The Potential of L-Methionine and Ethylene as Precursors of Aerobic Methane Emissions from Plants

by

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Abstract

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ABSTRACT

Earlier work has shown that plants emit aerobic methane, but it is unclear how this occurs on a metabolic level. The goal of this thesis is to determine the effects of ethylene on methane emissions, and to examine the potential of L-Methionine as a unique precursor. In addition to measurements of methane and ethylene, growth and physiological parameters were measured in addition to a metabolite profile to measure the content of 13 amino acids. While application of ethylene promoters and inhibitors had no effect on methane, high light decreased emissions whereas blue light increased them, and exogenous ethylene application significantly increased emissions. Relationships were shown between multiple amino acids and aerobic methane, suggesting that methionine is not a unique precursor. Experimental evaluation suggests that methane emissions were the product of reactive oxygen species-mediated methyl group cleavage from a number of compounds.

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"The more that you read,
The more things you will know.
The more that you learn,
The more places you'll go."

-Dr. Seuss

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1.0. INTRODUCTION

1.1. CLIMATE CHANGE

There is undeniable evidence that the Earth's climate has changed in the last century, to an extent that cannot be attributed to normal climate cycles (Wheeler & von Braun, 2013). Climate is defined as characteristic weather conditions at specific locations of the Earth's troposphere; it includes conditions such as air temperature, precipitation, atmospheric pressure, humidity, wind, sunlight, and cloud covering (UNFCCC, 2006). Climate change is a change in climatic patterns over a long period; while these changes once followed a natural cycle, modern climate change is in part due to human influence, fuelled by the increase in population and the rise in the combustion of fossil fuels (IPCC, 2013). Climate change has observable effects that leave people, societies, ecosystems, and even entire economies at risk, which is already evident. There has already been evidence of increased smog, species extinction, agricultural loss, and extreme climatic events (IPCC, 2013).

As a result of anthropogenic emissions, CO_2 levels have currently reached 407 μmol mol^{-1} and may surpass 700 μmol mol^{-1} by the end of the century (IPCC, 2013). Additionally, a warming of 0.65-1.06°C has already occurred in the period of 1880 to 2012, with the last 30 years being the warmest in nearly a millennium. From 2016 to 2035 alone, projections predict a further temperature increase of 0.3-0.7°C, and by 2100 it is expected that temperatures will rise at least another 1.5°C, and as much as 6.4°C (IPCC, 2013). Other effects of climate change include increased incidences of drought, alterations in light quantity and quality, changes in UVB irradiation, and modifications to ecosystem suitability for native species (IPCC, 2013).

From an agricultural perspective, negative effects of climate change are already noticeable (Deryng *et al*., 2014; Koralewski *et al*., 2015). Over the period of 1960-2013, losses of at least 2% were evident for major crops, such as wheat, rice, and corn, with temperate regions experiencing a greater loss than tropical ones. In contrast, yields of soybean increased marginally, but the negative effects of climate change will far outweigh the positive ones (IPCC, 2013). Since wheat, rice, and maize are among the six most widely grown crops in the world, accounting for 40% of global cropland and 55% of non-meat caloric intake, these effects may challenge future food security (Ingram *et al*., 2008; Wheeler & von Braun, 2013). This is evident by the 1-1.7% yield loss by every 1°C increase in temperature above 30°C (Lobell *et al*., 2011), and although cereal yields have recently stabilized, production levels are approximately 25% less than they need to be to meet agricultural demand by 2050 (Mwongera *et al*., 2014). Additionally, yield reductions of approximately 10% are expected in the future, and will exacerbate food security problems (IPCC, 2013), especially in developing countries where this is already an issue. Since crop breeding may not result in yield increases under climate change (Ingram *et al*., 2008), new and innovative methods, such as genetic engineering, are needed to increase yields in the future (Long & Ort, 2010).

Climate change may bring some benefit to certain parts of the world, including agricultural areas in northern latitudes above 55°; however, there is a small amount of land at this latitude that is suitable for agriculture (Ingram *et al*., 2008; Wheeler & von Braun, 2013), so this cannot compensate for the loss projected for other areas. On a local scale, changes in climate are likely to alter environmental suitability for the growth of certain

crops and may negatively affect local population diversity (Garcia *et al*., 2014). In addition to the direct effects of climate on crop yield, climate change may introduce new pests, diseases, and weeds to new environments (Ingram *et al*., 2008; Koralewski *et al*., 2015), due in part to shifting of optimal habitats towards the poles. It is imperative that more work is put into adaptation and mitigation strategies, including crop selection for desirable traits, such as resilience to both abiotic and biotic stresses, genetic engineering to introduce resilient genes, movement of crops to better-suited environments, and mitigation of the direct impact of agriculture on greenhouse emissions.

1.2. THE GREENHOUSE EFFECT

Solar radiation passes through the Earth's atmosphere, reaching the Earth's surface and leading to warming. It is then re-emitted back to the atmosphere as infrared radiation, where most of it is temporarily trapped by CO_2, water vapour, methane, and other greenhouse gases (GHGs). This process leads to warming of the lower atmosphere, which acts as a blanket around the Earth, keeping temperatures at levels that can sustain life (Galashev, 2011; Zhong & Haigh, 2013; Anderson *et al*., 2016). Without this natural effect, the average surface temperature would be approximately -21°C as opposed to the current 14°C (Zhong & Haigh, 2013; Anderson *et al*., 2016). The natural greenhouse effect has been enhanced since the industrial revolution, during which technological innovations increased dramatically (Angulo-Brown *et al*., 2009; Pearson & Foxon, 2012). This led to decreased cost, increased quality, and increased production output capabilities. As standards of living rose, so did urbanization, use of inexpensive coal, deforestation, and exportation of goods (Pearson & Foxon, 2012). These processes have led to an increased

use of fossil fuels, and therefore in GHG emissions, which have continued to rise ever since (Angulo-Brown *et al*., 2009; Pearson & Foxon, 2012).

In 2015, 195 countries came together at the summit of the United Nations Climate Change Conference in Paris to acknowledge the risks that climate change poses to humankind. A goal was established to reduce GHG emissions and keep total warming below 2°C, relative to pre-industrial levels (Pearson & Foxon, 2012; Anderson *et al*., 2016). It was estimated that this would require a 50% reduction in global GHG emissions by 2050, including a transition towards low-emission processes and renewable energy. While conversion to low-emission processes would require a significant economic investment, associated costs would be less than the cost of unmitigated climate change (Stern, 2007).

1.3. GREENHOUSE GASES

Greenhouse gases (GHGs) include compounds such as water vapour, carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) along with other trace gases, accounting for 36-70%, 9-26%, 4-9%, and 3-7% of GHGs, respectively (Yusuf *et al*., 2012). Of these, water vapor, CO_2 and CH_4 have been shown to be the main contributors to the rise in global surface temperatures (Yusuf *et al*., 2012), and recent emissions have exceeded known values for the past 800,000 years (IPCC, 2013). In developed countries, GHG emissions are relatively stagnant; the drastic increase is largely due to developing areas, which have a more rapid growing population and less-developed agricultural practices (Popp *et al*., 2010).

Carbon dioxide is the largest contributor to radiative forcing, with approximately 40% of emissions remaining in the atmosphere since 1750, while the other 60% has been taken

in by carbon sinks or reservoirs (IPCC, 2013). Increasing atmospheric GHGs have the potential for profound effects on agriculture; while studies have shown that biomass increases in response to CO_2 (Qaderi *et al*., 2013; Sakurai *et al*., 2014; Ackerman & Munitz, 2016), macro and micronutrients (Höby & Fangmeier, 2008) and overall protein content (Altenbach, 2012) have been shown to decrease in wheat, which may lead to a requirement for greater consumption for the same nutritional benefit.

Since 1970, global agricultural output has more than doubled, and agriculture now accounts for 25% of anthropogenic GHG emissions, in part due to the increase from 3.7 to over 7 billion people in the global population (Bennetzen *et al*., 2016). Recent evidence suggests that agriculture is becoming more efficient, with emission rates decreasing by 39% for crops and 44% for animal agriculture (Bennetzen *et al*., 2016). Non-CO_2 agricultural GHG emissions may be mitigated if meat consumption decreases; however, livestock protein contributes approximately 33% of dietary protein, which is important in areas of high malnutrition (Popp *et al*., 2010). As such, agriculture-based GHG emissions are expected to rise in the future, due to both a rising population and changing dietary preferences, with developing countries increasing consumption of meat and dairy products (Popp *et al*., 2010). Over the last 20 years, both animal and plant-based agriculture has contributed approximately 22% of total GHG emissions, increasing from 14% over the last hundred years (IPCC, 2013). Furthermore, agricultural N_2O emissions now account for 58% of total N_2O release, in part due to nitrogen fertilizers and manure storage and application (Popp *et al*., 2010).

1.4. PLANT RESPONSES TO CLIMATE CHANGE

Since plants are sessile organisms, they have evolved a number of coping mechanisms to persist in periods of environmental stress, including factors such as higher temperatures, elevated CO_2, increased ultraviolet-B radiation, drought, salinity, and altered light quantity or quality (Ahuja *et al.*, 2010). In response to chronic stress, plants can gradually acclimate, allowing for survival under subpar environments; however, extreme weather events do not allow for this same level of acclimation, often having drastic consequences for crop yield (Walter *et al.*, 2013). In response to chronic stress, plants may shift to northern latitudes, if the environments are suitable for their growth, exposing them to new environments with different average temperatures and light quality (Qaderi *et al*., 2015).

While studies examining individual environmental stress factors are abundant, few examine multiple factors and their interactions. Since stress factors occur in combination in natural environments, it is important to understand how plants respond to multiple stresses simultaneously (Ahuja *et al.*, 2010). Crop losses to multiple factors are often greater than observed with individual factors, and combining stress factors can lead to antagonistic or conflicting responses. Plant responses to climate change depend both on the species in question and the magnitude and combination of the applied stress (Qaderi *et al*., 2015), and it therefore proves difficult to predict the effects of multiple stress from the results of single-stress studies (Prasch & Sonnewald, 2015). Typically, responsive signal transduction pathways must be integrated in order for plants to adapt resource allocation between defense, growth, and reproduction for the greatest chance of survival. Signals usually include phytohormones such as abscisic acid (ABA), ethylene, jasmonic acid, and

salicylic acid; reactive oxygen species (ROS); lipid and metabolite signals; changes in redox status; altered protein composition, ion homeostasis; and changes to primary and secondary metabolism (Prasch & Sonnewald, 2015).

The most widely recognized physiological response to environmental stress in plants is reduced biomass; under stress conditions, plants allocate less resources to growth processes, and invest more into physiological responses to the stress (Wang & Frei, 2011). Plant exposure to stress leads to many physiological effects, including alterations in photosynthetic gas exchange and assimilate translocation (Morgan *et al.,* 2004), altered water uptake, evapotranspiration (Wang & Frei, 2011), nutrient uptake and translocation (Hu & Schmidhalter, 2005; Sánchez-Rodríguez *et al.*, 2010), antioxidant responses (Blokhina *et al.,* 2003; Apel & Hirt, 2004), programmed cell death, and alterations in gene expression and enzyme activity (Guo *et al.*, 2009). Persistence of chronic stress exposure can promote early flowering (Prasch & Sonnewald, 2015), thereby reducing yields and influencing chemical composition and subsequent quality of crops (Wang & Frei, 2011).

On a genetic level, environmental stress factors can influence the expression of thousands of genes (Wang & Frei, 2011). While individual stresses differ in their effects, there are some general trends among stress conditions. Many studies show an increase in antioxidant compounds such as phenolics and carotenoids under stress due to accumulation of ROS compounds (Wang & Frei, 2011). A single exposure to stress may lead to a decreased response to that same stress in the future; this occurs in part due to accumulation of metabolites that promote stress tolerance (Jansen *et al*., 2008).

From a biochemical perspective, seed carbohydrate biosynthesis is often decreased because of decreased starch accumulation and the inhibition of key enzymes involved in the synthesis of starches (Hurkman *et al.,* 2003; Wang & Frei, 2011). Stress factors also negatively affect the fatty acid composition of oil from oilseed crops such as canola; increases in saturation level were observed, resulting from a decreased proportion of polyunsaturated fatty acids and an increase in saturated fatty acids. Since polyunsaturated fatty acids, including linoleic acid, have health benefits in the human diets, this is important (Wang & Frei, 2011).

1.5. METHANE

Methane is the most abundant trace gas in the atmosphere (Wuebbles & Hayhoe, 2002), with a warming potential approximately 34x higher than CO_2 (Yusuf *et al*., 2012; Carmichael *et al*., 2014; Lattanzio *et al*., 2015), contributing approximately 25% of the climate forcing in the last quarter-millennia (Shindell *et al*., 2009). Following pre-industrial times, methane levels nearly tripled, rising steadily until the 1990's when levels began to decrease. Since 1999, methane has remained relatively constant, but anthropogenic sources appear to be increasing once more (Bousquet *et al*., 2006; IPCC, 2013), reaching an atmospheric concentration of 1803 ppb or 554 ± 55 Tg CH_4 y^{-1} (IPCC, 2013; Liu *et al.*, 2015).

Methane in the atmosphere facilitates warming of the Earth's climate because of a reaction with hydroxyl radicals to create water vapour, a primary greenhouse gas with a larger greenhouse potential than CO_2 (IPCC, 2013). Furthermore, atmospheric methane can have indirect effects on CO_2, ozone, sulphate aerosols, and the lifetime of

chlorofluorocarbons (CFCs; IPCC, 2013). Methane is a precursor to tropospheric ozone, referred to as "smog", formed through reactions with nitrogen oxides in the presence of sunlight. It can also absorb infrared radiation, contributing to increased global warming; therefore, the total contribution of methane emissions is far greater than the direct effects of methane alone (IPCC, 2013). Methane is considered a short-lived climate forcer; while it has a lifespan of only 12 years, it has significant immediate impacts, reacting to form water vapour in the stratosphere (Lattanzio *et al*., 2015).

Methane has accounted for approximately 18% of anthropogenic GHG emissions from 1970-2010 (IPCC, 2013), with 40% coming from natural sources and the other 60% from anthropogenic sources (Karakurt *et al*., 2012). The largest contributor of methane emissions is the agricultural industry, contributing approximately 50% of anthropogenic methane emissions because of enteric fermentation, manure management, rice paddies (Karakurt *et al*., 2012; Yusuf *et al*., 2012), and other agricultural sources, including aerobic methane emissions. Other large contributors are the energy (Karakurt *et al*., 2012; Yusuf *et al*., 2012) and waste management sectors (Karakurt *et al*., 2012). Methane sinks include dry soil oxidation and reaction with OH in the atmosphere (Wuebbles & Hayhoe, 2002).

1.5.1. Anaerobic methane emissions

Traditionally, methane is formed through a process called methanogenesis, occurring through decomposition of organic matter in low-nutrition, anoxic environments (Carmichael *et al*., 2014; Lattanzio *et al*., 2015). Natural sources of methane include wetlands, oceans, geological seepage, and termites, for a total of approximately 215 Tg CH_4 annually. In contrast, anthropogenic sources contribute around 430 Tg CH_4 annually

and include sources associated with fossil fuels, ruminants, waste and waste management, biomass burning, and rice cultivation (Lowe, 2006; Popp *et al*., 2010; Carmichael *et al*., 2014; Lattanzio *et al*., 2015).

1.5.2. Aerobic methane emissions

The methane budget was believed to contain all major methane sources; however, in 2006, Frank Keppler and his associates published a controversial paper in *Nature* on the role of plants in methane production (Keppler *et al*., 2006). They indicated that plants releasing aerobic methane, a previously unknown source that is unaccounted for in the methane budget. They examined C_3 and C_4 plants, eliminating possible microbial origin through leaf tissue sterilization using γ-radiation. Intact plants and detached leaves both emitted methane, and both dried and newly detached leaves were found to release methane, with typical emission rates ranging from 0.2-0.3 ng g^{-1} DM h^{-1}. Emissions were found to increase with both temperature and light levels (Keppler *et al*., 2006).

Following publication of this paper, significant debate ensued, and it was followed by refutations from several independent labs, who were unable to discern a significant rate of methane production (Dueck *et al*., 2007; Beerling *et al*., 2008; Wang *et al*., 2008). Using uniformly labelled ^{13}C plants, Dueck *et al*. (2007) measured methane emissions using continuous-flow gas exchange cuvettes, and found no emissions beyond usual background concentrations. Similarly, whole corn (*Zea mays* L.) plants measured under both light and dark (Beerling *et al*., 2008), along with detached leaves of corn were not found to emit methane (Kirschbaum *et al.*, 2008); however, for detached leaves, researchers collected tissue from the field and then transported them to the lab in plastic bags, without mention

of the length of time required for this process. This would increase the likelihood that the leaves were no longer physiologically active. Furthermore, using a whole-chamber method decreases the sensitivity of the system to detect small emissions.

In contrast to chamber-based methods, a study on a forest canopy measured every 2 hours for six weeks in 2007 failed to detect a significant foliar CH_4 source; however, this does not rule out the possibility of a weak source (Bowling *et al*., 2009). This would be consistent with the more conservative CH_4 estimates from plants, as opposed to the original estimates by Keppler *et al*. (2006). Furthermore, authors of this study believe that methane could be biophysical in nature as opposed to a metabolic product (Bowling *et al*., 2009). In contrast, canopy measurements of the Venezuelan forest yielded evidence of methane production (Crutzen *et al*., 2006); however, it is difficult to confirm that results from canopy studies are from the plants themselves as opposed to soil organisms.

1.5.2.1 Confirmation of the Phenomenon

While originally disputed, it is becoming increasingly clear that many species emit aerobic methane. Focus has now shifted to determining what conditions induce emissions from plants and establishing potential precursors along with pathways of production. Observed methane emissions cannot be bacterial in origin, as hydrogen (H_2), a substrate for methane production by anaerobic bacteria, was shown to have no significant effect on emissions (Wang *et al*., 2011b). Furthermore, emissions occurred from both fresh and dried organic matter, regardless of the presence of oxygen (Vigano *et al*., 2008). Methane production is non-enzymatic in nature, as there is a lack of correlation between leaf nitrogen content and methane emissions (Watanabe *et al*., 2012). Furthermore, methane release was found in

soil even above 70°C, beyond temperatures at which enzymes can function; however, some microbes can function at extreme temperatures and the possibility of extremophiles in the soil has not been ruled out (Hurkuck *et al*., 2012), but this can be avoided through soil sterilization or hydroponic growth systems.

The magnitude of aerobic emissions has been shown to vary between species; indeed, some species do not produce methane at all (Wang *et al*., 2008; Martel & Qaderi, 2017). In a study of 44 species, only 9 were found to emit methane, and some of these emissions were from the stem, likely a result of the transpirational stream. However, one species, *A. frigada*, a shrub, emitted methane continuously, indicating that emissions were not simply the result of methane stores in leaf air spaces (Wang *et al*., 2008). Emission rates of *A. frigada* were similar to the rates reported in the original paper by Keppler et al. (2006). Emissions were positively correlated with moisture content (Watanabe *et al*., 2012; Qaderi & Reid, 2014; Martel & Qaderi, 2017), and negatively correlated with leaf mass per area, which suggests that leaf thickness and structure may play a role in species-dependent differences in emissions (Watanabe *et al*., 2012). In some species, emissions were unaffected by aerobic or anaerobic conditions during incubation, while emissions in others increased in one over the other (Wang *et al*., 2011b).

1.5.2.2. Effects of environmental factors

Increases in plant-based emissions were found under environmental stress (McLeod *et al*., 2008; Vigano *et al*., 2008; Bruhn *et al*., 2009; Qaderi & Reid, 2009; Wang *et al*., 2009; Wang *et al*., 2011a, b; Bruhn *et al*., 2014; Qaderi & Reid, 2014; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al*., 2017; Martel & Qaderi, 2017), and since environmental stress

will increase with climate change, methane emissions may increase in the future as well, regardless of the mitigating effects of CO_2 (Qaderi & Reid, 2011). Aerobic methane production may account for 1.2-9% of the global methane budget, regardless of lack of inclusion in the IPCC reports (Carmichael *et al*., 2014). Since methane is a potent greenhouse gas, it is important to understand what conditions induce methane emissions from plants, along with the biochemical pathway for its production, to allow for the reduction of emissions through improved agricultural practices.

It was originally believed that methane emissions were stimulated only by UV-radiation and not visible light (Bruhn *et al*., 2014), but recent work has suggested that methane can be increased by altered visible light as well (Martel & Qaderi, 2017). UVB radiation (290 – 320 nm) is a greater stimulant of methane emissions than UVA (320 – 400 nm, Vigano *et al*., 2008; Bruhn *et al*., 2009), which may be the result of the higher-energy wavelength of UVB in comparison to UVA. UVB stimulated methane from both plant matter and structural components, including purified pectin, lignin, and cellulose (Vigano *et al*., 2008). In purified pectin, UVB radiation increased CH_4 emission in a positive linear relationship (McLeod *et al*., 2008); additionally, more recent work has suggested that methane emissions may also be correlated with light levels. Methane emissions from stinkweed capsules (*Thlapsi arvense* L.) were higher when incubated under bright light as opposed to low light (Qaderi & Reid, 2014), whereas higher light decreased methane emissions in sunflower (*Helianthus annuus* L.) and chrysanthemum (*Chrysanthemum coronarium* L.) in the vegetative stage. These differences may be the result of one study using capsules and the other using seedlings. Greater levels of methane were emitted from

seedlings in the late developmental stage (Qaderi & Reid, 2014), and seedlings may differ from vegetative plants in magnitude of emissions. Both low levels of blue light and red: far-red (R:FR) light increased methane emissions in comparison to the control in sunflower and chrysanthemum, and these emissions decreased over time, suggesting acclimation to environmental conditions (Martel & Qaderi, 2017).

Temperature has been shown to increase emissions exponentially from a variety of species (Keppler *et al.*, 2006; Vigano *et al.*, 2008; Bruhn *et al*., 2009). Higher temperatures, UVB radiation and water stress, both individually and in combination, increased methane emissions from six crop species, including faba bean, sunflower, pea, canola, wheat, and barley, with species-dependent variation (Qaderi & Reid, 2009). In a later study using the same species, elevated carbon dioxide decreased emissions from plants under temperature and water stress, whereas higher temperature (Qaderi & Reid, 2011, 2014) and water stress individually increased emissions (Qaderi & Reid, 2011, 2014; Han *et al*., 2017); however, emissions were still higher than in non-stressed plants, and CO_2 may only partially mitigate stress-induced amplification (Qaderi & Reid, 2011).

