported for Rumex (Rumex obtusifolius) (Van Staden and Wareing, 1972). On comparing the total isoprenoid cytokinins it was revealed that KAR1 was able to regulate the total isoprenoid cytokinins in the dark (Supplemental data, Supplemental Figure 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)/pathway(s) for lettuce seed germination, regulated by KAR1 and TMB (Wang et al., 2015).

The levels of aromatic cytokinin mT were low in SW- and KAR1-treated seeds compared to 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 (Kamínek et al., 1987) and to play a role in retarding senescence (Strnad, 1997).

The levels of indole-3-aspartate (IAAsp), the main naturally-occurring irreversible catabolite of IAA, was significantly higher in TMB-treated seeds under R-light compared to SW- and KAR₁-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 red 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 (approx. 33%) and proteins (about 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 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 KAR₁-treated seeds in comparison to TMB-treated seeds (Fig. 2 and Fig. 3). The increased protein content in TMB-treated seeds compared to the water control may be attributed to an increase in some germination inhibitory proteins in dark and FR-light which might have been suppressed in R-light. In this respect further investigations are necessary. KAR₁ 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. KAR₁ acted in a similar manner to GA, mediating the release of hydrolytic enzymes which hydrolyse 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 protein related to signalling and transport (Rehman et al.,
\[ \alpha -Amylase \] 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 seedlings by efficient starch mobilization (Sunmonu et al., 2016). TMB downregulates genes required for storage reserve mobilization (Soós et al., 2012). The present results showed a positive correlation (amylase: starch: \(R^2 = 0.32\); lipase: lipid: \(R^2 = 0.74\)) between elevation in hydrolytic enzymes (lipase and \(\alpha\)-amylase) and their substrates (lipids and starch). The germinated seeds showed greater mobilization of lipids and starch compared to 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 the abundance of transcripts encoding LIPOXYGENASE2 (LOX2) and ISOCITRATE LYASE (ICL) enzymes involved in the mobilization of lipids, were higher in germinated seeds than in both dry and ungerminated lettuce seeds. The increased \(\alpha\)-amylase and lipase activity in the lettuce seeds may be attributed to the relatively higher starch and lipid contents. In the present study, an increase in mobilization of food reserves with SW and KAR\(_1\) treatments and decrease in mobilization with TMB treatment was 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 the present 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 the present 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 growth rate of many plant species (Blank and Young, 1998).

**CONCLUSIONS**

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 signalling. 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 inter-conversion 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 absence of iZ and iZR. Further research is needed to identify the influence of smoke compounds on other growth regulators such as gibberellins (GA) and ethylene in phytochrome-regulated germination.

**MATERIALS AND METHODS**

**Plant Material**

*Lactuca sativa* L. cv Grand Rapids seeds were purchased from Stokes Seeds, U.S.A., (Lot # 212388). The seeds were checked for light sensitivity. Mature seeds of 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**

Smoke-water (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.

**Grand Rapids Bioassay**

For performing the 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 and counted under a green ‘safe light’ (0.5 μmol m$^{-2}$ s$^{-1}$) and were placed in 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 viz, smoke-water (SW, 1:2500 [v/v]), karrikinolide (KAR$_1$, 10$^{-7}$ M) and trimethylbutenolide (TMB, 10$^{-7}$ M), and distilled water (DW) was used as a control. The Petri plates were then wrapped in aluminium foil and placed in wooden light-proof boxes which were painted black inside, and the lid of the boxes were again wrapped in aluminium 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 red (R) light (660 nm) and another to far-red (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 seed, and four replicates with 200 mg seeds for each treatment were used for the biochemical determinations.

**Biochemical Determinations**

**Protein Estimation**

Total protein was estimated according to Bradford (1976) method with minor modifications, using bovine serum albumin (BSA) as a standard. Seed (200 mg) were homogenized in an ice-chilled mortar and pestle with 6 mL ice-cold phosphate-buffered saline (PBS) [8 g NaCl (137 mM), 0.2 g KCl (2.7 mM), 1.44 g Na₂HPO₄ (10 mM), 0.24 g KH₂PO₄ (1.8 mM) in 1 L of DW, (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 volume made up to 1 mL in all test tubes with PBS. 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 the Grand Rapids seeds using the method described by Sadasivam and Manickam (1996) with minor modifications. Seeds (200 mg) were extracted in 5 mL 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 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 properly diluted enzyme extract (from previous step) in a test tube and incubated at 27°C for 5 min. The reaction was stopped by the addition of 2 mL DNS 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 cooled in running tap water. Absorbance was recorded at 560 nm after the volume was made up to 5 mL by adding
1 mL distilled water. The standard curve was made using 0-100 µg maltose.