Grazing, herbivory, and extreme climatic events resulting in plant tissue damage may also increase methane emissions proportional to the degree of damage (Wang *et al*., 2009; 2011a). Hypoxic conditions, or environments with low oxygen supply, cause plant stress through metabolic by-product accumulation, and were shown to increase emissions with a linear trend (Wang *et al*., 2009). Anoxic conditions, a more severe oxygen-depleted state than hypoxia, also enhanced emissions in both intact and wounded leaves (Wang *et al*., 2011a).

Observed methane emissions may be in part due to reactive oxygen species (ROS), as a ROS scavenger decreased emissions whereas rose bengal, a generator of singlet oxygen, increased methane emissions from tobaco (*Nicotiana tabacum* L.) (McLeod *et al*., 2008). This has been confirmed in subsequent studies using hydrogen peroxide (H_2O_2), another ROS compound (Wang *et al.,* 2011b). In addition to surface stresses, stress on a molecular level may also increase plant emissions. Disrupting cytochrome c oxidase, part of the electron transport chain, increased emissions significantly in grape vine, (*Vitis vinifera* L.), sugar beet (*Beta vulgaris* L.), and tobacco (*Nicotiana tabacum*). Other disruptors of the ETC did not affect methane emissions, suggesting that there may something unique about this site (Wishkerman *et al*., 2011).

1.5.2.3. Potential precursors

Since the discovery of aerobic methane emissions, a wide number of potential precursors have been suggested; however, the pathway of methane production remains unknown. Stable isotope studies have shown that the methoxyl group of plant pectin can act as a precursor of methane (Keppler *et al*., 2008; Bruhn *et al*., 2009), whereas polygalacturonic acid, the control compound, did not emit methane unless methylated, in which case it emitted methane at rates similar to pectin. The liberation of methyl groups from plant pectin may lead to photodegradation of other carbons in the molecule (Keppler *et al*., 2008; Bruhn *et al*., 2009; Messenger *et al*., 2009), and the subsequent increase in methane emissions. In addition to pectin, lignin was also shown to emit methane (Hurkuck *et al*., 2012), with emissions increasing under aerobic conditions (Wang *et al*., 2011b), but cellulose did not emit quantifiable levels (Hurkuck *et al*., 2012).

While plant pectin has been a favored potential precursor, stable isotope studies have shown that it cannot account for all observed emissions from plants (Vigano *et al.*, 2010), and there must therefore be more than one precursor. In order to liberate the methyl groups, ROS may be involved (Messenger *et al*., 2009), and since ROS increase with environmental stress (Messenger *et a*l., 2009; Wang *et al*., 2009), this is in agreement with other work. In a study on 30 different species exposed to UVB radiation, there was no correlation between methane emissions, lignin content, and foliar methyl ester content. This indicates that either not all methyl ester groups are liberated upon irradiation, or that other compounds were also contributing to emission rates (Fraser *et al*., 2015). Studies examining plant pectin have shown that pectin methyl esterases decrease the rate of methane emission, but do not eliminate it entirely (Bruhn *et al*., 2009); similarly, addition of ROS scavengers decreased, but did not eliminate emissions (Messenger *et al*., 2009). These results reinforce the suggestion that pectin, through interactions with ROS, may only be a one of several contributors to methane emissions from plants (Bruhn *et al*., 2009).

Purified solutions of methylated carbohydrates, containing either methyl esters or acetyl esters, were found to emit methane under both light and dark conditions when hydroxyl radicals were added (Messenger *et al*., 2009). In vitro experiments showed that H_2O_2 induced methane emissions from ascorbic acid, but not choline chloride, chitin, pyruvic acid, acetylsalicylic acid, 2-methoxyphenol, vanillin or L-methionine, suggesting that not all molecules with a methyl group act as precursors (Althoff *et al*., 2010). Species variability could be a result of epidermal differences, which would allow different levels of UVB to penetrate into the leaf mesophyll (Fraser *et al*., 2015).

Epicuticular wax has been shown to emit methane under both oxic and anoxic conditions. Since a number of environmental stress conditions, including UVB radiation, induce the production of epicuticle wax (Fraser *et al*., 2015) , this may induce a feedback loop. The predominant compounds in wax were nonacosane and 15-nonacosanone, which could produce aerobic methane through photolytic rearrangement to produce a ketone, followed by cleavage of the ketone to make methane (Bruhn *et al*., 2014). Other proposed precursors include chlorophyll *a*, chlorophyll *b*, carotenoids, phsopholipids, amino acids, cutin, and suberin (Fraser *et al*., 2015). It has also been hypothesized that phosphatidylcholine and L-methionine may act as precursors of methane in plants (Wishkerman *et al*., 2011; Bruhn *et al*., 2012; Lenhart *et al*., 2015), but this will be examined further (see Chapter 3).

1.5.2.4. Exploration of alternative explanations

In addition to water transport via the transpirational stream, gas transport through plant tissue is crucial for any plant that inhabits water-heavy environments. However, gas movement in water is 10,000 times slower than gas movement in air, so this is a slow process (Colmer *et al*., 2003; Cao *et al*., 2008). Since methane is slightly soluble in water, regardless of its lack of polarity, it is dissolved in water, taken up by the transpirational stream and could theoretically be the source of methane emissions from plants (Nisbet *et al*., 2009; Watanabe *et al*., 2012). This dissolved methane would originate from soil methanogens, and transported through the same pores that allow for the transport of ethylene and carbon dioxide (Nisbet *et al*., 2009). It is unlikely that dissolved methane makes a significant contribution to observed methane emissions, however; according to

Henry's law of partial pressures (Watanabe *et al*., 2012), the magnitude of emissions is greater than expected through the transpirational stream.

An alternative explanation for aerobic methane emissions is aerenchyma, low-resistance pathways that allow transport of gasses such as O_2, CO_2, ethylene, and methane (CH_4) through leafy tissues (Colmer *et al*., 2003; Cao *et al*., 2008); however, many of the species examined generally do not form aerenchyma, especially when watered to field capacity. Methane emissions from plants cannot be explained through methanogenic archaea living in the xylem. Analyses have not found genes related to the Archaeal enzymes responsible for methanogenesis, or the genes necessary to conduct methylphosphonate decomposition, a process carried out by many bacteria (Nisbet *et al*., 2009). In a study on grey poplar grown under sterile conditions, researchers ruled out the possibility of microbial methane origin (Brüggemann *et al*., 2009), and if plants do produce methane it would be by a previously unknown biochemical pathway (Nisbet *et al.*, 2009).

Alternatively, methane production could be the product of breakdown of cellular material, caused by high-stress conditions (Nisbet *et al*., 2009) that induce formation of ROS. It has yet to be ruled out that ROS may be the primary cause of methane emissions from methylated compounds, as opposed to a novel biochemical pathway; however, since ROS are diverse compounds with a wide variety of effects, more work is needed to discern whether aerobic methane emissions are the result of a unique biochemical pathway or methyl cleavage from a variety of compounds.

1.6. PLANT METABOLISM

Plant metabolism is defined as the sum of all chemical reactions that occur in any individual organism, and metabolites are typically divided into primary and secondary metabolites (Wahid, 2007). Primary metabolites are essential for the viability of the organism, and include compounds such as amino acids, nucleotides, sugars, and phospholipids (Tenenboim & Brotman, 2016). Secondary metabolites, on the other hand, are essential for the viability of the organism within the context of its environment, but are non-essential to proper cellular functioning (Tenenboim & Brotman, 2016). Primary metabolites are used as building blocks in biosynthetic pathways that lead to the production of secondary metabolites. While primary metabolites are common across the entire plant kingdom, secondary metabolites are often found to be associated with a specific taxonomic grouping of plants. It is currently believed that there are over 200,000 plant secondary metabolites which serve an array of functions, including defence, pollinator attraction, and stress signalling (Pavarini *et al.*, 2012; Jay-Allemand *et al.*, 2015).

1.7. *BRASSICA NAPUS*

Several decades ago, Canadian plant breeders began searching for a new oilseed crop that would be suited to the Canadian environment. By 1974, they produced an improved version of rapeseed that removed undesirable compounds, such as erucic acid, and they named this plant canola, a combination of **Can**adian **O**il, **L**ow **A**cid (Woods *et al*., 1991). There are now three species that are considered canola; *Brassica napus*, *Brassica rapa*, and a canola-quality Indian mustard, *Brassica juncea* (Woods *et al*., 1991; Goodwin, 2005). These plants were tested in Winnipeg and Saskatoon, with each site yielding suitable individual plants

(Steffanson & Storgaard, 1969). Following oil harvest, the remaining seed parts are processed into a high-protein animal feed (Goodwin, 2005), and are also being examined as a potential biofuel crop; in comparison to fossil fuels, canola fuel would reduce greenhouse gas emissions by up to 90% (Canola Council of Canada, 2017). Canola is a cool season crop that is commonly grown in Canada and Northern Europe. It is highly sensitive to temperature fluctuations in the flowering stage, with higher temperatures (>30°C) resulting in yield losses or flower infertility (Warland *et al*., 2006; Kutcher *et al*., 2010).

Canola is a dicot plant with two cotyledons that emerge from the soil; the first true leaves develop approximately 4-8 days following emergence. Canola plants grow first in the rosette stage, with the stem elongation period occurring after, until about 40 days post-emergence, by which the plant has reached its maximum height of 1-2 meters, depending on environmental factors. By day 50, canola plants generally develop flowers, which open by day 60, and will soon be self-pollinated. By day 80, the pods have reached their final size, and within 10 more days the seeds are ripe, with harvesting occurring around day 99 (Canola Council of Canada 2017). Each canola pod is small and thin, containing approximately 15-45 seeds, characterized by their dark brown colour at maturity (Goodwin, 2005; Canola Council of Canada 2017), and with seeds accounting for approximately 23-31% of dry mass (Canola Council of Canada, 2017). In Saskatchewan, canola is seeded in May, where it begins to bolt and flower in late June (Kutcher *et al*., 2010).

Canola seeds contain 44% oil, with the oil being the healthiest in the world (Goodwin, 2005). Canola was developed specifically for its nutritional qualities, with low levels of

erucic acid in the oil, reduced glucosinolates in the meal, and low saturated fat content (Goodwin, 2005; Canola Council of Canada 2017). Approximately 93% of the fats found in canola are monounsaturated and polyunsaturated fats, and the oil is also a rich source of omega fatty acids and Vitamin E. Canola is a light and clear oil, and is the oil of choice for many chefs around the world. (Canola Council of Canada 2017).

Canola now contributes $19.3 billion to the Canadian economy, generating nearly 249,000 jobs. In the past decade alone, economic contribution of canola has nearly doubled, with the greatest economic benefits occurring predominantly in Western Canada, where the environment is best suited for its production. Saskatchewan is the leading producer of canola (LMC International, 2013), accounting for nearly 44% of Canadian canola production (Goodwin, 2005), followed by Alberta and Manitoba, respectively (LMC International, 2013).

In addition to the aforementioned qualities of canola, it was chosen for this thesis because previous work has shown it to emit methane (Qaderi & Reid, 2011, 2014). Furthermore, it grows relatively easily and rapidly, allowing for collection of numerous leaves for analysis, and is an important contributor to the Canadian economy, making research on canola both relevant and important.

1.8. OBJECTIVES AND HYPOTHESES

This thesis had three main objectives; (i) to investigate the potential of ethylene, a product of the methionine pathway, as a precursor of methane; (ii) to assess whether the primary metabolite L-methionine is a unique precursor of aerobic methane emissions; and (iii) to determine whether there is an effect of environmental factors on these parameters.

Based on previous work, it was hypothesized that ethylene promoters would increase, and ethylene inhibitors would decrease, aerobic methane emissions from plants, and that L-methionine would be a unique precursor for methane emissions from plants. It was also hypothesized that environmental factors would significantly influence aerobic methane emissions, but whether or not they increased or decreased would depend on the individual factor.

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Chapter 2: The effects of endogenous ethylene on methane emissions from plants

2.1 INTRODUCTION

As discussed previously (see section 1.5, page 8), plants release aerobic methane (Keppler *et al.*, 2006). While still under debate, a potential precursor for aerobic methane emissions from plants is L-methionine (Lenhart *et al.*, 2015), but exact pathways remain unknown. L-methionine (L-Met), a sulphur-containing amino acid, leads to the production of ethylene (C_2H_4), a gaseous plant hormone, through the third and fourth carbons of L-Met. Ethylene synthesis occurs with S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) acting as intermediates, using the enzymes SAM synthetase, ACC synthase, and ACC oxidase (Amir, 2008). Following ethylene production, the sulphur and sulphur-bound methyl group are recycled back into methionine through the Yang cycle (Amir, 2008; Hopkins & Hüner, 2009).

While not required for regular vegetative growth, ethylene is important in both development of roots and shoots and the ripening of fruits. Furthermore, it is important in the stress response, and can be produced in large quantities (Zhou *et al.*, 2013; Habben *et al.,* 2014). Ethylene has a number of regulatory roles in plants, including many growth and development processes under abiotic stress, but these will be discussed in more detail in subsequent chapters. Ethylene is present in gas form, and can be emitted to the surrounding atmosphere, including release into the soil during stress-induced ethylene accumulation (Zhou *et al.,* 2013; Habben *et al.*, 2014). Oxidative stress has been shown to increase the emissions of ethylene from tobacco plants (*Nicotiana tabacum* L.) under UVB stress (McLeod *et al.*, 2008). Once synthesized, ethylene acts as a ligand, binding to receptors in

the endoplasmic reticulum to trigger a cascade of responses (Habben *et al*, 2014). In general, ethylene contributes to a decrease in overall yield of cereals when under abiotic stress conditions (Habben *et al*., 2014).

In canola (*Brassica napus*), a transgenic species was created that expressed the ACC deaminase gene from bacteria, which reduced ethylene production. These transgenic canola plants were found to have smaller siliques, which in turn caused a smaller number of seeds as well as a reduction in seed size. Transgenic canola also had a reduction in gibberellin A1 and A4, along with indole-3-acetic acid. When ethephon, an ethylene promoter, was added, proper seed size was restored (Walton *et al*., 2012). Ethephon application also increased growth, gas exchange, and accumulation of nitrogen in canola-quality Indian mustard (*Brassica juncea*; Khan *et al*., 2008); however, addition of ethephon to the wild-type counterpart canola impeded proper development of seeds, implying that too much ethylene can have a negative effect on canola (Walton *et al.,* 2012).

Earlier research has shown that ethylene production can either be increased or decreased using a number of chemical treatments. Methyl jasmonate application on both apple (*Mallus pumila* L., Misczcak *et al*., 1995) and plums (*Prunus salicina* L., Ozturk *et al*., 2014) increased ethylene evolution; however, in apple the effects had a time-dependent response, with the greatest effect occurring 24 hours after application (Ozturk *et al*., 2014). Ethephon is an ethylene-releasing compound that has commercial effects in fruit ripening; in canola-quality Indian mustard (*Brassica juncea* L., Khan *et al*., 2008) and Arabidopsis (*Arabidopsis thaliana* L., Zhang and Wen, 2010), ethephon application at various treatments significantly increased ethylene evolution. Similarly, ethylene evolution was

increased by kinetin application in mung bean (*Phaseolus aureus* L., Lau and Yang 1975, 1976), and by indole-3-acetic acid application in pea (*Pisum sativum* L., Fuchs and Lieberman, 1968; Johnstone *et al*., 2005). Ethylene evolution was decreased, on the other hand, by application of 2,4-dinitrophenol in mung bean (Yu *et al*., 1980) and apple (Murr and Yang, 1974), and by application of silver nitrate in sweet cherry (*Prunus avium* L., Sgamma *et al*., 2015) and a number of other plants (see Kumar *et al*., 2016).

If ethylene acts as a precursor of methane in plants, it would be expected that emissions of both would increase concurrently; for example, periods of fruit ripening, in which ethylene is produced in abundance, may also increase methane emissions as well, but aerobic methane emissions have yet to be studied in the context of fruit development. Ethylene also increases in times of stress, and methane has been shown to increase under environmental stress as well. Both ethylene and L-methionine should be examined further as potential precursors of aerobic methane emissions from plants. The original hypotheses of this experiment were; (i) that ethylene promoters would increase, but ethylene inhibitors would decrease, ethylene evolution; (ii) aerobic methane emissions would increase with increased ethylene evolution; and (iii) plant growth and development would be affected by the application of ethylene promoters and inhibitors, but this would depend in part on their concentrations.

2.2. METHODS

2.2.1. Plant material and growth conditions

Seeds of canola (*Brassica napus* L., cv. 6056 CR, BrettYoung Seeds, Winnipeg, Manitoba) were germinated in 100 x 15 mm Petri dishes with blue germination paper (Anchor Paper

Co., St. Paul, MN) for 7 days. Seedlings of uniform size were potted in a mixture of peat moss, Perlite and Vermiculite (2:1:1, v/v/v), with pellets of slow-release fertilizer (N-P-K, 14-14-14). To produce enough seedlings of uniform size, four seedlings were sown per pot and then thinned to one at the onset of the experiment. Pots were transferred to a growth chamber (model ATC26, Conviron, Controlled Environments, Winnipeg, MB, Canada), set to a temperature regime of 22/18°C on a 16 h photoperiod. The photosynthetic photon flux density (PPFD), measured at the shoot apex using a quantum LI-250A radiometer/photometer (LI-COR Biosciences, Lincoln, NE) was 300 µmol photon m^{-2} s^{-1}. Light was supplied by a mixture of incandescent lamps (Luminus, Conglom Inc., St-Laurent, Québec, Canada) and cool white fluorescent bulbs (Master TL-D-58 W/840, Philips, Amsterdam, Netherlands). Plants were left to acclimate for 7 days, until the emergence of the first true leaves, and were then randomly assigned to one of the 19 experimental conditions.

Plants were applied with either an ethylene promoter (Met-J; methyl jasmonate (Misczcak *et al*., 1995; Ozturk *et al*., 2014), Eth; Ethephon (Khan *et al*., 2008; Zhang and Wen, 2010), Kin; kinetin (Lau and Yang 1975, 1976), IAA; indole-3-acetic acid (Fuchs and Lieberman, 1968; Sitbon *et al*., 1999; Johnstone *et al*., 2005)), or an ethylene inhibitor (2,4-DNP; 2,4-dinitrophenol (Murr *et al*., 1975; Yu *et al*., 1980), $AgNO_3$; silver nitrate (Sgamma *et al*., 2015; Kumar *et al*., 2016)), and each at 3 different concentrations. With the control (no chemical application), there was a total of 19 experimental conditions, consisting of: (1) control plants (no chemical application); (2) 10^{-5} M Met-J; (3) 5 x 10^{-5} M Met-J; (4) 10^{-4} M Met-J; (5) 10^{-4} M Eth; (6) 5 x 10^{-4} M Eth; (7) 10^{-3}M Eth; (8) 10^{-5} M Kin;

(9) 10^{-4} M Kin; (10) 10^{-3} M Kin; (11) 10^{-5} M IAA; (12) 10^{-4} M IAA; (13) 10^{-3} M IAA; (14) 10^{-5} M 2,4-DNP; (15) 5 x 10^{-5} M 2,4-DNP; (16); 10^{-4} M 2,4-DNP; (17) 10^{-5} M $AgNO_3$; (18) 5 x 10^{-5} M $AgNO_3$; (19) 10^{-4} M $AgNO_3$. Plants were grown under experimental conditions for 21 days, with bi-weekly rotation within the chambers to reduce the effects of positional differences. Experiments were conducted three times for statistical reliability, with the chambers rotated each time to minimize potential effects from any small, inherent differences among them.

2.2.2. Measurement of methane and ethylene emissions

Methane and ethylene emissions were determined using a protocol similar to the method commonly used for ethylene emissions (Liu *et al*., 1990; Emery *et al*., 1994; Qaderi & Reid, 2009, 2011, 2014). From each growth condition, four leaves, each of approximately 0.2 g fresh mass (FM), were detached and immediately incubated for 20 minutes within 3-mL syringes flushed with CH_4-free air. Samples were incubated within the chambers, following which 1-mL of gas was collected and injected manually into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian 3900 Gas Chromatograph; Varian Canada, Mississauga, Canada), stocked with a capillary column (Carboxen 1006 PLOT, 30m x 0.53 mm ID, Supelco, Bellefonte, PA). The injector temperature was set to 200°C and the detector to 230°C, with helium as the carrier gas at 10 ml min^{-1}. The temperature gradient was: 1 min isothermal heating at 35°C, followed by an increase of 24°C min^{-1} until the oven ramp reached a temperature of 225°C, for a total run time of 18 min. Methane and ethylene were identified based on their retention times (~2.8 min and 11.5 min, respectively), and were quantified on the basis of standard curves derived from the

injection of known quantities of methane (CH_4, expressed in ng g^{-1} DM h^{-1}, where DM is dry mass) and ethylene (C_2H_4, expressed in pmol g^{-1} FM h^{-1}, where FM is fresh mass). Methane was calculated based on dry mass following 72 h of drying in an oven at 60°C, whereas ethylene was calculated from fresh mass immediately following injection.

2.2.3. Plant growth and dry mass accumulation

Following 21 days of experimental growth, three plants of average height (extreme outliers, such as plants that were dying, were removed from the pool and then three plants were randomly chosen from those that remained) from each growth condition were harvested to determine growth parameters, including plant height, stem diameter, leaf number and area, growth rate, and dry mass accumulation. Stem diameter was determined using a Digimatic caliper (Mitutoyo Corp., Kanagawa, Japan), and stem height was determined using a ruler. To determine leaf area and dry mass accumulation, plant parts were dried for up to 120 h at 60°C in a forced air Fisher Isotemp® Premium oven (model 750F, Fisher Scientific, Nepean, Canada), following which aboveground and belowground biomass were determined using an analytical balance (model ED244s, Sartorius, Goettingen, Germany). Leaf area was determined for dried leaves using a ΔT leaf area meter (Delta-T Devices, Cambridge, UK). These measurements were used to calculate growth indices, including shoot-root mass ratio, shoot DM (dry mass):root DM; leaf mass per area (g m^{-2}), leaf DM:leaf area; leaf mass ratio, leaf DM:plant DM; and leaf area ratio (cm^2 g^{-1}), leaf area:plant DM.

2.2.4. Chlorophyll fluorescence

A Fluorpen FP 100 portable fluorometer (Photon Systems Instruments, Drasov, Czech Republic) was used to determine chlorophyll fluorescence for at least three fully-grown leaves from each condition. Photosynthetic electron transport was measured under light conditions to determine the effective quantum yield of PSII (ϕPSII), following which leaves were dark-adapted within fluorometer clamps for 30 min and measurements were taken of maximum quantum yield of PSII (F_v/F_m), non-photochemical quenching (qNP; (F_m-F_m') – 1) and photochemical quenching (qP; F_q'/F_v'; Baker, 2008). The saturating light pulse was delivered at 2100 µmol photons m^{-2} s^{-1} for 1 s.