**Lipase Activity**

Lipase activity was assayed using the method of Itaya and Ui (1965) with minor modifications. Grand Rapids seeds (200 mg) were homogenised with 2 mL borate buffer (0.2 M, pH 7.2) containing 20% (w/v) PVP and centrifuged at 10,000 × g for 20 min. To 100 µL supernatant, 1 mL substrate [0.98% (w/v) NaCl, 5 g gum acacia, 5 mL 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. Afterwards, 6 mL chloroform and 2 mL sodium phosphate buffer (0.66 mM, pH 6.2) was added and allowed to settle for 30 min at room temperature. The lower layer was separated and 3 copper triethanolamine reagents (1 M triethanolamine, 1 N acetic acid, 6.45% [w/v] copper nitrate) were mixed with it and re-incubated for next 30 min.

Thereafter, in the lower layer, 100 µL 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 DM (dry mass) of seeds.

**Estimation of Starch**

Starch content was estimated using the method described by Sadasivam and Manickam (1996) with minor modifications. Grand Rapids seeds (200 mg) were homogenised in hot ethanol (80%) to remove sugars. The homogenate was centrifuged at 3,000 × g for 15 min, and the residue retained and repeatedly washed with hot ethanol (80%) till the washings did not give colour 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 water and 3 mL 52% 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 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 colour changed from green to dark green. The standard curve was made using 0–100 µg glucose.
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 hydrolysed in a boiling water bath for 3 h with 3 mL 2.5 N HCl and then cooled to room temperature. The hydrolysed 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 anthrone reagent was added after the volume was made up to 1 mL using DW. 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 colour changed from green to dark green. The standard curve was prepared using 0-100 µg glucose.

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 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 pre-weighed 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 (mg g⁻¹) of fresh seed.

Estimation of Phytohormones

After appropriate treatment with SW, KAR₁ 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 modified Bieleski buffer (60% methanol, 10% formic acid and 30% H2O) with a cocktail of stable isotope-labelled internal standards (0.25 pmol of cytokinin bases, ribosides, N-glucosides, and 0.5 pmol of cytokinin O-glucosides, nucleotides per sample) for determination of endogenous cytokinins. The extracts were purified using a combination of C18 (1 mL/30 mg) and MCX (1 mL/30 mg)
cartridges (Dobrev and Kamínek, 2002). Eluates were evaporated to dry, dissolved in 30 μL 10% methanol and analysed by the separation methods described in Svačinová et al. (2012).

To determine levels of auxins and abscisic acid, 1 mL 50 mM sodium phosphate buffer (pH 7.0) was used as the extraction solution with isotope-labelled internal standards (5 pmol of \[^{13}C_6\]-IAA, \[^{13}C_6\]-IAAsp, \[^{13}C_6\]-oxIAA, \[^{13}C_6\]-IAGlu, [D6]-ABA per sample) added before homogenization and purified using HLB (1 mL/30 mg) cartridges. Eluates were evaporated to dry, subsequently dissolved in 30 μL 10% methanol and analysed by methods described by Novák et al. (2012). The endogenous levels of phytohormones were determined using an ultra-high performance liquid chromatography (Acquity UPLC® I-Class System; Waters, Milford, Ma, USA) coupled to a triple quadrupole mass spectrometer with an electrospray interface (Xevo TQ-S, Waters, Manchester, UK). 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) (Genstat 17th Edition). Correlation was calculated using MS excel software programme.

Supplemental Data

Supplemental Figure S1. Endogenous 2-C-methyl-D-erythritol 4-phosphate pathway derived and mevalonate pathway derived isoprenoid cytokinin content.