2.2.5. Photosynthetic pigments

Concentrations of chlorophyll (Chl) *a*, Chl *b*, and carotenoids were determined by punching four leaf discs from each of three fully-grown leaves from each condition, and placing them in a 12-mL vial with 5 mL of dimethyl sulfoxide (VWR International Inc, Misssssauga, Canada). Discs were incubated for 24 hours under dark conditions, following which absorbance values were read using a UV-visible spectrophotometer (model Ultraspec 3100 pro, Biochrom Ltd., Cambridge, UK). Absorbance values were measured at 664, 648, and 470 nm, and these values were used to calculate the concentrations of Chl *a*, Chl *b,* carotenoids, total Chl, and the ratio between Chl *a*/*b* (Chappelle *et al*., 1992).

2.2.6. Moisture content

Moisture content (%) was determined by excising leaves, immediately taking the fresh mass, and then drying the tissue in a forced air Fisher Isotemp® Premium oven for 72h.

Dry mass was determined and the moisture content was then calculated on the difference between fresh and dry mass.

2.2.7. Flavonoids and nitrogen balance index

Flavonoids and nitrogen balance index (NBI) were determined using the Dualex Scientific® (Dualex Scientific, Force-A, OrsayCedex, France). The Dualex determines flavonoid levels using a type of LogFER chlorophyll fluorescence measurement, conducting a quantitative comparison of light delivered to and emitted from the leaf. NBI is a patented calculation that estimates the levels of nitrogen nutrition by establishing a ratio of chlorophyll to flavonoids. A higher NBI indicates alterations to the allocation of carbon/nitrogen in leaves due to N-deficiency (Cerovic *et al*., 2012).

2.2.8. Data analysis

The overall effects of ethylene promoters and inhibitors, and their concentration, were determined on plant emissions and growth and physiological parameters of canola using a two-way analysis of variance. The differences between chemical concentrations were determined using a one-way analysis of variance using Scheffé's multiple-comparison procedure at the 5% confidence level (SAS Institute, 2011). Significant results were presented in figures, whereas non-significant results were presented in tables.

2.3. RESULTS

2.3.1. Methane and ethylene emissions

While absolute values of methane emissions were affected by chemical treatment, these were not significant on the basis of the one-way ANOVA, and emissions were not

influenced by concentration (Table 2.1). Different concentrations of the same chemical had no significant effects based on the one-way ANOVA. Most of the chemical treatments resulted in higher methane emissions than the control (Fig. 2.1), but there appeared to be no differences between the promoters (Fig. 2.1A-D) and inhibitors (Fig. 2.1E-F).

Ethylene emissions were significantly affected by both promoter and inhibitor application along with the interaction between chemical and concentration (Table1.2). Ethephon (Fig. 2.2B) had the greatest effect on ethylene emissions, with emissions as much as 10x greater than other treatments. The other promoters and inhibitors did not significantly influence ethylene emissions based on the one-way ANOVA (Fig. 2.2A-F), and although absolute values of ethylene emissions were increased by most chemical treatments, including the inhibitors, standard errors were large and this was not significant on the basis of the one-way ANOVA (Fig. 2.2).

Table 2.1. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on methane and ethylene emissions of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of Variation	df	MS	F	*P*
Methane Emissions				
Chemical (C)	6	574.42	2.34	**0.0490**
Concentration (M)	2	452.78	1.84	0.1712
C x M	12	146.15	0.59	0.8343
Error	41	245.16		
Ethylene Emissions				
Chemical (C)	6	4976543.70	41.74	**<0.0001**
Concentration (M)	2	674559.64	5.66	**0.0068**
C x M	12	714058.66	5.99	**<0.0001**
Error	41	119219.30		

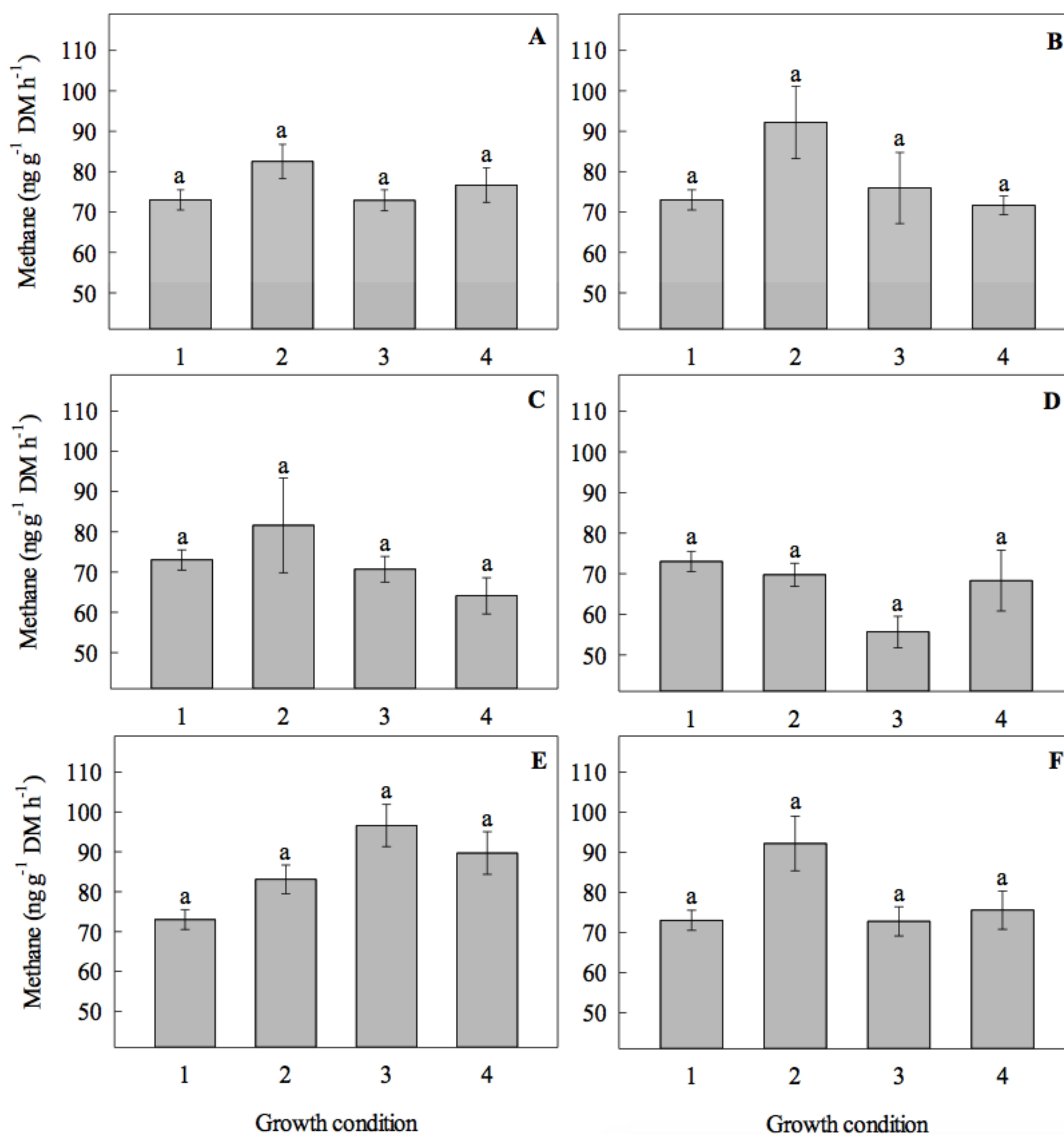

Fig. 2.1. Methane emissions from canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Error bars depict ± 1 SE (n = 3), and bars surmounted by different letters are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure.

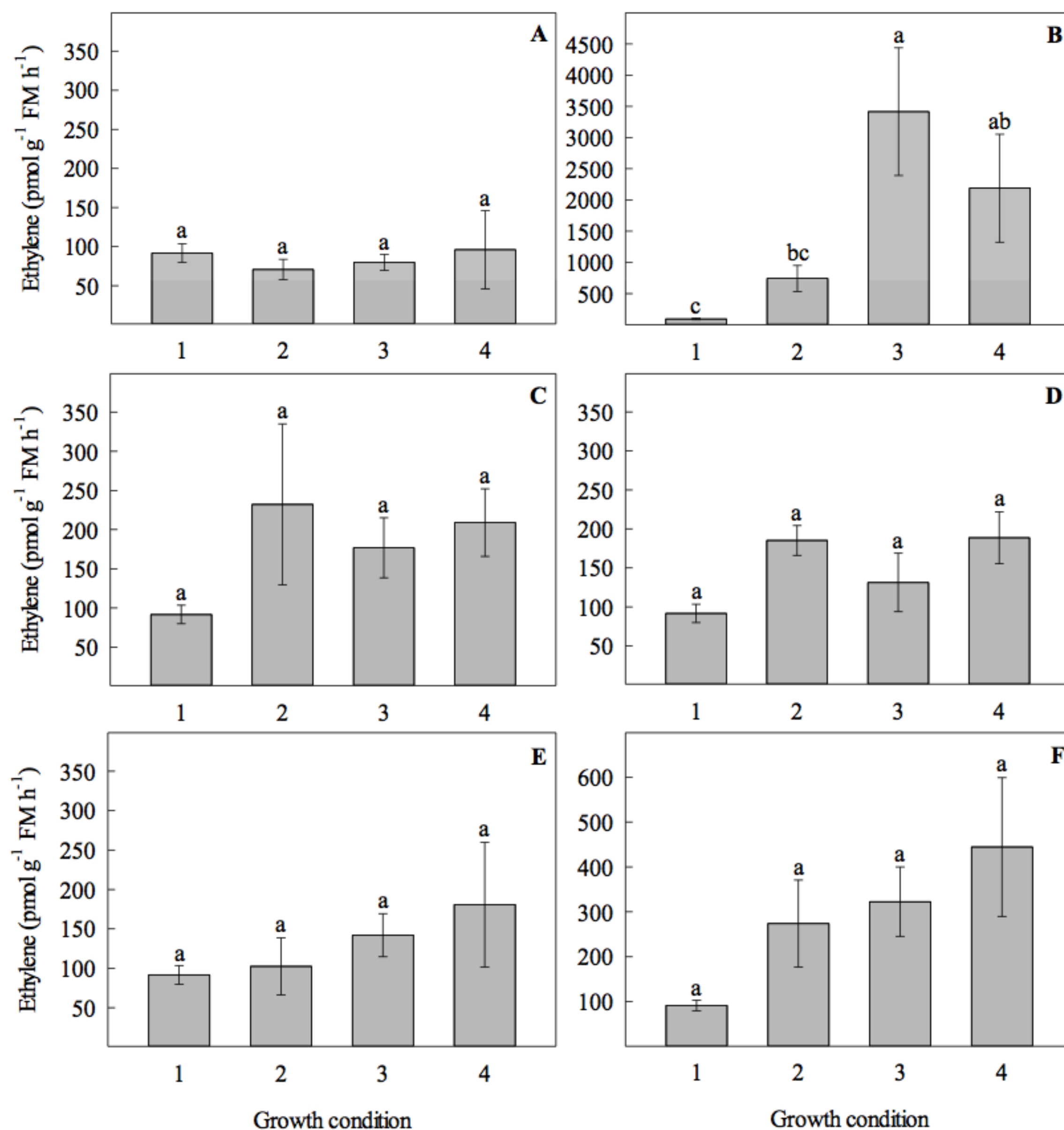

Fig. 2.2. Ethylene emissions from canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

2.3.2. Plant growth and dry mass accumulation

All parameters of plant growth and dry mass accumulation were significantly influenced by the chemical treatment (Table 2.2). None of these parameters were affected by the chemical concentration according to the two-way ANOVA. According to the one-way ANOVA, height was significantly increased by ethephon and silver nitrate (Fig. 2.3B, F). Increases in the absolute height were also caused by kinetin and indole-3-acetic acid (Fig. 2.3C-D), whereas methyl jasmonate and 2,4-dinitrophenol resulted in decreases in the absolute height (Fig. 2.3A, E), but none of these values were significantly different. Leaf number was significantly increased by the highest concentration (10^{-3} M) of ethephon in comparison to the control, but differences were not observed between concentrations of any other chemical application (Table 2.3).

Leaf area was significantly decreased by high concentration (10^{-4} M) of methyl-jasmonate; similarly, stem diameter also decreased in response to all concentrations of methyl jasmonate and medium and high concentrations ($5 x 10^{-5}$ M and 10^{-4} M, respectively) of 2,4-dinitrophenol (Table 2.3). Also, 2,4-dinitropenol resulted in a decrease in the absolute value of leaf area, whereas ethephon, kinetin, indole-3-acetic acid and silver nitrate increased the absolute value of leaf area, but these effects were not statistically significant. On the other hand, growth rate was significantly decreased by methyl jasmonate and 2,4-dinitrophenol, whereas it was increased by ethephon.

On the basis of the two-way ANOVA, all parameters of dry mass accumulation were significantly affected by chemical application, but not by the concentration of promoters and inhibitors applied (Table 2.4). Methyl jasmonate, indole-3-acetic acid, and 2,4-

dinitrophenol decreased total mass (Fig.2.4A, D-E), leaf mass, and stem mass (Table 2.5) according to the one-way ANOVA (Fig. 2.4A, D-E), whereas these parameters were not significantly affected by ethephon, kinetin or silver nitrate (Fig. 2.4B-C, F; Table 2.5). Growth indices were significantly influenced by chemical treatment according to the two-way ANOVA (Table 2.6), but there were no statistically significant differences among concentrations of each chemical based on the one-way ANOVA (Table 2.7). Absolute values of LMA and S:R ratio were decreased by all chemical treatments, whereas the absolute value of LAR was increased by ethephon, kinetin, and silver nitrate, but these results were not significant (Table 2.7).

Table 2.2. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on growth of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of variation	df	MS	F	*P*
Final stem height				
Chemical (C)	6	1903.54	16.83	**<0.0001**
Concentration (M)	2	29.78	0.26	0.77
C x M	12	107.99	0.95	0.51
Error	41	113		
Stem diameter				
Chemical (C)	6	11.8	29.07	**<0.0001**
Concentration (M)	2	0.51	1.27	0.2921
C x M	12	0.19	0.49	0.91
Error	41	0.41		
Leaf area				
Chemical (C)	6	53727.45	14.84	**<0.0001**
Concentration (M)	2	596.13	0.16	0.85
C x M	12	1471.39	0.41	0.95
Error	41	3620.87		
Leaf number				
Chemical (C)	6	0.93	2.11	0.07
Concentration (M)	2	0.33	0.75	0.48
C x M	12	0.55	1.26	0.28
Error	41	0.44		
Growth rate				
Chemical (C)	6	4.10	15.38	**<0.0001**
Concentration (M)	2	0.07	0.27	0.77
C x M	12	0.25	0.95	0.51
Error	41	0.27		

Table 2.3. Effects of ethylene promoters and inhibitors on growth of canola plants applied with either an ethylene promoter (methyl jasmonate, Met-Jas; ethephon, Eth; kinetin, Kin; or indole-3-acetic acid, IAA) or an ethylene inhibitor (2-4-dinitrophenol, 2-4-DNP or silver nitrate, SN), each at four different concentrations (control, no application, low, medium, or high). Values represent the average (at least 3 replications per trial) ± 1 standard error (SE). Means followed by different letters within each chemical and parameter are significantly different according to Scheffé's multiple-comparison procedure at the 5% level.

Growth condition	Stem diameter (mm)	Leaf number ($plant^{-1}$)	Leaf area (cm^2 $plant^{-1}$)	Growth rate (mm d^{-1})
Control	3.30 ± 0.14a	8.83 ± 0.20a	105.6 ± 11.1a	0.90 ± 0.07a
Met-Jas, low	2.40 ± 0.10b	8.88 ± 0.30a	78.8 ± 12.9ab	0.63 ± 0.08ab
Met-Jas, medium	1.90 ± 0.13bc	9.12 ± 0.35a	54.9 ± 7.8ab	0.57 ± 0.09ab
Met-Jas, high	1.57 ± 0.14c	8.13 ± 0.35a	32.8 ± 3.1b	0.39 ± 0.08b
Control	3.30 ± 0.14a	8.83 ± 0.20b	105.6 ± 11.1a	0.90 ± 0.07b
Eth, low	4.44 ± 0.20a	9.17 ± 0.29ab	239.8 ± 21.4a	1.99 ± 0.20ab
Eth, medium	3.51 ± 0.34a	9.33 ± 0.27ab	159.4 ± 24.5a	2.71 ± 0.21a
Eth, high	3.96 ± 0.10a	10.17 ± 0.32a	227.4 ± 19.4a	1.87 ± 0.30ab
Control	3.30 ± 0.14a	8.75 ± 0.20a	105.6 ± 11.1a	0.90 ± 0.07a
Kin, low	3.96 ± 0.22a	8.75 ± 0.28a	167.8 ± 27.6a	1.21 ± 0.20a
Kin, medium	3.88 ± 0.15a	9.49 ± 0.27a	165.7 ± 22.5a	1.69 ± 0.23a
Kin, high	3.58 ± 0.28a	8.88 ± 0.33a	179.1 ± 22.9a	1.32 ± 0.17a
Control	3.30 ± 0.14a	8.83 ± 0.20a	105.6 ± 11.1a	0.90 ± 0.07a
IAA, low	3.69 ± 0.27a	8.99 ± 0.22a	162.6 ± 25.3a	1.26 ± 0.19a
IAA, medium	3.93 ± 0.26a	8.63 ± 0.24a	184.9 ± 25.3 a	1.32 ± 0.18a
IAA, high	3.87 ± 0.29a	8.44 ± 0.20a	149.3 ± 18.5a	1.93 ± 0.21a
Control	3.30 ± 0.14a	8.83 ± 0.20a	105.6 ± 11.1a	0.90 ± 0.07a
DNP, low	2.77 ± 0.20ab	9.22 ± 0.30a	79.6 ± 13.6a	0.83 ± 0.09ab
DNP, medium	2.33 ± 0.06b	8.61 ± 0.38a	64.2 ± 7.0a	0.53 ± 0.06ab
DNP, high	2.16 ± 0.10b	8.89 ± 0.29a	51.8 ± 6.6a	0.47 ± 0.06b
Control	3.30 ± 0.14a	8.83 ± 0.20a	105.6 ± 11.1a	0.90 ± 0.07b
SN, low	5.57 ± 0.21a	8.42 ± 0.26a	275.8 ± 16.4a	1.92 ± 0.17ab
SN, medium	5.53 ± 0.42 a	9.17 ± 0.30a	270.8 ± 28.5a	2.35 ± 0.24a
SN, high	5.22 ± 0.29a	8.25 ± 0.82a	259.9 ± 27.4a	1.87 ± 0.15ab

Table 2.4. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on dry mass accumulation of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of variation	df	MS	F	*P*
Leaf mass				
Chemical (C)	6	0.23	15.57	**<0.0001**
Concentration (M)	2	0.01	0.47	0.63
C x M	12	0.02	1.39	0.21
Error	41	0.01		
Stem Mass				
Chemical (C)	6	0.06	13.61	**<0.0001**
Concentration (M)	2	0.00	0.16	0.85
C x M	12	0.00	0.31	0.98
Error	41	0.00		
Root Mass				
Chemical (C)	6	0.02	9.19	**<0.001**
Concentration (M)	2	000	0.09	0.92
C x M	12	0.00	0.95	0.46
Error	41	0.00		
Total Mass				
Chemical (C)	6	0.71	15.95	**<0.0001**
Concentration (M)	2	0.01	0.25	0.78
C x M	12	0.04	0.96	0.50
Error	41	0.24		

Table 2.5. Effects of ethylene promoters and inhibitors on dry mass accumulation of canola plants applied with either an ethylene promoter (methyl jasmonate, Met-Jas; ethephon, Eth; kinetin, Kin; or indole-3-acetic acid, IAA) or an ethylene inhibitor (2-4-dinitrophenol, 2-4-DNP or silver nitrate, SN), each at four different concentrations (control, no application, low, medium, or high). Values represent the average (at least 3 replications per trial) ± 1 standard error (SE). Means followed by different letters within each chemical and parameter are significantly different according to Scheffé's multiple-comparison procedure at the 5% level.

Growth condition	Leaf mass (mg)	Stem mass (mg)	Root mass (mg)
Control	674.0 ± 55.0a	228.0 ± 20.0a	130.7 ± 24.0a
Met-Jas, low	336.5 ± 48.0b	68.5 ± 10.0b	55.0 ± 9.0a
Met-Jas, medium	220.6 ± 37.0b	41.8 ± 7.0b	102.6 ± 4.9a
Met-Jas, high	131.7 ± 15.0b	23.1 ± 2.0b	24.0 ± 2.0a
Control	674.0 ± 55.0a	228.0 ± 0.02a	130.7 ± 24.0a
Eth, low	483.1 ± 106.0a	247.2 ± 0.043a	166.9 ± 35.0a
Eth, medium	319.3 ± 37.0a	168.9 ± 0.086a	85.5 ± 15.0a
Eth, high	403.2 ± 46.0a	237.8 ± 0.010a	100.7 ± 11.0a
Control	674.0 ± 55.0a	228.0 ± 0.02 a	130.7 ± 24.0a
Kin, low	364.2 ± 47.0b	166.6 ± 0.032a	103.6 ± 21.0a
Kin, medium	356.7 ± 37.0b	154.7 ± 0.024a	91.4 ± 15.0a
Kin, high	441.5 ± 56.0b	142.9 ± 0.022a	110.1 ± 18.0a
Control	674.0 ± 55.0a	228.0 ± 0.02 a	130.7 ± 24.0a
IAA, low	346.3 ± 44.0b	146.6 ± 0.026a	106.1 ± 16.0a
IAA, medium	430.1 ± 59.0b	168.1 ± 0.026a	140.9 ± 24.0a
IAA, high	281.2 ± 31.0b	160.7 ± 0.024a	96.2 ± 13.0a
Control	674.0 ± 55.0a	228.0 ± 0.02 a	130.7 ± 24.0a
DNP, low	326.7 ± 50.0b	77.3 ± 0.015b	49.8 ± 72.0b
DNP, medium	229.6 ± 29.0b	48.9 ± 0.008b	46.8 ± 45.0b
DNP, high	282.2 ± 26.0b	59.9 ± 0.008b	40.8 ± 47.0b
Control	674.0 ± 55.0a	228.0 ± 0.02 a	130.7 ± 24.0a
SN, low	545.3 ± 79.0a	242.2 ± 0.023a	193.5 ± 28.0a
SN, medium	556.8 ± 54.0a	270.7 ± 0.037a	177.3 ± 24.0a
SN, high	761.3 ± 111.0a	267.3 ± 0.038a	231.1 ± 33.0a

Table 2.6. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on growth indices of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing. LMA, leaf mass per area (g m^{-2}); LMR, leaf mass ratio; LAR, leaf area ratio (cm^2 g^{-1}); S:R mass ratio, shoot to root mass ratio.

Source of Variation	df	MS	F	*P*
LMA				
Chemical (C)	6	746.00	21.29	<0.0001
Concentration (M)	2	18.59	0.06	0.77
C x M	12	19.4	0.35	0.99
Error	41	72.16		
LMR				
Chemical (C)	6	0.04	8.62	<0.0001
Concentration (M)	2	0.00	0.05	0.95
C x M	12	0.00	0.49	0.91
Error	41	0.00		
LAR				
Chemical (C)	6	28234.92	8.06	<0.0001
Concentration (M)	2	1390.80	0.40	0.67
C x M	12	1386.75	0.40	0.96
Error	41	3502.35		
S:R mass ratio				
Chemical (C)	6	8.41	8.41	<0.0001
Concentration (M)	2	0.05	0.05	0.96
C x M	12	0.19	1.19	0.32
Error	41	0.16		

Table 2.7. Effects of ethylene promoters and inhibitors on growth indices of canola plants were applied with an ethylene promoter (methyl jasmonate, Met-Jas; ethephon, Eth; kinetin, Kin; or indole-3-acetic acid, IAA) or an ethylene inhibitor (2-4-dinitrophenol, 2-4-DNP or silver nitrate, SN), each at four different concentrations (control, no application, low, medium, or high). Values represent the average (at least 3 replications per trial) ± 1 standard error (SE). Means followed by different letters within each chemical and parameter are significantly different according to Scheffé's multiple-comparison procedure at the 5% level. LMA, leaf mass per area (g m^{-2}); LMR, leaf mass ratio; LAR, leaf area ratio (cm^2 g^{-1}); S:R mass ratio, shoot to root mass ratio.

Growth condition	LMA	LMR	LAR	S:R mass ratio
Control	37.91 ± 2.60a	0.65 ± 0.01a	181.00 ± 13.20a	1.97 ± 0.33a
Met-Jas, low	41.60 ± 1.92a	0.74 ± 0.01a	180.10 ± 7.80a	1.26 ± 0.06a
Met-Jas, medium	40.50 ± 1.83a	0.68 ± 0.05a	172.20 ± 16.00a	1.00 ± 0.15a
Met-Jas, high	41.30 ± 2.24a	0.74 ± 0.02a	180.70 ± 7.20a	1.00 ± 0.08a
Control	37.91 ± 2.60a	0.65 ± 0.01a	181.00 ± 13.20a	1.97 ± 0.33a
Eth, low	20.30 ± 3.40b	0.53 ± 0.02a	311.30 ± 29.90a	1.70 ± 0.12a
Eth, medium	25.70 ± 3.70ab	0.58 ± 0.12a	271.70 ± 96.70a	2.22 ± 0.82a
Eth, high	19.00 ± 3.00b	0.53 ± 0.02a	329.20 ± 30.50a	2.58 ± 0.23a
Control	37.91 ± 2.60a	0.65 ± 0.01a	181.00 ± 13.20a	1.97 ± 0.33a
Kin, low	25.80 ± 12.00a	0.61 ± 0.12a	271.40 ± 84.00a	1.70 ± 0.6a
Kin, medium	26.80 ± 4.60a	0.62 ± 0.11a	283.20 ± 18.20a	1.76 ± 0.7a
Kin, high	27.10 ± 10.40a	0.66 ± 0.06a	265.50 ± 69.00a	1.33 ± 0.51a
Control	37.91 ± 2.60a	0.65 ± 0.01a	181.00 ± 13.20a	1.97 ± 0.33a
IAA, low	26.90 ± 12.20a	0.61 ± 0.10a	264.30 ± 90.80a	1.28 ± 0.49a
IAA, medium	27.10 ± 10.90a	0.61 ± 0.09a	261.20 ± 88.80a	1.31 ± 0.38a
IAA, high	23.50 ± 3.00a	0.56 ± 0.12a	88.10 ± 1.60a	1.62 ± 0.59a
Control	37.91 ± 2.60a	0.65 ± 0.01b	181.00 ± 13.20a	1.97 ± 0.33a
DNP, low	42.50 ± 2.60a	0.72 ± 0.01a	174.00 ± 9.60a	1.51 ± 0.07a
DNP, medium	46.40 ± 2.34a	0.71 ± 0.01a	156.00 ± 6.90a	1.11 ± 0.11a
DNP, high	42.90 ± 2.30a	0.74 ± 0.01a	174.60 ± 8.70a	1.45 ± 0.07a
Control	37.91 ± 2.60a	0.65 ± 0.01a	181.00 ± 13.20b	1.97 ± 0.33a
SN, low	19.40 ± 2.40b	0.54 ± 0.02a	312.30 ± 26.30a	1.42 ± 0.15a
SN, medium	21.40 ± 1.10b	0.57 ± 0.02a	270.60 ± 9.50ab	1.53 ± 0.11a
SN, high	28.30 ± 2.04b	0.60 ± 0.01a	222.90 ± 16.30ab	1.25 ± 0.13a

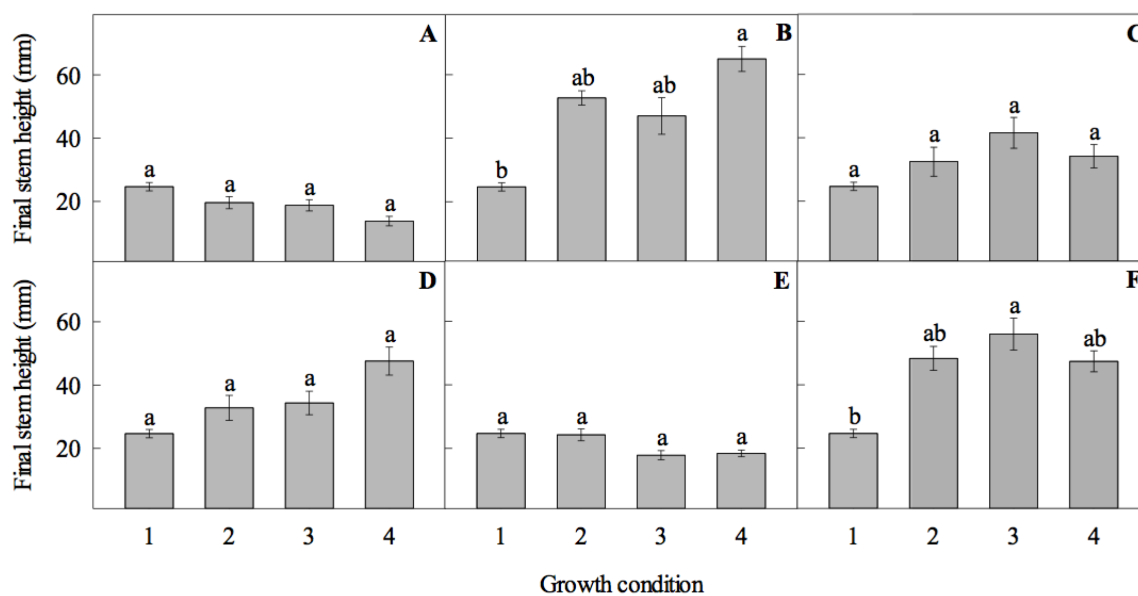

Fig. 2.3. Final stem height of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

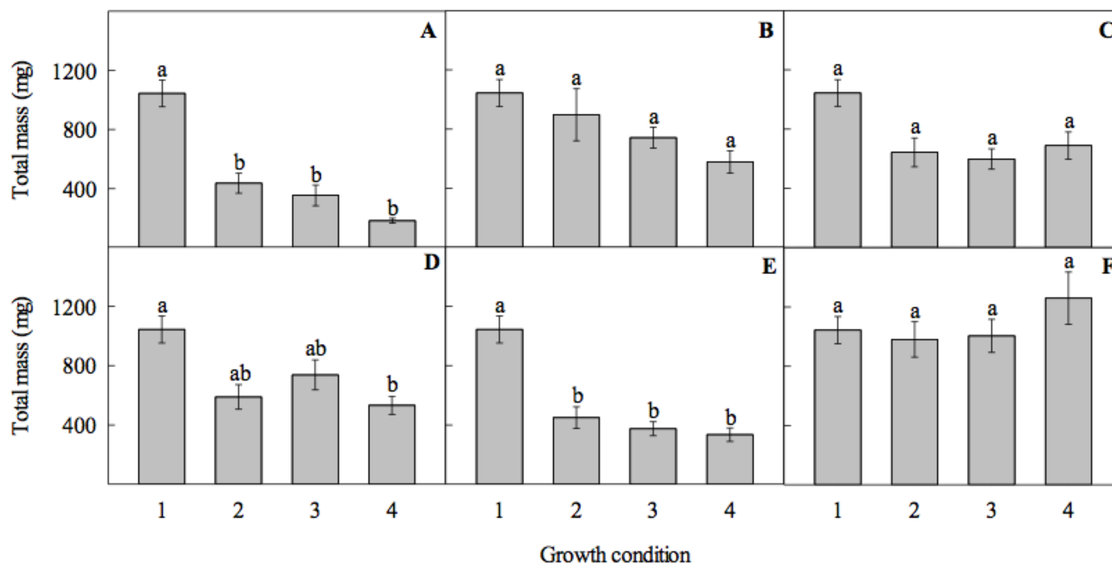

Fig. 2.4. Total mass of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

2.3.3. Chlorophyll fluorescence

Effective quantum yield of PSII (ϕPSII) and maximum quantum yield of PSII (F_v/F_m) were significantly affected by the promoters and inhibitors (two-way ANOVA), whereas non-photochemical quenching and photochemical quenching were unaffected (Table 2.8). The one-way ANOVA showed no significant differences among concentrations of any promoter or inhibitor and for any parameter (Table 2.9).

Table 2.8. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on chlorophyll fluorescence of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of Variation	df	MS	F	*P*
ϕPSII				
Chemical (C)	6	0.09	3.11	**0.0132**
Concentration (M)	2	0.02	0.72	0.4928
C x M	12	0.03	1.00	0.4664
Error	41	0.03		
F_v/F_m				
Chemical (C)	6	0.02	4.38	**0.0017**
Concentration (M)	2	0.00	0.36	0.6975
C x M	12	0.00	0.39	0.9596
Error	41	0.01		
qNP				
Chemical (C)	6	0.08	1.59	0.1744
Concentration (M)	2	0.08	1.68	0.1996
C x M	12	0.04	0.83	0.6204
Error	41	0.05		
qP				
Chemical (C)	6	0.02	1.98	0.0901
Concentration (M)	2	0.00	0.12	0.8881
C x M	12	0.01	1.38	0.2149
Error	41	0.01		

Table 2.9. Effects of ethylene promoters and inhibitors on chlorophyll fluorescence of canola. Plants were applied with either an ethylene promoter (methyl jasmonate, Met-Jas; ethephon, Eth; kinetin, Kin; or indole-3-acetic acid, IAA) or an ethylene inhibitor (2-4-dinitrophenol, 2-4-DNP or silver nitrate, SN), each at four different concentrations (control, no chemical application, low, medium, or high). Values represent the average (at least 3 replications per trial) ± 1 standard error (SE). Means followed by different letters within each chemical and parameter are significantly different according to Scheffé's multiple-comparison procedure at the 5% level. ϕPSII, effective quantum yield of PSII; F_v/F_m, maximum quantum yield of PSII; qNP, non-photochemical quenching; qP, photochemical quenching.

Growth condition	ϕPSII	F_v/F_m	qNP	qP
Control	0.76 ± 0.01a	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
Met-Jas, low	0.50 ± 0.20a	0.52 ± 0.10a	0.72 ± 0.26a	0.15 ± 0.09a
Met-Jas, medium	0.58 ± 0.17a	0.53 ± 0.18a	0.75 ± 0.31a	0.12 ± 0.01a
Met-Jas, high	0.68 ± 0.13a	0.61 ± 0.14a	0.92 ± 0.22a	0.19 ± 0.05a
Control	0.76 ± 0.01ab	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
Eth, low	0.79 ± 0.01a	0.70 ± 0.04a	1.04 ± 0.36a	0.23 ± 0.12a
Eth, medium	0.76 ± 0.01ab	0.69 ± 0.03a	0.65 ± 0.13a	0.21 ± 0.09a
Eth, high	0.76 ± 0.01b	0.67 ± 0.02a	1.08 ± 0.20a	0.25 ± 0.11a
Control	0.76 ± 0.01a	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
Kin, low	0.77 ± 0.02a	0.68 ± 0.02a	0.96 ± 0.24a	0.23 ± 0.07a
Kin, medium	0.76 ± 0.02a	0.66 ± 0.02a	0.97 ± 0.09a	0.17 ± 0.07a
Kin, high	0.76 ± 0.02a	0.70 ± 0.03a	1.02 ± 0.22a	0.21 ± 0.08a
Control	0.76 ± 0.01a	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
IAA, low	0.70 ± 0.10a	0.62 ± 0.10a	0.89 ± 0.35a	0.19 ± 0.10a
IAA, medium	0.76 ± 0.02a	0.66 ± 0.02a	0.99 ± 0.26a	0.19 ± 0.08a
IAA, high	0.76 ± 0.02a	0.67 ± 0.03a	0.90 ± 0.18a	0.21 ± 0.04a
Control	0.76 ± 0.01a	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
DNP, low	0.68 ± 0.12a	0.63 ± 0.02a	0.86 ± 0.42a	0.17 ± 0.05a
DNP, medium	0.54 ± 0.21a	0.58 ± 0.15a	0.87 ± 0.41a	0.19 ± 0.07a
DNP, high	0.71 ± 0.14a	0.63 ± 0.13a	0.98 ± 0.37a	0.19 ± 0.06a
Control	0.76 ± 0.01a	0.67 ± 0.01a	1.08 ± 0.04a	0.22 ± 0.02a
SN, low	0.77 ± 0.02a	0.68 ± 0.02a	1.03 ± 0.20a	0.21 ± 0.08a
SN, medium	0.77 ± 0.01a	0.70 ± 0.03a	1.00 ± 0.29a	0.23 ± 0.09a
SN, high	0.78 ± 0.02a	0.70 ± 0.03a	0.90 ± 0.28a	0.20 ± 0.09a

2.3.4. Photosynthetic pigments

Chlorophyll *a*, *b*, total chlorophyll and carotenoids were significantly affected by the promoters or inhibitors, their concentrations, and the interactions between the two, whereas the ratio between chlorophyll *a*/*b* was only affected by chemical application (Table 2.10). Chl *a* and *b* were significantly decreased by methyl jasmonate, kinetin, 2,4-dinitrophenol, and silver nitrate, but were unaffected by ethephon and increased indole-3-acetic acid (Fig. 2.5A-F; Fig. 2.6A-F). Overall, chlorophyll *a* and *b* have similar trends, and this is trend was also evident in total chlorophyll content (Fig. 2.7A-F) and carotenoid content (Fig. 2.8A-F). The highest overall chlorophyll content occurred with medium concentrations of ethephon (Fig. 2.5B), whereas the lowest content occurred with low concentrations of 2,4-dinitrophenol (Fig. 2.5E). Because of the similar patterns between chlorophyll *a* and *b*, the chl *a*/*b* ratio was not affected by any treatment on the basis of the one-way ANOVA (Table 2.11), though chemical application had a significant effect (two-way ANOVA; Table 2.10). This is evident by the differences in chl *a*/*b* ratio among promoters and inhibitors; silver nitrate resulted in the highest ratio, and ethephon the lowest (Table 2.11).

Table 2.10. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on photosynthetic pigments of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of Variation	df	MS	F	*P*
Chlorophyll *a*				
Chemical (C)	6	1.76	20.51	**<0.0001**
Concentration (M)	2	0.59	6.95	**0.0025**
C x M	12	0.40	4.67	**<0.0001**
Error	41	0.09		
Chlorophyll *b*				
Chemical (C)	6	0.17	14.64	**<0.0001**
Concentration (M)	2	0.10	8.87	**0.0006**
C x M	12	0.03	2.41	**0.0180**
Error	41	0.01		
Total Chlorophyll				
Chemical (C)	6	2.92	22.61	**<0.0001**
Concentration (M)	2	1.19	9.20	**0.0005**
C x M	12	0.63	4.84	**<0.0001**
Error	41	0.13		
Chlorophyll *a*/*b*				
Chemical (C)	6	0.32	5.31	**0.0004**
Concentration (M)	2	0.14	2.37	0.1060
C x M	12	0.06	1.00	0.4630
Error	41	0.06		
Carotenoids				
Chemical (C)	6	0.07	15.11	**<0.0001**
Concentration (M)	2	0.02	3.60	**0.0364**
C x M	12	0.02	4.12	**0.0003**
Error	41	0.00		

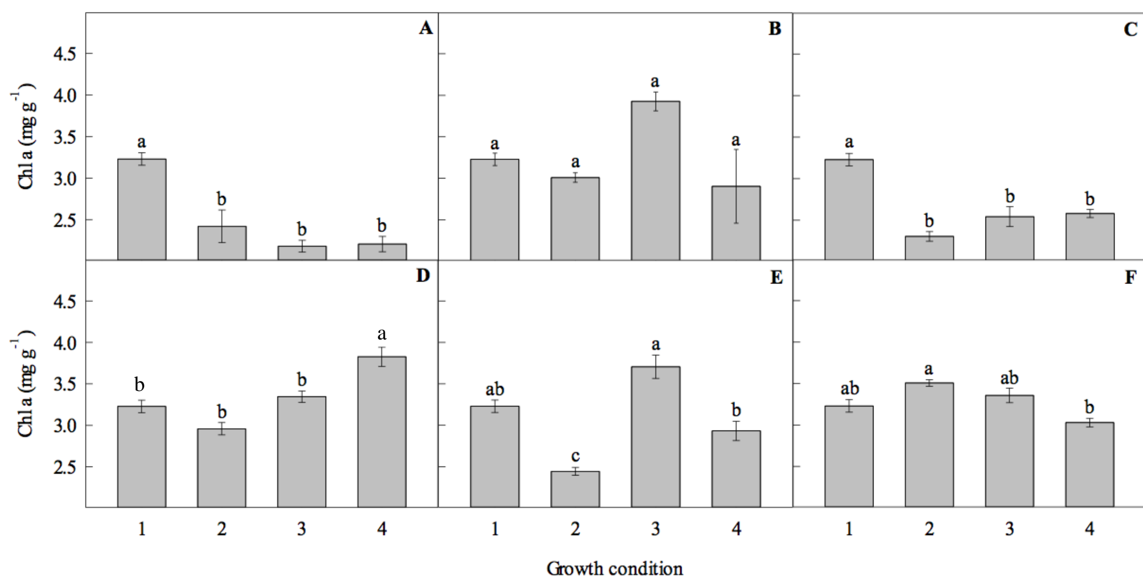

Fig. 2.5: Chlorophyll *a* content of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

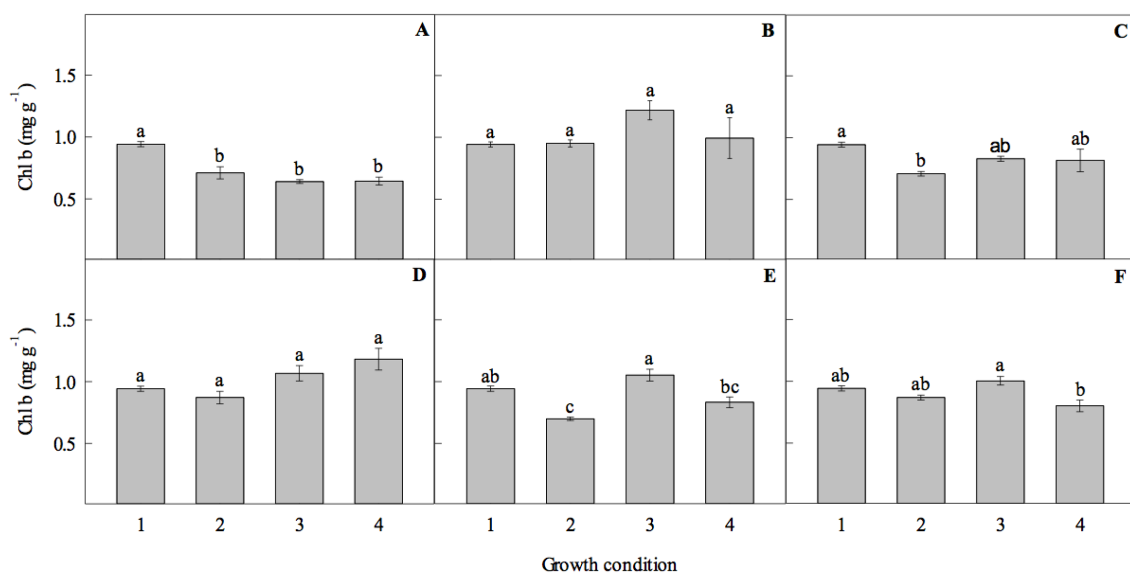

Fig. 2.6: Chlorophyll *b* content of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

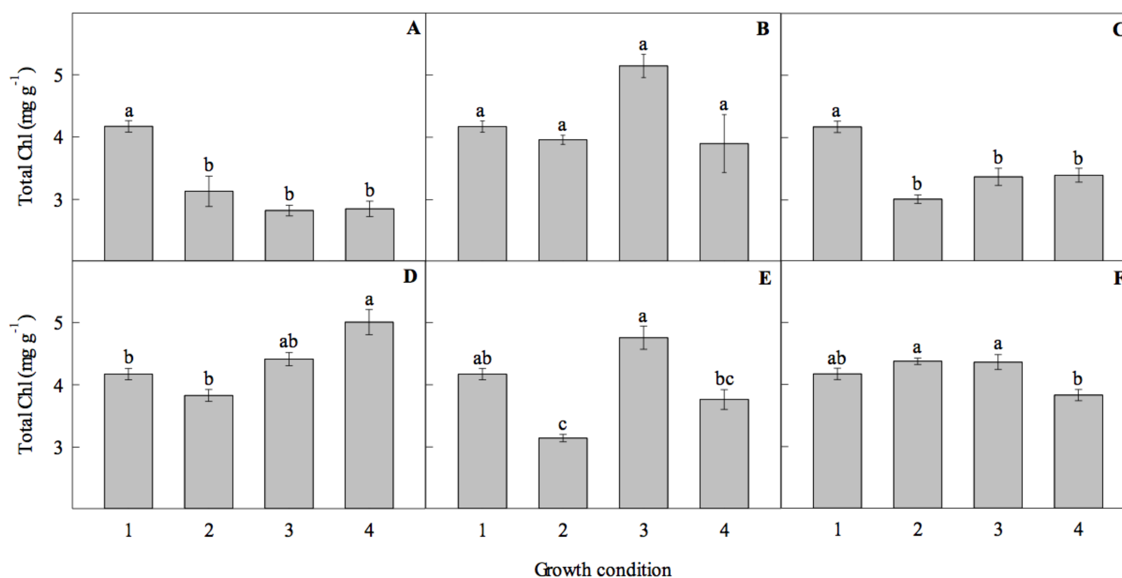

Fig. 2.7: Total chlorophyll content of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

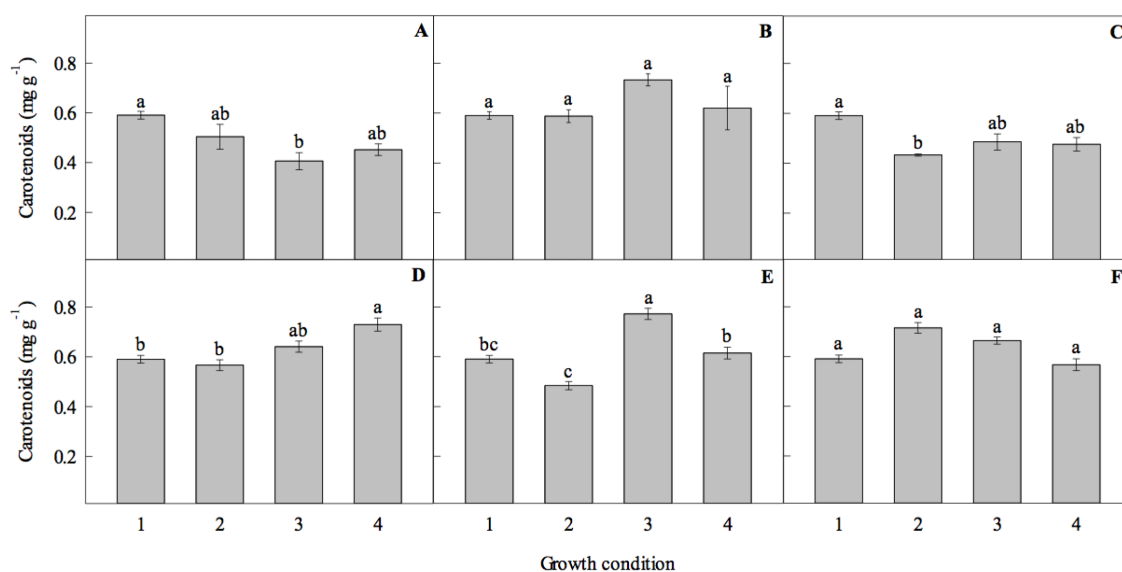

Fig. 2.8: Carotenoid content of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

2.3.5. Moisture content

Moisture content was significantly influenced by chemical application (two-way ANOVA; Table 2.12), but no statistically significant differences were observed among growth conditions (Table 2.11; Fig. 2.6A-F). Several trends were observed, though; kinetin, 2,4-dinitrophenol, and silver nitrate decreased the absolute value of moisture content with increasing concentration, whereas moisture content of methyl-jasmonate and kinetin, low and medium concentrations mildly decreased moisture, but these results were not statistically significant (Fig. 2.6A-F).