Supplemental Figure S2. Total endogenous isoprenoid cytokinin content in Grand Rapids lettuce seeds incubated for 24 h at 25°C.
of incubation in the dark, seeds were exposed to respective red or far red light treatments for 1 h and were replaced in the dark.

Supplemental Figure S2. Total endogenous isoprenoid cytokinin content (pmol g\(^{-1}\)) in 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 respective red or far red light treatments for 1 h and were replaced in the dark.

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Conflict of interest
It is declared that the authors have no conflict of interest in the publication of this article. Neither the manuscript nor its main contents have been published or submitted elsewhere.

Table Legends
Table 1: Endogenous isoprenoid cytokinin content (pmol g\(^{-1}\)) in 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 respective red or far red light treatments for 1 h and were replaced in the dark.

Table 2: Endogenous aromatic cytokinin and auxin content (pmol g\(^{-1}\)) in 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 respective red or far red light treatments for 1 h and were replaced in the dark.

Figure Legends
Figure 1. Effect of smoke-water (SW), karrikinolide (KAR$_1$), and (trimethylbutenolide) TMB on germination ($n = 4$) and abscisic acid (ABA, $n = 3$) levels in 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 respective red or far red light treatments 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 ($P < 0.05$).

Figure 2. Influence of SW, KAR$_1$ and TMB on $\alpha$-amylase activity (A–C), and starch, sugar and protein content (D–F) in 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 respective red or far red light treatments 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 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 respective red or far red light treatments for 1 h and were replaced in the dark. Bars (lipase± SE) and symbols (lipids ± SE) for each light condition(s) with different letters are significantly different according to Bonferroni correction ($P < 0.05$).

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Fig. 1 Gupta et al.
Fig. 2 Gupta et al.
**Fig. 3 Gupta et al.**
| Cytokinin (pmol g⁻¹) | Treatment | Dark | Red | Far red |
|---------------------|-----------|------|-----|--------|
| **Riboside**        |           |      |     |        |
|                     |           |      |     |        |
| IZR5'MP             | trans-Zeatin riboside-5'-monophosphate |      |     |        |
| Control             | –         | 2.5 ± 0.1 a | –   | –      |
| SW                  | –         | 2.3 ± 0.1 a | –   | –      |
| KAR₁                | –         | 3.5 ± 0.1 a | 1.5 ± 0.1 a | – |
| TMB                 | –         | –      | –   | –      |
| **Riboside**        |           |      |     |        |
|                     |           |      |     |        |
| IZR                 | trans-Zeatin riboside |      |     |        |
| Control             | –         | 12.3 ± 0.3 a | 14.3 ± 1.1 a | 14.7 ± 1.8 a |
| SW                  | –         | 5.3 ± 0.2 b | 3.8 ± 0.2 b | 7.5 ± 0.5 b |
| KAR₁                | –         | 5.9 ± 0.3 b | 4.4 ± 0.3 b | 7.7 ± 0.2 b |
| TMB                 | –         | 6.1 ± 0.4 b | 7.1 ± 1.6 b | 5.7 ± 0.4 b |
| **Base**            |           |      |     |        |
|                     |           |      |     |        |
| IZ                  | trans-Zeatin |      |     |        |
| Control             | –         | 1.8 ± 0.1 b | 1.1 ± 0.0 c | 1.1 ± 0.0 c |
| SW                  | –         | 4.3 ± 0.3 a | 3.7 ± 0.3 b | 4.6 ± 0.0 b |
| KAR₁                | 0.19 ± 0.1 a | 5.8 ± 0.2 a | 3.2 ± 0.2 b | 7.3 ± 0.3 a |
| TMB                 | –         | 4.0 ± 0.1 a | 5.8 ± 0.5 a | 5.9 ± 0.4 b |
| **Glucoside**       |           |      |     |        |
|                     |           |      |     |        |
| IZOG                | trans-Zeatin-O-glucoside |      |     |        |
| Control             | 86.7 ± 7.8 a | 46.5 ± 3.5 a | 52.4 ± 3.7 a |         |
| SW                  | 61.4 ± 1.3 ab | 49.9 ± 0.1 a | 49.7 ± 2.6 a |         |
| KAR₁                | 61.1 ± 1.7 b | 51.3 ± 3.6 a | 49.5 ± 3.4 a |         |
| TMB                 | 61.2 ± 0.2 ab | 55.8 ± 2.4 a | 49.1 ± 2.3 a |         |
| **Riboside-**       |           |      |     |        |
|                     |           |      |     |        |
| IZOG                | trans-Zeatin-O-glucoside riboside |      |     |        |
| Control             | 1.5 ± 0.1 a | 1.3 ± 0.1 a | 1.3 ± 0.0 a |         |
| SW                  | 1.5 ± 0.1 a | 1.5 ± 0.1 a | 1.2 ± 0.1 a |         |
| KAR₁                | 1.8 ± 0.1 a | 1.0 ± 0.1 a | 1.2 ± 0.1 a |         |
| TMB                 | 1.5 ± 0.1 a | 1.2 ± 0.1 a | 1.2 ± 0.1 a |         |
| **O-glucoside**     |           |      |     |        |
|                     |           |      |     |        |
| IZ7G                | trans-Zeatin-7-glucoside |      |     |        |
| Control             | 10.2 ± 0.4 a | 7.8 ± 0.4 a | 9.5 ± 0.5 ab |         |
| SW                  | 9.4 ± 0.5 a | 9.2 ± 0.6 a | 9.7 ± 0.3 a |         |
| KAR₁                | 9.4 ± 1.0 a | 8.2 ± 0.5 a | 8.1 ± 0.4 bc |         |
| TMB                 | 9.0 ± 0.7 a | 9.2 ± 0.5 a | 7.8 ± 0.5 c |         |
| **9-glucoside**     |           |      |     |        |
|                     |           |      |     |        |
| IZ9G                | trans-Zeatin-9-glucoside |      |     |        |
| Control             | –         | –    | –   | –      |
| SW                  | –         | –    | –   | –      |
| KAR₁                | –         | –    | –   | –      |
| TMB                 | –         | –    | –   | –      |