2.3.6. Flavonoids and NBI

Both flavonoids and nitrogen balance index (NBI) were not significantly influenced by chemical treatment, concentration, or the interaction between the two (Table 2.12). On the basis of one-way ANOVA, no differences were found among growth conditions for any chemical treatment (Table 2.11).

Table 2.11. Effects of ethylene promoters and inhibitors on protective compounds and nitrogen balance index (NBI) of canola. Plants were applied with either an ethylene promoter (methyl jasmonate, Met-Jas; ethephon, Eth; kinetin, Kin; or indole-3-acetic acid, IAA) or an ethylene inhibitor (2-4-dinitrophenol, 2-4-DNP or silver nitrate, SN), each at four different concentrations (control, no chemical application, low, medium, or high). Values represent the average (at least 3 replications per trial) ± 1 standard error (SE). Means followed by different letters within each chemical and parameter are significantly different according to Scheffé's multiple-comparison procedure at the 5% level.

Growth condition	Chl *a*/*b*	NBI	Flavonoids (μg cm^{-2})
Control	3.42 ± 0.05a	79.7 ± 4.7a	0.46 ± 0.04a
Met-Jas, low	3.39 ± 0.05a	93.9 ± 28.9a	0.55 ± 0.02a
Met-Jas, medium	3.40 ± 0.03a	63.4 ± 5.0a	0.54 ± 0.03a
Met-Jas, high	3.43 ± 0.03a	74.3 ± 6.4a	0.51 ± 0.03a
Control	3.42 ± 0.05a	79.7 ± 4.7a	0.46 ± 0.04a
Eth, low	3.18 ± 0.08a	79.2 ± 7.9a	0.50 ± 0.05a
Eth, medium	3.26 ± 0.11a	81.7 ± 5.1a	0.45 ± 0.03a
Eth, high	3.21 ± 0.46a	85.6 ± 9.6a	0.47 ± 0.07a
Control	3.42 ± 0.05a	79.7 ± 4.7a	0.46 ± 0.04a
Kin, low	3.26 ± 0.08a	80.5 ± 6.7a	0.48 ± 0.05a
Kin, medium	3.06 ± 0.09a	89.6 ± 9.2a	0.46 ± 0.06a
Kin, high	3.31 ± 0.28a	90.7 ± 7.6a	0.45 ± 0.06a
Control	3.42 ± 0.05a	79.7 ± 4.7a	0.46 ± 0.04a
IAA, low	3.44 ± 0.18a	73.4 ± 7.4a	0.45 ± 0.05a
IAA, medium	3.17 ± 0.16a	90.1 ± 10.2a	0.46 ± 0.05a
IAA, high	3.29 ± 0.15a	91.3 ± 5.3a	0.38 ± 0.03a
Control	3.42 ± 0.05a	79.7 ± 4.7a	0.46 ± 0.04a
DNP, low	3.50 ± 0.04a	86.9 ± 10.5a	0.43 ± 0.03a
DNP, medium	3.53 ± 0.04a	106.1 ± 7.8a	0.41 ± 0.02a
DNP, high	3.53 ± 0.06a	60.1 ± 7.8a	0.51 ± 0.03a
Control	3.42 ± 0.05ab	79.7 ± 4.7a	0.46 ± 0.04a
SN, low	4.04 ± 0.08a	92.3 ± 8.0a	0.45 ± 0.06a
SN, medium	3.35 ± 0.05b	77.1 ± 7.4a	0.55 ± 0.06a
SN, high	3.83 ± 0.17ab	72.5 ± 5.7a	0.48 ± 0.04a

Table 2.12. Analysis of variance for effects of ethylene promoters (methyl jasmonate, ethephon, kinetin, or indole-3-acetic acid) and inhibitors (2,4-dinitrophenol or silver nitrate), along with chemical concentration (none, low, medium, or high), on flavonoids, nitrogen balance index, and moisture content of canola (*Brassica napus*, cv. 6056 CR) plants grown under experimental conditions for 21 days beginning 14 days after sowing.

Source of Variation	df	MS	F	*P*
Moisture Content				
Chemical (C)	6	6.68	4.64	**0.0011**
Concentration (M)	2	0.86	0.59	0.5568
C x M	12	1.51	1.04	0.4289
Error	41	1.44		
Flavonoids				
Chemical (C)	6	0.01	0.41	0.8650
Concentration (M)	2	0.00	0.06	0.9461
C x M	12	0.01	0.23	0.9959
Error	41	0.02		
NBI				
Chemical (C)	6	96.92	0.17	0.9842
Concentration (M)	2	208.90	0.36	0.6999
C x M	12	500.25	0.86	0.5898
Error	41	580.40		

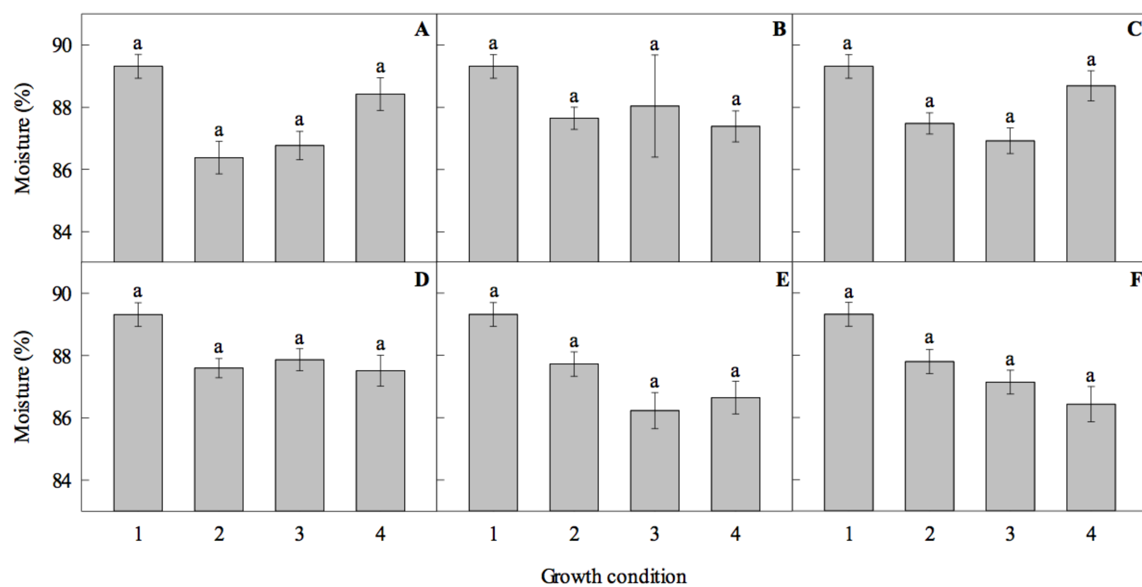

Fig. 2.9. Moisture content of canola (*Brassica napus*) plants applied with either an ethylene promoter ((A), methyl jasmonate, (B) ethephon, (C) kinetin, (D) indole-3-acetic acid) or inhibitor ((E) 2,4-dinitrophenol, (F) silver nitrate), each at four different concentrations (1, control; 2, low; 3, medium; 4, high). Otherwise, as per Fig. 2.1.

2.4. DISCUSSION

There is mounting evidence that plants release methane (CH_4) emissions, but it remains unknown how these emissions are produced on a biochemical level. While several precursors, including pectin (Keppler *et al*., 2008; McLeod *et al*., 2008) and L-methionine (Lenhart *et al*., 2015) have been suggested, no precursor alone can account for the entirety of methane emissions. The purpose of this study was to determine whether promoters or inhibitors of ethylene emissions influenced aerobic methane emissions from canola. Since L-methionine (L-met) leads to the production of ethylene, a gaseous plant hormone, it was hypothesized that L-met-derived methane emissions may come from ethylene.

Results from this study show that aerobic methane emissions were not significantly influenced by any of the chemical treatments. As expected, ethylene emissions were significantly increased by ethephon, which has commercial use and is readily metabolized to ethylene, but none of the other chemical treatments significantly affected ethylene according to statistical analysis. While this was unexpected, their roles in ethylene production have only been loosely examined in the literature, and mostly on fruit (Fuchs and Lieberman, 1967; Lau and Yang, 1975; Murr *et al*., 1975; Lau and Yang, 1976; Yu *et al*., 1980; Misczcak *et al*., 1995; Sitbon *et al.*, 1999; Johnstone *et al.*, 2005; Khan *et al*., 2008; Zhang and Wen, 2010; Ozturk *et al*., 2014; Sgamma *et al*., 2015; Kumar *et al*., 2016), with concentrations varying greatly among species. Since these compounds, with the exception of ethephon (Walton *et al*., 2012) have never been studied on canola, there may be a species-dependent response to these chemical treatments, much like there is for methane. In support of this, one study on peaches (*Prunus persica*) showed a decrease in

ethylene emissions in response to methyl jasmonate, though it increases ethylene in most species (Soto *et al*., 2012). Furthermore, there is evidence that promotion and inhibition of ethylene by most of these compounds is a short-lived response (Ozturk *et al*., 2014), and the peak response may not have been observed at the time emissions were measured.

This experiment yielded few interesting results. A number of physiological parameters were measured to determine whether the plants were affected on a physical level and had experienced stress. No significant differences in these parameters means that the plants were not significantly affected by the chemical treatment. Plants applied with methyl jasmonate had decreased leaf area, stem diameter, and growth rate, whereas those applied with silver nitrate had increased growth rate (Table 2.3). Methyl jasmonate, kinetin, indole-3-acetic acid, and 2,4-dinitrophenol decreased plant biomass (Table 2.5), indicating that plants were experiencing stress conditions, but these results were not reflected in the growth indices, as only silver nitrate decreased LMA but no other chemical treatment affected any of the growth indices (Table 2.7). Ethephon application mildly decreased ϕPSII, but other parameters were unaffected by chemical treatment. Since the majority of factors were unaffected by chemical treatment, this could indicate either that concentrations were insufficient to induce visible responses, or that the plant was not effectively taking up the chemical solution. Alternatively, differences may have appeared if plants were grown for a longer period of time; however, a growth time of 28 days has been sufficient in earlier experiments (Martel & Qaderi 2016, 2017). Since chemical treatments were applied at the shoot apical meristem, it is possible that the solutions evaporated off or dripped off the plant before being taken up, though this method has been

successful in earlier studies on salicylic acid application to pea (*Pisum sativum*) plants (Martel & Qaderi, 2016). In order to determine whether or not this was an effective method, further studies will examine the effects of chemical application on biochemical parameters, including amino acid content.

While it is well understood that aerobic methane emissions respond to environmental factors (Vigano *et al*., 2008; Bruhn *et al*., 2009; Wang *et al*., 2009; Wang *et al*., 2011a, b; Qaderi & Reid, 2009, 2011, 2014; Liu *et al*., 2015; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al*., 2017; Martel & Qaderi, 2017), it is poorly understood how plants respond to chemical treatments. One earlier study showed that disruption of cytochrome c oxidase, complex IV of the electron transport chain, increased methane emissions by two orders of magnitude, whereas other disruptions did not exhibit this same effect (Wishkerman *et al*., 2011). This indicates that methane emissions are sensitive to very specific biochemical changes, which may explain the lack of distinct responses to the chemical applications used.

The results of this study were important in ruling out an obvious effect of ethylene promoters and inhibitors on methane emissions. However, since these experiments were performed under control conditions, it was hypothesized that the effect of these chemicals may be marginal in the absence of environmental stimuli. To test this, one promoter (kinetin), and one inhibitor (silver nitrate) were chosen to use in further studies. Since it is known that environmental factors influence methane emissions, chemical treatment in conjunction with these factors may induce a response, which can be measured on both biochemical and physiological levels.

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3.0. EFFECTS OF TEMPERATURE AND LIGHT QUANTITY ON METHANE EMISSIONS FROM PLANTS

3.1. INTRODUCTION

Methane (CH_4) is a potent greenhouse gas that has contributed approximately 25% of the climate forcing in the last quarter-millennia (Shindell *et al*., 2009), due to a warming potential at least 34 times higher than CO_2 (Carmichael *et al*., 2014). Though atmospheric levels appeared to level off, methane concentration is now increasing again, reaching 1803 ppb (Liu *et al*., 2015). This is problematic due to the reaction of methane with hydroxyl radicals, creating water vapour, a primary greenhouse gas (IPCC, 2014). Methane, water vapour and other greenhouse gases lead to warming of the Earth's surface along with increased climatic events that can damage agricultural crops and negatively impact yields. These changes may also lead to migration of vegetation towards the poles, exposing them to differences in environmental factors, such as altered temperatures or light intensity (Jaakola and Hohtola, 2010; Qaderi *et al*., 2015).

Studies over the last decade have made it clear that plants are releasing aerobic methane emissions, and these emissions are influenced by a number of environmental factors (McLeod *et al*., 2008; Vigano *et al*., 2008; Bruhn *et al*., 2009; Qaderi and Reid, 2009; Wang *et al*., 2009; Qaderi & Reid, 2011; Wang *et al*., 2011a, b; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al*., 2017; Martel & Qaderi, 2017) as discussed in chapter 1, section 1.5 (page 8). While several precursors have been suggested, with evidence to support each, it remains unknown how this occurs on a chemical level. One of the suggested precursors is methionine (Met, Lenhart *et al*., 2015), one of 20 amino acids, and one of the 9 that humans and livestock cannot synthesize (Amir, 2008). Since humans can't synthesize

methionine, it is consumed from plants, but not all plants are plentiful in methionine. Legumes, such as soybean, pea, bean, alfalfa, and lentils, and cereal crops, are low in amino acids, such as methionine and cysteine, both of which contain sulfur (Hesse, 2004; Amir, 2008).

Methionine has many important roles in both plants and humans (Hesse, 2004; Amir, 2008). In plants, methionine is crucial for mRNA translation and is formed through the aspartate cycle along with lysine, threonine, and isoleucine (Hesse, 2004; Amir, 2008). The first precursor of L-Methionine is s-adenosylmethionine (SAM), which then goes on to produce ethylene. SAM is a primary methyl donor (Hesse, 2004; Amir, 2008) for a variety of important compounds, such as ethylene, vitamin B1, and dimethylsulphonioproprionate, which acts as an osmoprotectant. Methionine also gives rise to s-methylmethionine (SMM), which is thought to be the mobile storage unit for methionine. Parts of methionine can be recycled in plants, through either the methyl cycle or the Yang cycle (Amir, 2008).

It had previously been hypothesized that phosphatidylcholine and L-methionine may be precursors of methane in plants (Wishkerman *et al*., 2011; Bruhn *et al*., 2012). Indeed, bacteria have also been shown to produce methane as well, with L-methionine as a precursor (Kamat *et al.,* 2013), but the possibility of L-methionine (Met) as a precursor of methane in plants had not been examined beyond in vitro experiments (Althoff *et al.,* 2010). Lenhart *et al.* (2015) used isotopic labelling of the methyl thiol group of Met to track the carbon and determined that the emitted methane had the same isotopic signature. Physical stress, including leaf compressing, also promoted release of methane, with a 3-fold increase from non-stressed leaves. However, this study acknowledges that this is not direct evidence

that Met is a biological precursor; it is more likely that methionine gets oxidized in the presence of reactive oxygen species (ROS), leading to the formation of methane from the methyl thiol group (Lenhart *et al.,* 2015). Overall the potential of Met as a precursor has not been examined beyond the stable isotope study; additionally, it is unknown whether other amino acids may play a role in emissions as well. Amino acids are susceptible to small changes in environmental conditions, which may lead to influences on emissions.

Due to an increase in atmospheric greenhouse gases, temperature has risen 0.65-1.06°C from 1880 to 2012, and in the next 20 years a further increase of 0.3-0.70°C is projected, reaching as high as 6.4°C by the end of the century (IPCC, 2014). Light is one of the most important environmental factors that determine plant growth and success (Estell *et al*., 2016; Fazal *et al*., 2016), and can be influenced by factors such as time of day and cloud covering. Light intensity, duration and quality are all components of light that can affect plant development (Suzuki *et al*., 2014). All plants have photoreceptors, including phytochromes for red (R) and far-red (FR) light and cryptochromes and phototropins for blue light, that can detect aspects of light through a process called photomorphogenesis (Kopsell *et al*., 2015).

Temperature can have drastic effects on plant metabolism, leading to the production of heat shock proteins that cause a cascading effect (Qureshi *et al*., 2007). Under high night temperature, temperature-sensitive cultivars of rice (*Oryza sativa* L.) had increased concentrations of nine amino acids, including glycine, serine, valine, aspartic acid, asparagine, glutamine, glutamic acid, lysine, and proline, whereas tolerant or intermediate cultivars did not (Glaubitz *et al*., 2015). No difference was found in overall protein content,

suggesting that other proteins may be decreasing due to competition for resources (Glaubitz *et al*., 2015). All measured amino acids, including methionine, tyrosine, and tryptophan, also increased in castor bean (*Ricinus communis* L.) grown under elevated temperature (Ribeiro *et al*., 2014, 2015). In soybean (*Glycine max* L.) seedlings, content of most amino acids was relatively unchanged by higher temperatures with the exception of methionine, which increased with increased temperature (Wolf *et al*., 1982). Conversely, high temperature in potato (*Solanum tuberosum* L.) reduced the majority of primary metabolites, including all amino acids, tricarboxylic acid (TCA) cycle intermediates succinate, fumarate, and citrate, and all N-containing compounds (Hancock *et al*., 2014), whereas glucose-6- and fructose-6-phosphate were reduced in both potato and castor bean (Hancock *et al*., 2014; Ribeiro *et al*., 2014). Proline increases both drastically and consistently to a number of environmental stresses (Claussen, 2005); however, when multiple stress factors, such as higher temperatures and drought stress, are used simultaneously, proline content decreases (Mittler, 2006).

Both light quantity and quality can influence plant morphology, development, and synthesis of both primary and secondary metabolites (Ahmad *et al*., 2016), often acting in a species-specific manner (Estell *et al*., 2016). Furthermore, light is a critical component of cellular differentiation, including chloroplast development, leaf expansion, and initiation of flowering (Hemm *et al*., 2004). Low light typically inhibits plant growth and productivity through inhibition of gas exchange (Zavala & Ravetta, 2001; Fan *et al*., 2013), whereas high light intensity can damage the photosynthetic apparatus (Lichtenthaler *et al*., 2007; Fan *et al*., 2013). Higher light intensities can decrease photosynthetic efficiency as

a result of oxidative damage to photosystem (PS)II; this occurs following saturation of carbon fixation, which leads to accumulation of excitation energy in the chloroplast, increasing generation of ROS (Qureshi *et al*., 2007). Light activates many important metabolites, such as phenolic compounds, which can act as antimicrobial or antifungal agents along with ROS scavengers (Ouzounis *et al*., 2014). Furthermore, carotenoids, important tetraterpenoid pigments, are in close association with the thylakoid membranes of chloroplasts; through interactions with PSI and PSII complexes, carotenoids assist in energy capture along with dissipation of extra energy as heat (Kopsell *et al*., 2015).

If light is too weak, photosynthetic capacity is diminished, which can lead to etiolation, characterized by longer, weaker stems with reduced chlorophyll content; however, excessive light results in photoinhibition, and both of these extremes can impair proper development and lead to extreme changes in the metabolic balance (Darko *et al*., 2014). Under high light, most amino acids were found to increase in Arabidopsis leaves (Florez-Sarasa *et al*., 2012; Jänkänpää, 2012) and roots (Hemm *et al*., 2004). In Arabidopsis leaves, increases were mostly observed in phenylalanine, alanine, and isoleucine, with extreme increases in glycine. This could suggest that conversion of glycine to serine was inhibited by high light (Florez-Sarasa *et al*., 2012). In Buckwheat (*Fagopyrum tataricum* L.), amino acids such as proline, serine, glutamine, threonine, alanine, phenylalanine, leucine, valine, aspartic acid, and histidine were higher under a 16h photoperiod as opposed to shaded, low-light conditions (Peng *et al*., 2015). On the basis of controlled lighting, LED irradiated ginseng roots (*Panax ginseng*) had higher sucrose and lower amino acids than those grown in fluorescent lighting (Park *et al*., 2013), indicating that the spectrum of light used in

controlled-environment chambers may influence results. Results can also be influenced by the time of day the leaves are harvested; since amino acids operate on a diurnal cycle, plants harvested in the morning may not be comparable to plants harvested in the late afternoon (Florez-Sarasa *et al*., 2012).

Overall sugar content was higher under high light conditions in Arabidopsis (Florez-Sarasa *et al*., 2012; Jänkänpää *et al.,* 2012); similarly, increased sugar content was observed in Cat's whiskers (*Orthosiphon stamineus* L.) (Ibrahim *et al*., 2012) and increases were also found in non-structural carbs in Arabidopsis (Jänkänpää *et al.,* 2012) and flowering plant kacip fatimah (*Labisia pumila* L.; Ibrahim *et al.*, 2014). Conversely, six of eight measured sugars, including glucose and fructose, were decreased in Buckwheat (Peng *et al*., 2015). Most primary metabolites that are found to increase under light conditions are those that can be readily metabolized into compounds that are important in stress acclimation (Jänkänpää *et al.,* 2012). For example, observed increases in aromatic amino acids, as described above, could be used as precursors for hormones, glucosinolates, alkaloids, lignins, and flavonoids and anthocyanins, and these compounds are sometimes stored for later use. However, it has been shown that stored metabolites, including carbohydrates and primary antioxidants, may be depleted if the light stress becomes too severe (Brunetti *et al*., 2015).

It is evident that environmental factors, such as temperature and light, influence both aerobic methane emissions and primary metabolites in plants, but it remains poorly understood how aerobic methane emissions are related to primary metabolites. Furthermore, interactive effects of stress factors remain poorly understood, though

evidence suggests that tailored responses occur to the combination of environmental stresses. These responses, occurring only from the combination of stress factors, cannot be inferred from results of individual stress factor experiments, and therefore multiple-factor studies are important (Ramegowda & Senthil-Kumar, 2014).

The purposes of this study were (i) to observe the effects of temperature and light, both individually and through the interactions between these two factors, on aerobic methane emissions from canola (*Brassica napus*); (ii) to determine whether environmental factors lead to observable effects of ethylene promoter and inhibitor application; and (iii) to examine a number of amino acids as potential precursors of methane emissions. It was hypothesized that temperature would increase methane emissions whereas a high light intensity of levels just above that found in tropical regions (Poorter *et al*., 2016) would decrease it, and that aerobic methane emissions would be related to multiple amino acids. Temperatures were chosen to represent the current average temperature value (22/18°C), whereas the higher temperatures represent the possible average temperature by the end of the century if temperatures increase by the maximum predicted value of 6.4°C (28/24°C, IPCC, 2013). The light values were chosen because the lower light represents the average light level that plants are grown in a growth chamber setting; the higher light intensity, in contrast, is a value slightly higher than the greatest values found in subtropical and temperature regions during the summer, representing a maximum light level to which plants may reasonably encounter (Poorter *et al*., 2016).

3.2. METHODS

3.2.1. Plant material and growth conditions

Seeds of canola (*Brassica napus* L., cv. 6056 CR, BrettYoung Seeds, Winnipeg, Manitoba) were germinated and potted as described in chapter 2, section 2.2.1 (see page 36). Pots were transferred to a growth chamber (model PGR15, Conviron, Controlled Environments Ltd., Winnipeg, Manitoba, Canada), set to the same temperature regime of 22/18°C on a 16 h photoperiod, with the PPFD as described in section 2.2.1 (see page 36). Light was supplied by a mixture of incandescent lamps (Lumens 40W/120V, Sonepar, Laval, Quebec, Canada) and cool white fluorescent bulbs (Sylvania Pentron FP39/841/HO/ECO, USA). Plants were left to acclimate for 7 days until the emergence of the first true leaves, and were then randomly assigned to one of the four experimental conditions; lower temperature (22/18°C) with a low light intensity (300 μmol photon m^{-2} s^{-1}), lower temperature with a higher light intensity (600 μmol photon m^{-2} s^{-1}), higher temperature (28/24°C) with a low light intensity, and higher temperature with a higher light intensity. Within each chamber, three trays were placed, and plants were assigned to one of three chemical treatments; the control, with no chemical application, application of an ethylene promoter (Kinetin, 10^{-4} M), or application of an ethylene inhibitor (silver nitrate, 10^{-4} M), with 100 μl of the chemical solution applied every other day, for a total of 12 experimental conditions. Plants were grown for 21 days under experimental conditions, with bi-weekly pot rotation to reduce the effects of any potential positional differences. The experiment was replicated three times to ensure statistical reliability, during which the chambers were reversed for temperature and light treatments.

3.2.2. Measurement of methane and ethylene emissions

Measurement of methane and ethylene emissions were conducted as described in Chapter 2, section 2.2.2 (see page 38).

3.2.3. Plant growth and biomass accumulation

Plant growth and biomass accumulation was determined, and growth indices calculated from these values, as described in Chapter 2, section 2.2.3 (see page 39).

3.3.4. Gas exchange

Measurements of net CO_2 assimilation (A_N; µmol CO_2 m^{-2} s^{-1}), transpiration (E; mmol H_2O m^{-2} s^{-1}), and stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) were taken from a minimum of three grown leaves of average size from each condition, using a LI-COR portable photosynthesis system (model LI-6400XT, LI-COR Biosciences, Lincoln, Nebraska, USA). The LI-COR was calibrated with a CO_2 concentration of 400 µmol mol^{-1}, and the flow rate was adjusted to 400 ml s^{-1}. The light emitted from the diode was changed for each light condition to reflect the condition in which the plants were grown (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}). In order to calculate the instantaneous water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O), net CO_2 assimilation (A_N) was divided by transpiration (*E*; Topbjerg *et al*., 2015).

3.2.5. Chlorophyll fluorescence

Chlorophyll fluorescence was measured as described in Chapter 2, section 2.2.4 (see page 39).

3.2.6. Photosynthetic pigments

Photosynthetic pigments were determined as described in Chapter 2, section 2.2.5 (see page 40).

3.2.7. Moisture content

Moisture content was calculated as described in Chapter 2, section 2.2.6 (see page 40).

3.2.8. Epicuticular wax

Leaf surface waxes were removed from at least three fully-grown leaves from each experimental condition. Following measurement of fresh mass and leaf area, each leaf was immediately submerged into 20 ml of trichloromethane for 30 s, as per the procedure detailed in Qaderi *et al*. (2002). The solution was evaporated fully, after which the mass of the residue was determined gravimetrically. The quantity of epicuticular wax per leaf was calculated in terms of leaf surface area (μg mg^{-1} FM).

3.2.9. Flavonoids and nitrogen balance index

Flavonoids and nitrogen balance index (NBI) were measured as described in Chapter 2, section 2.2.7 (see page 41).

3.2.10. Metabolite profiling

Metabolite profiling was conducted according to a modified version of the protocol from Yang and Bernards (2007). After 21 days of experimental growth, leaves of uniform age and size were excised with one clean cut and immediately frozen by submersion in liquid nitrogen. They were lyophilized until dry (up to 96 hours) using a freeze dryer (VirTis

BenchTop Pro with Omnitronics^{TM} – 3L, SP Scientific, Gardiner, New York, USA), and ground into a fine powder using a mortar and pestle. Then, 10 mg of dried plant tissue was weighed and transferred into 4 ml vials, following which 2.1 ml of methanol/water/chloroform (4:1:2, v/v/v) was added along with 40 µl of triacontane (1mg ml^{-1} in chloroform) as a non-polar internal standard. Samples were vortexed (using Mini Vortexer MV 1; IKA Works, Inc., Wilmington, North Carolina, USA) for 45s and then incubated in a 50°C water bath for 1h. Solutions were cooled to room temperature, and 0.9 ml of distilled water with 20 µl of ribitol (2 mg/ml in water) was added to each vial. Vials were vortexed for 45 s more and then centrifuged (Sorvall Legend RT, Thermo Scientific, Bellemonte, Pennsylvania, USA) for 30 min at 4°C at 2900 x *g*. The polar (aqueous) layer was collected, and the non-polar (chloroform) layer was discarded. The polar layer was evaporated by lyophilization, and the resultant sediment was re-dissolved directly into 50 µl methoxyamine-HCl (20mg/ml in pyridine) at 50°C until totally re-dissolved (~1 h), following which they were further derivatized through addition of 50 µl of MSTFA + 1% TMCS (Thermo Scientific, Bellemonte, Pennsylvania, USA) and incubated for another 1 h at 50°C.

Samples (1 µl) were injected in split mode into the GC-MS (Agilent 7820A-5977E, Agilent Technologies, Santa Clara, California, USA) equipped with a capillary column (HP-5MS, 30 m x 0.250 mm ID, Agilent Technologies, Santa Clara, California, USA) with the following temperature program: after a 5 min delay at 70°C, the oven temperature was increased to 310°C at 5°C min^{-1}. It was held at 310°C for 6 minutes, cooled to 70°C, and then held for 5 more minutes, for a total analysis time of 64 minutes. High purity helium

gas at a flow rate of 1 ml min^{-1} was used as a carrier gas. Chromatograms were analyzed, with compounds identified using both an internal library and external standards. Amino acids that could not be confirmed through both internal and external standards were not used for quantification. Amino acids were quantified on the basis of standard curves derived from the injection of known quantities of pure amino acid solution, which were evaporated and derivatized as described above.

3.2.10. Data analysis

The overall effects of temperature, light quantity, and chemical application, along with their interactions, were determined on growth and physiological characteristics of canola grown under lower (22/18°C) or higher (28/24°C) temperatures, with lower (300µmol photon m^{-2} s^{-1}) or higher (600 µmol photon m^{-2} s^{-1}) light intensity, applied with either no chemical, an ethylene promoter (kinetin, 10^{-4} M), or an ethylene inhibitor (silver nitrate, 10^{-4} M), using ANOVA for split-split-plot design, which accounts for the presence of multiple manipulated factors, with each factor creating a split within the whole plot (SAS Institute, 2011). For this analysis, temperature regimes were treated as the main plot, light intensity as the subplot, and chemical treatments as the split subplot, and trials as the replications (Hinkelman and Kempthorne, 2008). To determine differences among the 12 experimental treatments, Scheffé's multiple-comparison procedure at the 5% confidence level was used; data from all replicates were used for this one-way ANOVA. Pearson's correlation coefficient at the 5% confidence interval was used to determine relationships between parameters (SAS Institute, 2011).

3.3. RESULTS

3.3.1. Emissions

Although the values of ethylene emissions were lower with increased light quantity, this was not statistically significant according to the one-way ANOVA (Table 3.1), as only plants grown under higher temperatures with lower light intensity and no chemical application had significantly greater ethylene evolution than plants grown under higher temperatures with higher light intensity and silver nitrate application (Fig. 3.1A). Absolute values of emissions were higher with higher temperatures, but this was not significant according to split-split-plot analysis (Table 3.1; see appendix A1). There was no significant effect of chemical application on ethylene emissions, and no observable trend on the basis of the one-way ANOVA (Fig. 3.1). Methane emissions, on the other hand, were significantly influenced by light, and the interaction between T x L, but not temperature or chemical or any other interactions among factors. Higher light intensity decreased methane emissions (Table 3.1; see appendix A1). On the basis of the interaction between T x L, plants grown under low temperatures with lower light intensity had the highest methane emissions, whereas plants grown under higher temperatures and higher light intensity had the lowest emissions (Fig. 3.1).

Table 3.1. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on methane and ethylene emissions of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Ethylene	Methane
Temperature (T)	1	0.2	0.5
Main plot error	2	-	-
Light Intensity (L)	1	44.9**↓	379.7****↓
T x L	1	0.0	19.6*
Subplot error	4	-	-
Chemical (C)	2	4.4*	1.82
T x C	2	2.4	0.79
L x C	2	1.7	0.99
T x L x C	2	0.3	1.63
Split-subplot error	16	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

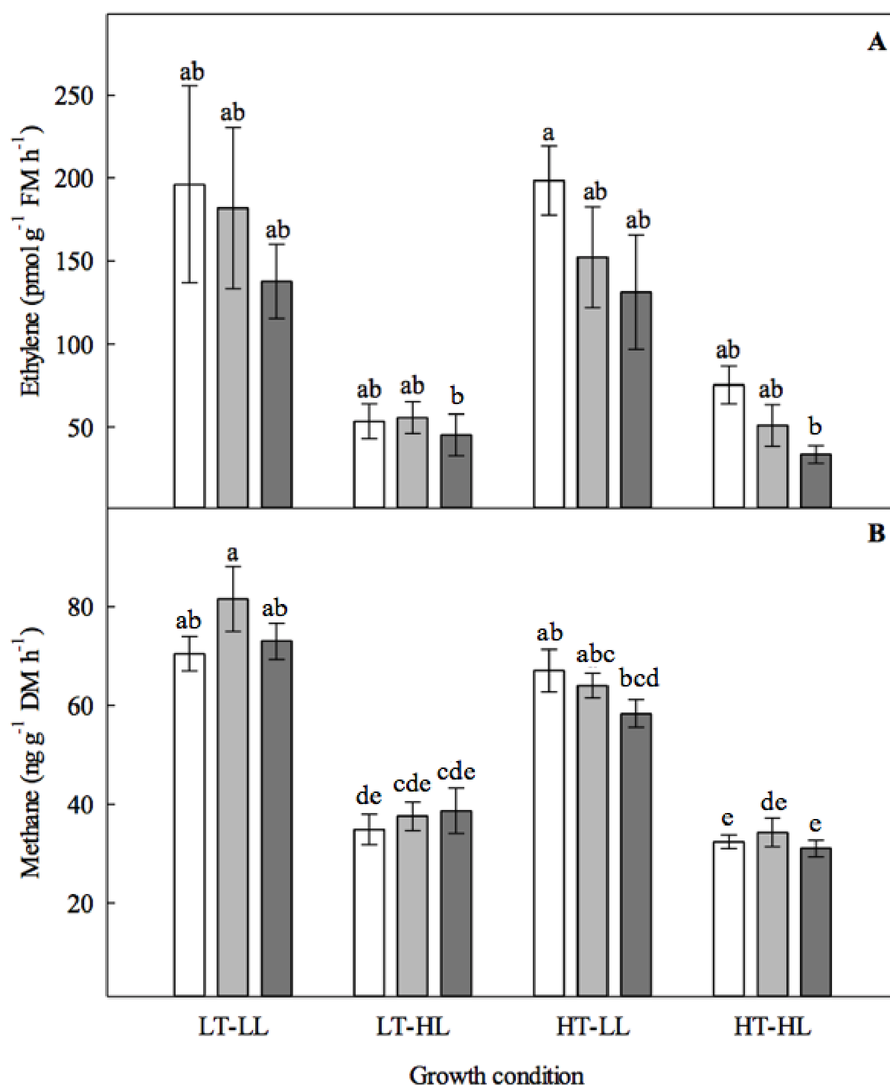

Fig. 3.1. Ethylene and methane emissions from canola plants grown under two temperatures (lower, 22/18°C; higher, 28/24°C), two light intensities (lower, 300 µmol photon m^{-2} s^{-1}; higher, 300 µmol photon m^{-2} s^{-1}), and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). Data are means ± SE (n = 3), and bars surmounted by different letters are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison test. (A) ethylene emissions; (B) methane emissions.

3.3.2. Plant growth and development

Final stem height and leaf number were significantly affected by light intensity, but not by temperature, chemical application, or the interactions between or among factors. Canola plants grown under lower temperatures had a greater final height and leaf number than plants grown under higher temperatures (Table 3.2; see Appendix A1). There were no significant effects of chemical application or interactions among any of the factors. Leaf area was not significantly influenced by any of the factors, but there was a significant interaction between T x C (Table 3.2). This interaction showed that plants grown under lower temperatures at lower light intensity with no chemical application had the largest leaves, whereas plants grown under higher temperatures with higher light intensity and silver nitrate application had the smallest leaves. These results, however, were not significant according to the one-way ANOVA (Fig. 3.2). Stem diameter was significantly affected only by light intensity, but not any of the other factors or their interactions. Stem diameter was significantly increased by light intensity (Table 3.2), but this was not significant according to the one-way ANOVA (Fig. 3.2).

Table 3.2. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on growth of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Final stem height	Leaf number	Leaf area	Stem diameter
Temperature (T)	1	5.5	9.1	0.0	12.2
Main plot error	2	-	-	-	-
Light Intensity (L)	1	148.4***↓	7.3*↓	0.0	16.4*↑
T x L	1	1.2	1.8	6.5	0.3
Subplot error	4	-	-	-	-
Chemical (C)	2	0.7	0.4	1.65	2.9
T x C	2	1.1	1.0	6.8**	0.5
L x C	2	1.6	1.3	3.1	2.1
T x L x C	2	0.8	0.1	3.1	0.9
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

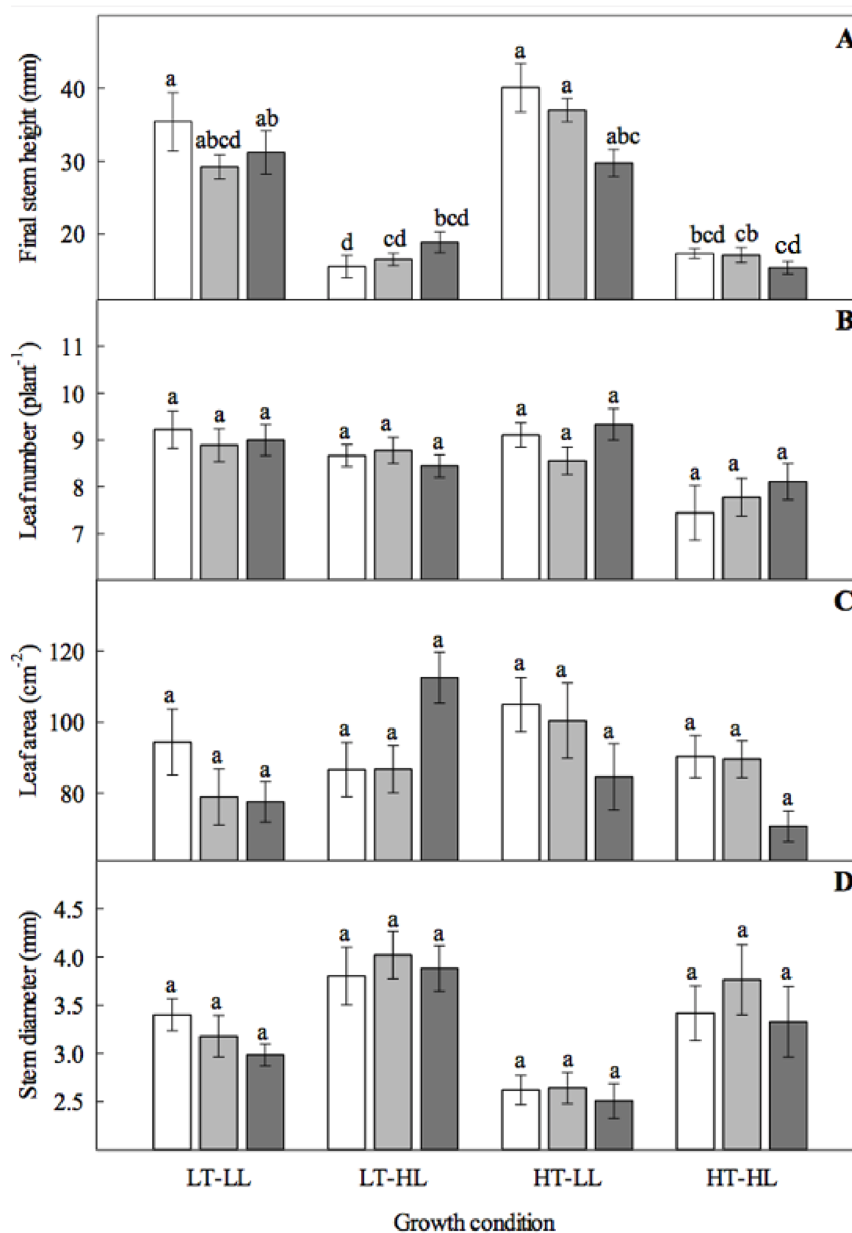

Fig. 3.2. Growth and development of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) final height; (B) leaf number; (C) leaf area; (D) stem diameter. Otherwise, as per Fig. 3.1.

3.3.3. Biomass accumulation

Leaf mass and total mass were significantly influenced by light intensity, and the interactions between T x C and among T x L x C (Table 3.3). Both leaf and total mass were significantly increased by higher light intensity, but were not significantly influenced by other parameters (see Appendix A1). On the basis of the interactions among factors, plants grown under lower temperature with higher light had the highest biomass, and plants grown under higher temperatures and higher light intensity had the lowest biomass; however, these were not significant according to the one-way ANOVA (Fig. 3.3). Stem mass was significantly increased by temperature and light intensity, but not by chemical application, and was also affected by the interaction between T x C (Table 3.3). Stem mass was highest in plants grown under low temperature with high light intensity and silver nitrate application, and lowest in plants grown under higher temperatures with high light intensity and silver nitrate application (Fig. 3.3). Root mass was not significantly influenced by the main factors or their interactions (Table 3.3).