Mean values for each cytokinin type and light condition in a column with different letter(s) are significantly different according to Bonferroni correction ($P < 0.05$). Hyphens (–) represent values below detection levels. Treatments: smoke water (SW; 1°C 2500 μW), karrkinolide (KAR; 10⁻⁷ M), trimethylbutenolide (TMB; 10⁻⁷ M).
Table 2. Endogenous aromatic cytokinin and auxin content (pmol g\(^{-1}\)) in 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 respective red or far red light treatments for 1 h and were replaced in the dark.

| Treatment | Dark | Red light | Far-red light | Dark | Red light | Far-red light |
|-----------|------|-----------|---------------|------|-----------|---------------|
|           | Cytokinin (pmol g\(^{-1}\)) | | | Auxin (pmol g\(^{-1}\)) | | |
| mT meta-topolin | | | | IAAsp Indole-3-aspartate | | |
| 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 ab | 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 ab | 5.6 ± 0.3 a | 46.3 ± 0.1 a | 115.6 ± 15.3 a | 50.6 ± 3.5 a |
| mTR meta-topolin riboside | | | | IAAGlu Indole-3-glutamate | | |
| Control   | - | - | - | - | - | - |
| SW        | - | - | - | - | - | - |
| KAR\(_1\) | - | - | - | - | - | - |
| TMB       | - | - | - | - | - | - |
| mT7G meta-topolin-7-glucoside | | | | IAAox 2-oxindole-3-acetic acid | | |
| Control   | - | - | - | - | - | - |
| SW        | - | - | - | - | - | - |
| KAR\(_1\) | - | - | - | - | - | - |
| TMB       | - | - | - | - | - | - |
| mT9G meta-topolin-9-glucoside | | | | | | |
| Control   | - | - | - | - | - | - |
| SW        | - | - | - | - | - | - |
| KAR\(_1\) | - | - | - | - | - | - |
| TMB       | - | - | - | - | - | - |

Mean values for each aromatic cytokinin and auxin in a column with different letter(s) are significantly different according to Bonferroni correction (\(P < 0.05\)). Hyphens (–) in the columns represents values below detection levels. Treatments: smoke-water (SW; 1:2500, v/v), karrikinolide (KAR\(_1\); 10\(^{-7}\) M), trimethylbutenolide (TMB; 10\(^{-7}\) M).
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