Table 3.3. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on biomass accumulation of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Leaf mass	Stem mass	Root mass	Total mass
Temperature (T)	1	0.1	32.5*↓	1.0	1.4
Main plot error	2	-	-	-	-
Light Intensity (L)	1	9.4*↑	6.3*↑	5.7	8.3*↑
T x L	1	0.2	0.0	0.8	0.0
Subplot error	4	-	-	-	-
Chemical (C)	2	0.8	2.6	1.7	0.5
T x C	2	6.6**	3.9*	2.7	6.5**
L x C	2	3.4	2.2	1.1	3.1
T x L x C	2	5.6*	3.2	2.5	5.7*
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

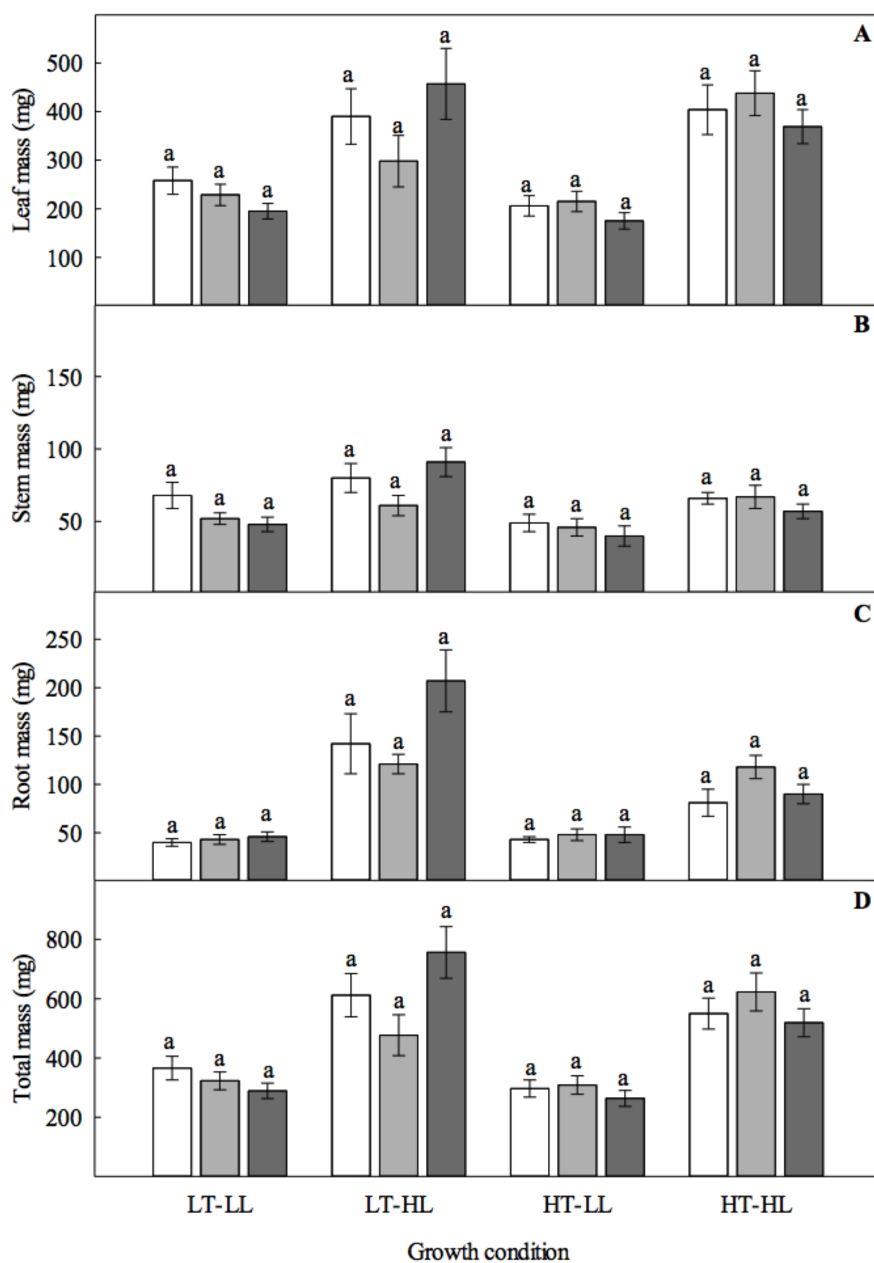

Fig. 3.3. Biomass accumulation of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) leaf mass; (B) stem mass; (C) root mass; (D) total mass. Otherwise, as per Fig. 3.1.

3.3.4. Growth indices

LMA and LAR were significantly affected by light intensity and the interactions between T x C and L x C, and among T x L x C (Table 3.4). LMA was increased by light intensity whereas LAR was decreased by light intensity (Table 3.4; see Appendix A2). While not significant on the basis of split-split-plot analysis, LAR was increased by temperature. The interactions among factors showed that plants grown under higher temperatures with higher light intensity and silver nitrate application had the highest LMA, and plants grown under higher temperatures and lower light, regardless of chemical application, had the lowest LMA; however, this was not significant according to the one-way ANOVA (Fig. 3.4A). Similarly, plants grown under higher temperatures with higher light intensity and silver nitrate application had the highest LAR, and plants grown under lower temperatures with high light intensity and silver nitrate had the lowest LAR (Fig. 3.4B). LMR was not significantly influenced by the main factors or their interactions, whereas S:R mass ratio was affected only by chemical application, with control plants having the highest S:R mass ratio and plants applied with silver nitrate the lowest S:R mass ratio (Table 3.4; Fig. 3.4). Neither LMR nor S:R mass ratio had significant differences according to the one-way ANOVA (Fig. 3.4).

Table 3.4. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on growth indices of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	LMA	LMR	LAR	S:R mass ratio
Temperature (T)	1	1.1	3.6	10.9	0.1
Main plot error	2	-	-	-	-
Light Intensity (L)	1	14.4*↑	4.3	43.1**↓	7.5
T x L	1	2.4	20.8*	7.0	2.9
Subplot error	4	-	-	-	-
Chemical (C)	2	0.8	1.9	1.0	4.5*
T x C	2	5.1*	0.4	6.6**	0.4
L x C	2	4.8*	0.3	4.4*	0.0
T x L x C	2	3.9*	0.3	1.8*	2.6
Split-subplot error	16	-	-	-	-

$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$

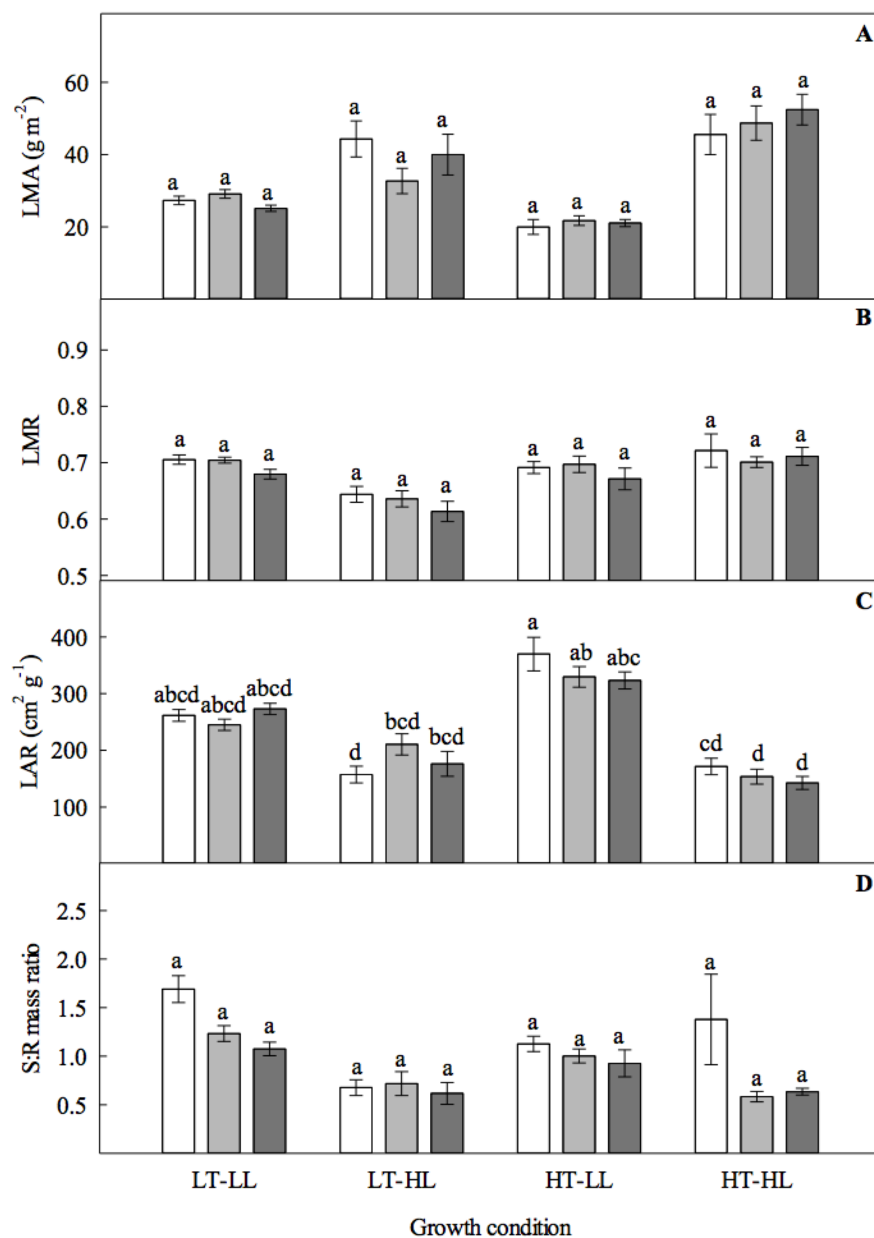

Fig. 3.4. Growth indices of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) LMA, leaf mass per area; (B) LMR, leaf mass ratio; (C) LAR, leaf area ratio; (D) S:R ratio, shoot to root mass ratio. Otherwise, as per Fig. 3.1.

3.3.5. Gas exchange

Net CO_2 assimilation (A_N) and water use efficiency (WUE) were significantly affected by all the main factors as well as by their interactions (Table 3.5). A_N was significantly decreased by temperature and silver nitrate, but increased by light intensity, whereas WUE was increased by temperature and light intensity and decreased by kinetin (Table 3.5; see Appendix A2). A_N was highest under lower temperatures with high light and kinetin application, but lowest under lower temperatures with low light and silver nitrate application (Fig. 3.5). WUE was highest in plants grown under higher temperatures with lower light intensity and silver nitrate application but lowest in plants grown under lower temperatures with lower light intensity and silver nitrate application. Both transpiration (E) and stomatal conductance (g_s) were significantly influenced by temperature, light intensity and the interactions between T x C, and among T x L x C, and only E was influenced by the interaction between L x C (Table 3.5). Transpiration was decreased by temperature and increased by light intensity. Interactions among the main factors indicated that the highest transpiration rate occurred under low temperatures with low light intensity and no chemical application, whereas the lowest transpiration occurred under higher temperatures with higher light intensity and no chemical application (Fig. 3.5). Stomatal conductance, on the other hand, was decreased by both temperature and light intensity, and the highest g_s occurred under lower temperatures with low light intensity and no chemical application, and the lowest occurred under higher temperatures with high light intensity and no chemical application (Fig. 3.5).

Table 3.5. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on gas exchange of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	A_N	*E*	g_s	WUE
Temperature (T)	1	30.8*↓	45.1*↓	44.3*↓	202.8**↑
Main plot error	2	-	-	-	-
Light Intensity (L)	1	538.9****↑	5.1*↑	6.6	13.5*↓
T x L	1	919.1****	0.4	0.0	410.0****
Subplot error	4	-	-	-	-
Chemical (C)	2	26.2**** – ↓	14.4***– ↓	5.6* – –	4.5* – –
T x C	2	23.4****	49.6****	24.3****	18.1****
L x C	2	16.3****	6.0*	0.4	6.5**
T x L x C	2	16.4****	54.0****	12.1***	30.9****
Split-subplot error	16	-	-	-	-

**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

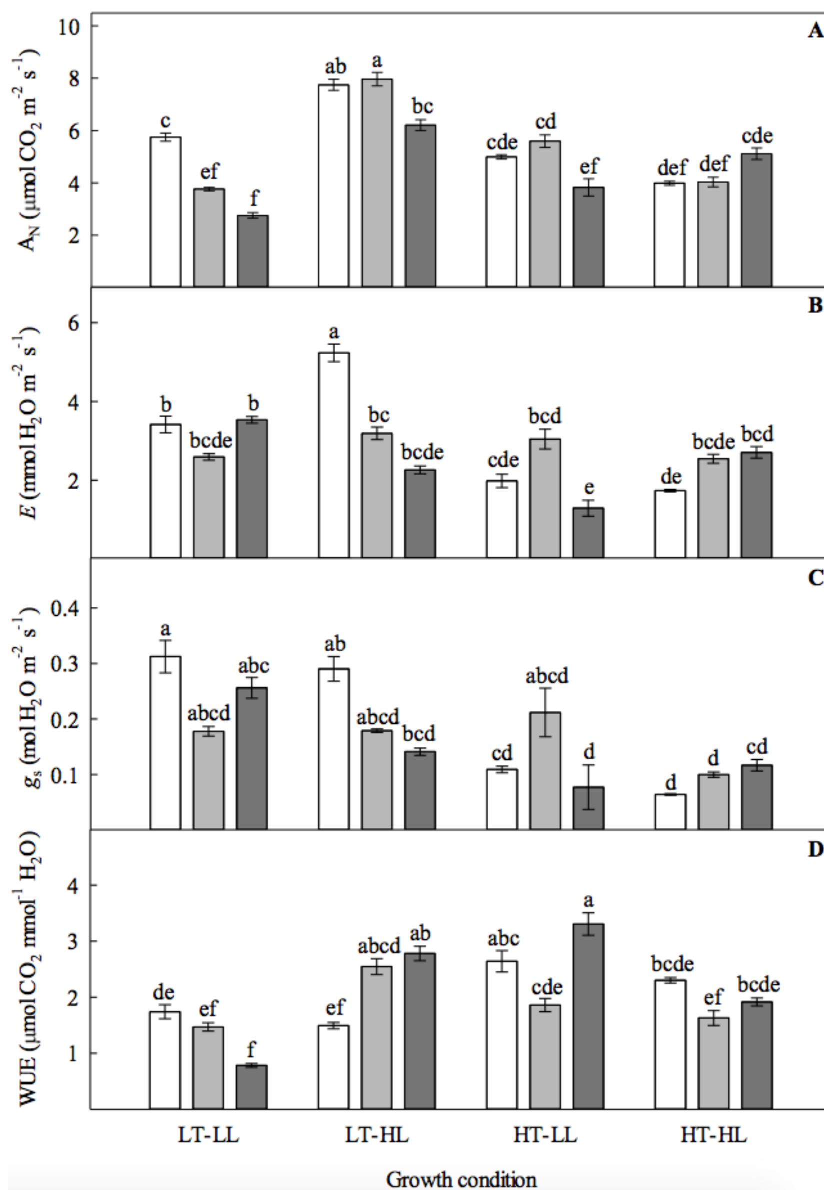

Fig. 3.5. Gas exchange of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) A_N, net CO_2 assimilation; (B) E, transpiration; (C) g_s, stomatal conductance; (D) WUE, water use efficiency. Otherwise, as per Fig. 3.1.

3.3.6. Chlorophyll fluorescence

The effective quantum yield of PSII (ϕPSII) was not significantly affected by any environmental factor or their interactions. Maximum quantum yield of PSII (F_v/F_m), non-photochemical quenching (qNP) and photochemical quenching were all significantly affected by light intensity, with F_v/F_m and qP decreased in response to high light, and qNP increased under high light (Table 3.6; see Appendix A3). F_v/F_m and qP were also significantly affected by the interaction among T x L x C, but this was only significant on as per the one-way ANOVA for qP. The highest qP was found in plants grown under higher temperatures with higher light intensity and kinetin application, whereas the lowest was under lower temperatures with lower light intensity and no chemical application (Fig. 3.6).

Table 3.6. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on chlorophyll fluorescence of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	ϕPSII	F_v/F_m	qNP	qP
Temperature (T)	1	0.9	0.0	15.8	0.3
Main plot error	2	-	-	-	-
Light Intensity (L)	1	0.9	15.6*↓	7.8*↑	125.3***↑
T x L	1	0.1	0.1	1.3	0.1
Subplot error	4	-	-	-	-
Chemical (C)	2	1.3	0.0	1.2	2.2
T x C	2	0.1	2.6	1.5	2.3
L x C	2	2.7	1.5	0.2	2.1
T x L x C	2	1.2	3.9*	1.8	4.3*
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

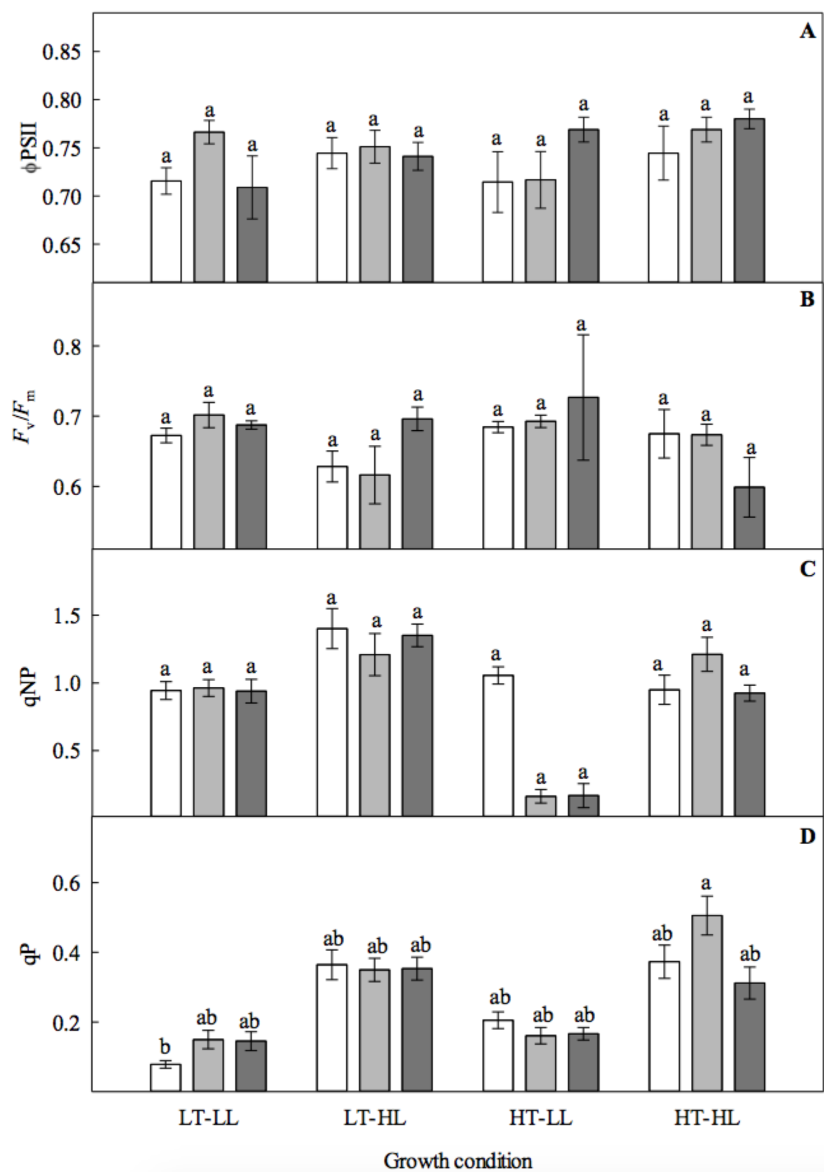

Fig. 3.6. Chlorophyll fluorescence of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, white), an ethylene promoter (kinetin, light gray), or ethylene inhibitor (silver nitrate, medium gray). (A) ϕPSII, effective quantum yield of PSII; (B) F_v/F_m, maximum quantum yield of PSII; (C) qNP, non-photochemical quenching; (D) qP, photochemical quenching. Otherwise, as per Fig. 3.1.

3.3.7. Photosynthetic pigments

All parameters of photosynthetic pigments were affected only by light intensity, with significant decreases in response to increased light (see Appendix A2). None of the photosynthetic pigments were significantly affected by temperature, chemical application, or their interactions (Table 3.7); however, on the basis of two-way ANOVA, chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids were decreased by high light (Table 3.7), but these results were not significant according to the one-way ANOVA (Fig. 3.7).

Table 3.7. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on photosynthetic pigments of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b* ratio	Carotenoids
Temperature (T)	1	0.1	0.3	0.1	0.0	1.1
Main plot error	2	-	-	-	-	-
Light Intensity (L)	1	27.0**↓	26.9**↓	27.2**↓	9.3*↓	10.7*↓
T x L	1	0.3	0.6	0.4	1.6	0.2
Subplot error	4	-	-	-	-	-
Chemical (C)	2	0.4	0.2	0.4	3.3	1.2
T x C	2	0.3	0.7	0.4	3.6	0.3
L x C	2	0.9	0.5	0.8	0.8	1.8
T x L x C	2	1.4	0.6	1.2	2.0	2.4
Split-subplot error	16	-	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

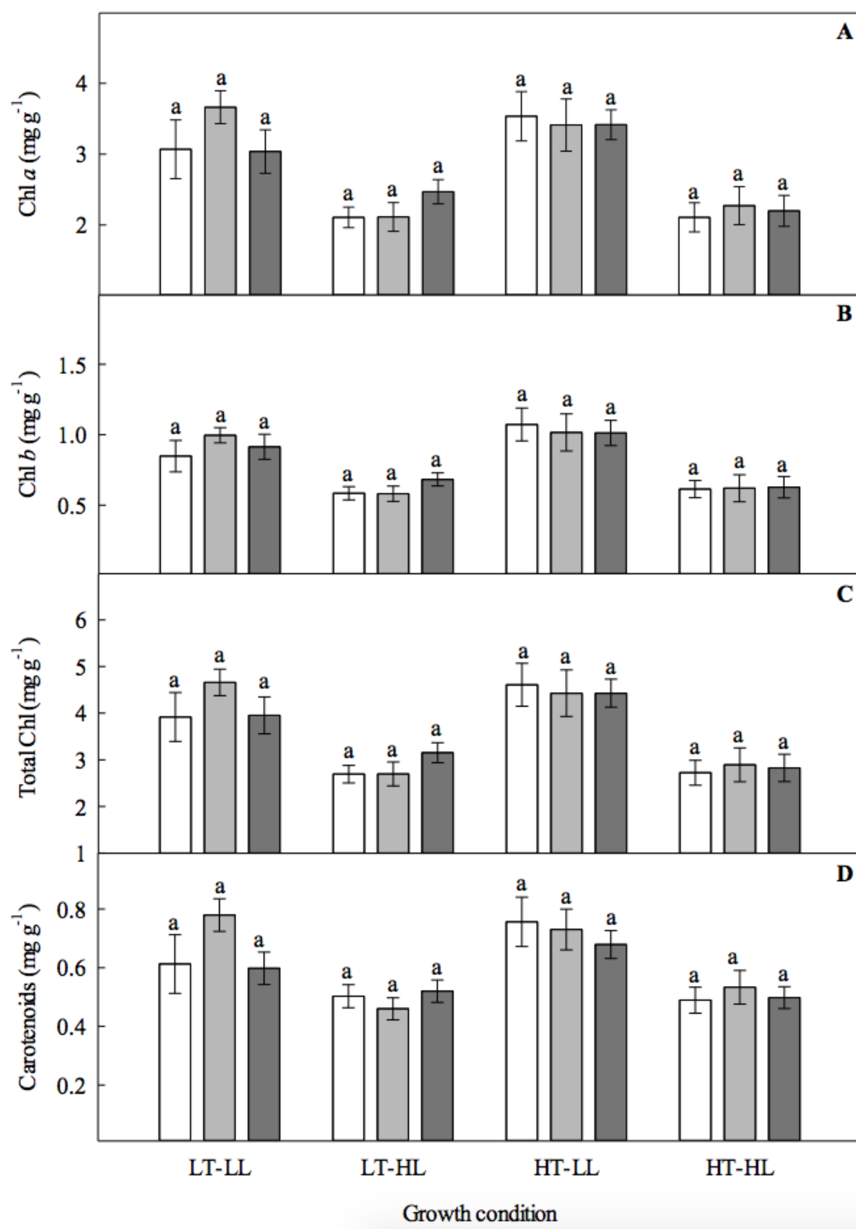

Fig. 3.7. Photosynthetic pigments of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) chlorophyll (Chl) *a*; (B) Chl *b*; (C) total Chl; (D) carotenoids. Otherwise, as per Fig. 3.1.

3.3.8. Moisture content

Moisture content was significantly increased by higher light intensity, but was unaffected by temperature, with no significant interactions between or among factors (Table 3.8).

3.3.9. Epicuticular wax

Wax content per leaf fresh mass was unaffected by any parameter or the interactions between or among them (Table 3.8; Fig. 3.8B).

3.3.10. Nitrogen balance index and flavonoids

While the absolute value of nitrogen balance index (NBI) was increased by temperature, this was not significant on the basis of one-way ANOVA. NBI was significantly decreased by light intensity (Table 3.8l; see Appendix A4), but was not affected by any of the interactions. Flavonoids were also increased in response to light intensity, and were also significantly affected by the interaction between L x C (Table 3.8), which indicated that the highest flavonoid content occurred in plants grown under lower temperatures with higher light intensity and kinetin application, and the lowest flavonoids occurred in plants grown under higher temperatures with lower light intensity and no chemical application (Fig. 3.8).

Table 3.8. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on nutrient balance, moisture content, and protective compounds of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Moisture	NBI	Flavonoids	Wax
Temperature (T)	1	0.0	2.5	1.9	1.3
Main plot error	2	-	-	-	-
Light Intensity (L)	1	17.3*↓	25.2**↓	30.6**↑	0.1
T x L	1	0.0	0.2	0.7	0.1
Subplot error	4	-	-	-	-
Chemical (C)	2	0.3	1.0	6.0*– –	0.1
T x C	2	0.3	1.0	2.6	0.0
L x C	2	0.4	2.4	6.2*	1.0
T x L x C	2	1.8	0.6	2.2	0.5
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

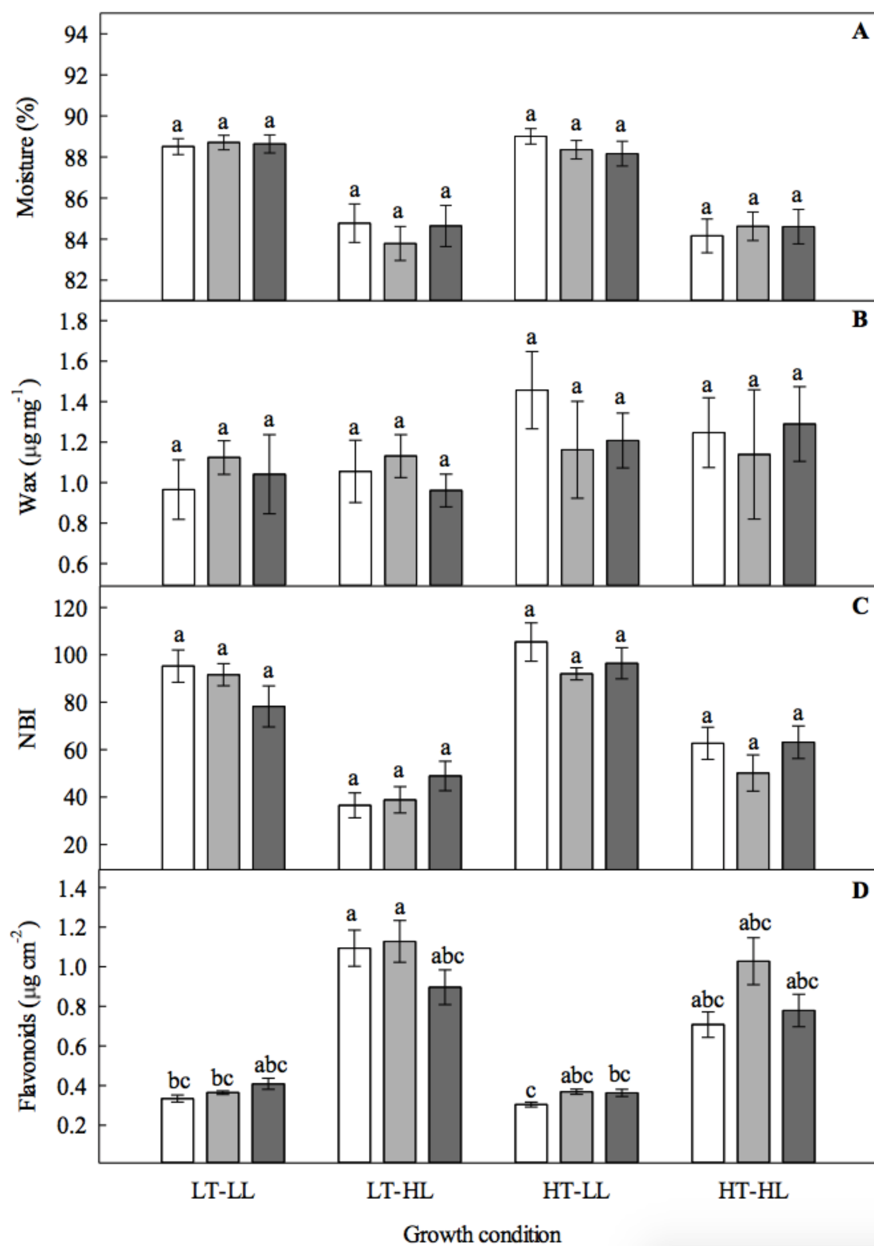

Fig. 3.8. Moisture content, nutrient balance, and protective compounds of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) moisture content; (B) NBI, nitrogen balance index; (C) epicuticular wax; (D) flavonoids. Otherwise, as per Fig. 3.1.

3.3.11. Metabolite profiling

Metabolite profiling led to the identification of 13 amino acids, based on both internal and external standards. On the basis of individual factors (see Appendix A5), temperature decreased content of two amino acids (Ala and Tyr), increased five amino acids (Asp, Ile, Leu, Pro, Val), but had no effect on the remaining six amino acids (Gly, Lys, Met, Phe, Ser, Thr). High light decreased content of three amino acids (Ala, Asp, Met) increased nine amino acids (Gly, Ile, Leu, Lys, Pro, Ser, Thr, Tyr, Val), but had no effect on the remaining two (Leu, Phe). In respect to the control plants, kinetin application decreased two amino acids (Ala, Leu), increased eight amino acids (Asp, Lys, Phe, Pro, Ser, Thr, Tyr, Val) and had no effect on the remaining three (Gly, Ile, Met). Silver nitrate application decreased three amino acids (Ala, Leu, Thr), increased four amino acids (Asp, Lys, Tyr, Thr), but had no effect on the remaining seven (Gly, Ile, Met, Phe, Pro, Ser, Thr) (Tables 3.9 – 3.11).

Interactions among factors were significant for the majority of amino acids, but the effects of these interactions varied greatly among individual amino acids (see Fig. 3.9 – 3.12). For methionine, the greatest content occurred in control plants grown under lower temperatures with low light intensity, and the lowest methionine content occurred in control plants grown under higher temperatures with high light intensity. For Ala, the highest content occurred in plants grown under lower temperatures with low light intensity; for Gly, Ile, Leu, Pro and Val, the highest amino acid content occurred in plants grown under higher temperatures with high light intensity, and for Lys, Ser, and Tyr, the highest amino acid content occurred in plants grown under lower temperatures with high light intensity. Chemical application had no significant influence on these interactions (Fig. 3.9

– 3.12), and for Gly, Ile, and Thr, differences were not significant according to the one-way ANOVA (Figs. 3.9C, 3.10A, 3.12B, respectively).

Table 3.9. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on content of amino acids alanine (Ala), aspartic acid (Asp), glycine (Gly), isoleucine (Ile), and leucine (Leu) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Ala	Asp	Gly	Ile	Leu
Temperature (T)	1	86.6*↓	17.5*↑	7.6	9.0	13.3
Main plot error	2	-	-	-	-	-
Light Intensity (L)	1	7.7*↓	2.2	55.1**↑	5.3	0.6
T x L	1	38.4**	0.2	7.1	3.6	25.9**
Subplot error	4	-	-	-	-	-
Chemical (C)	2	91.9****↓↓	8.7**↑↑	0.7	1.7	5.4*↓↓
T x C	2	37.5****	18.3****	10.1**	4.7*	1.0
L x C	2	3.3	1.6	0.2	4.0*	4.4*
T x L x C	2	63.5****	9.1**	0.5	1.8	28.2****
Split-subplot error	16	-	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Table 3.10. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on content of amino acids lysine (Lys), methionine (Met), phenylalanine (Phe), and proline (Pro) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Lys	Met	Phe	Pro
Temperature (T)	1	3.9	0.4	39.3*–	97.3*↑
Main plot error	2	-	-	-	-
Light Intensity (L)	1	25.8**↑	42.3**↓	1.7	8.5*↑
T x L	1	24.3**	14.6*	1.0	8.9*
Subplot error	4	-	-	-	-
Chemical (C)	2	29.8****–↑	0.8	5.7*↑–	15.0***↑–
T x C	2	7.2**	23.1****	0.3	20.1****
L x C	2	32.2****	40.7****	1.7	26.8****
T x L x C	2	11.8***	11.3***	0.2	14.8***
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Table 3.11. Summary of split-split-plot ANOVA (*F* value) for effects of temperature, light intensity, and chemical application on content of amino acids serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Ser	Thr	Tyr	Val
Temperature (T)	1	0.0	0.7	15.2	13.8
Main plot error	2	-	-	-	-
Light Intensity (L)	1	12.7*↑	2.8	10.0*↑	38.0**↑
T x L	1	21.5**	0.1	20.2*	7.9*
Subplot error	4	-	-	-	-
Chemical (C)	2	5.7*– –	5.2*– –	13.6***↑↑	20.7****↑↑
T x C	2	6.9**	0.4	0.4	4.3*
L x C	2	6.6**	6.6**	13.3***	7.7**
T x L x C	2	19.7****	3.1	8.4**	8.6**
Split-subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

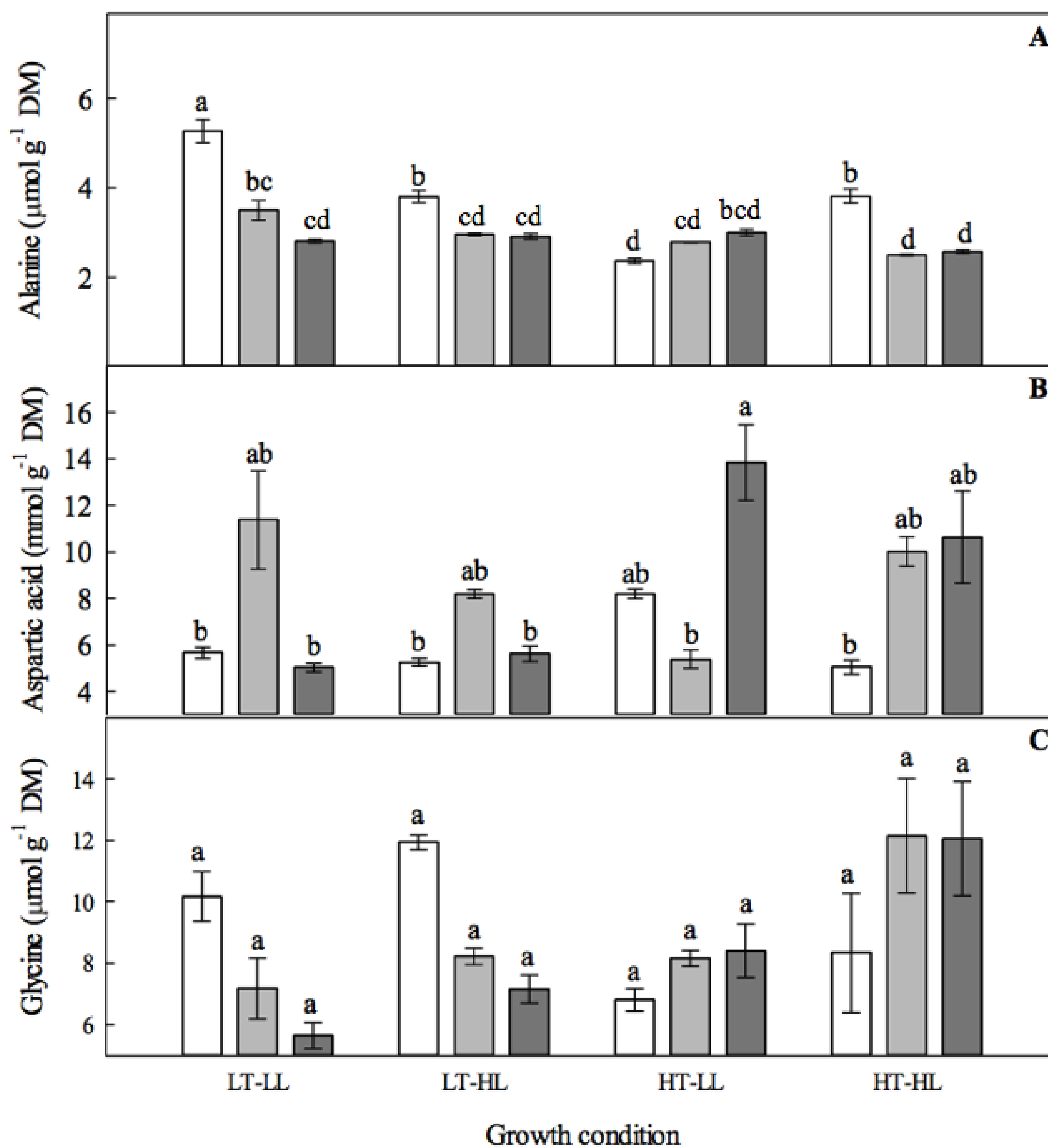

Fig. 3.9. Alanine, aspartic acid, and glycine content of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) alanine; (B) aspartic acid; (C) glycine. Otherwise, as per Fig. 3.1.

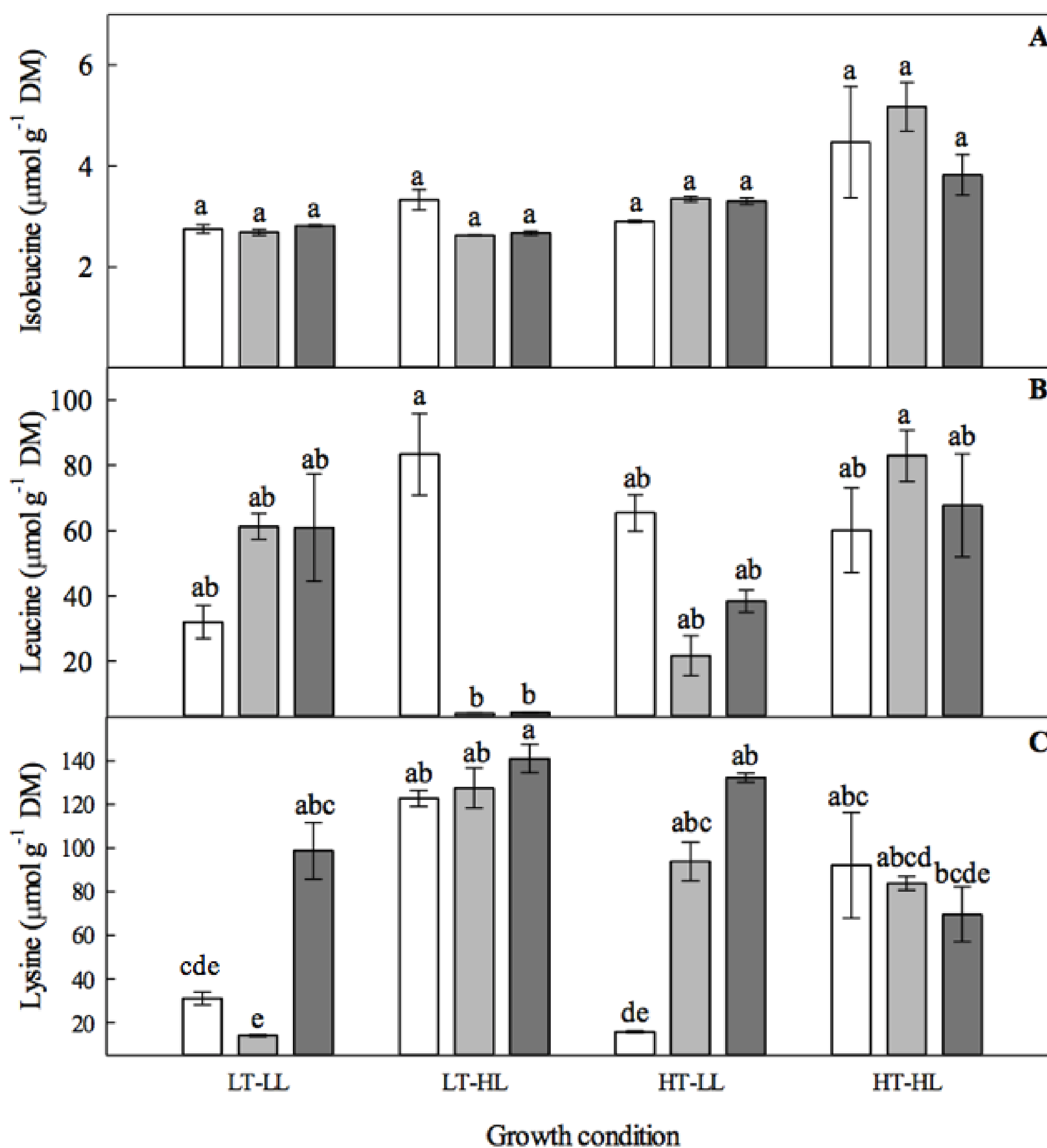

Fig. 3.10. Isoleucine, leucine, and lysine content of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) isoleucine; (B) leucine; (C) lysine. Otherwise, as per Fig. 3.1.

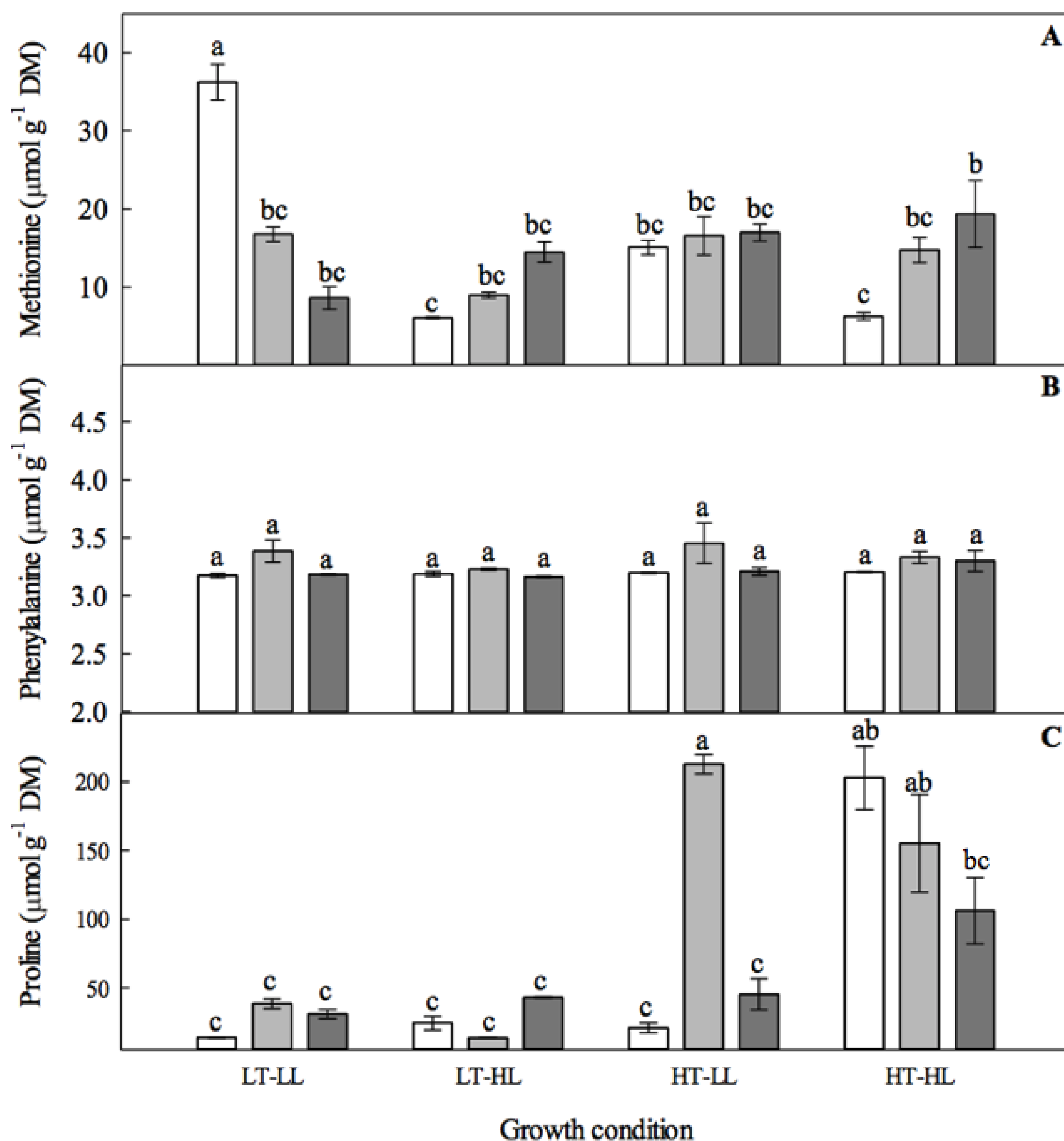

Fig. 3.11. Methionine, phenylalanine, and proline content of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) methionine; (B) phenylalanine; (C) proline. Otherwise, as per Fig. 3.1.

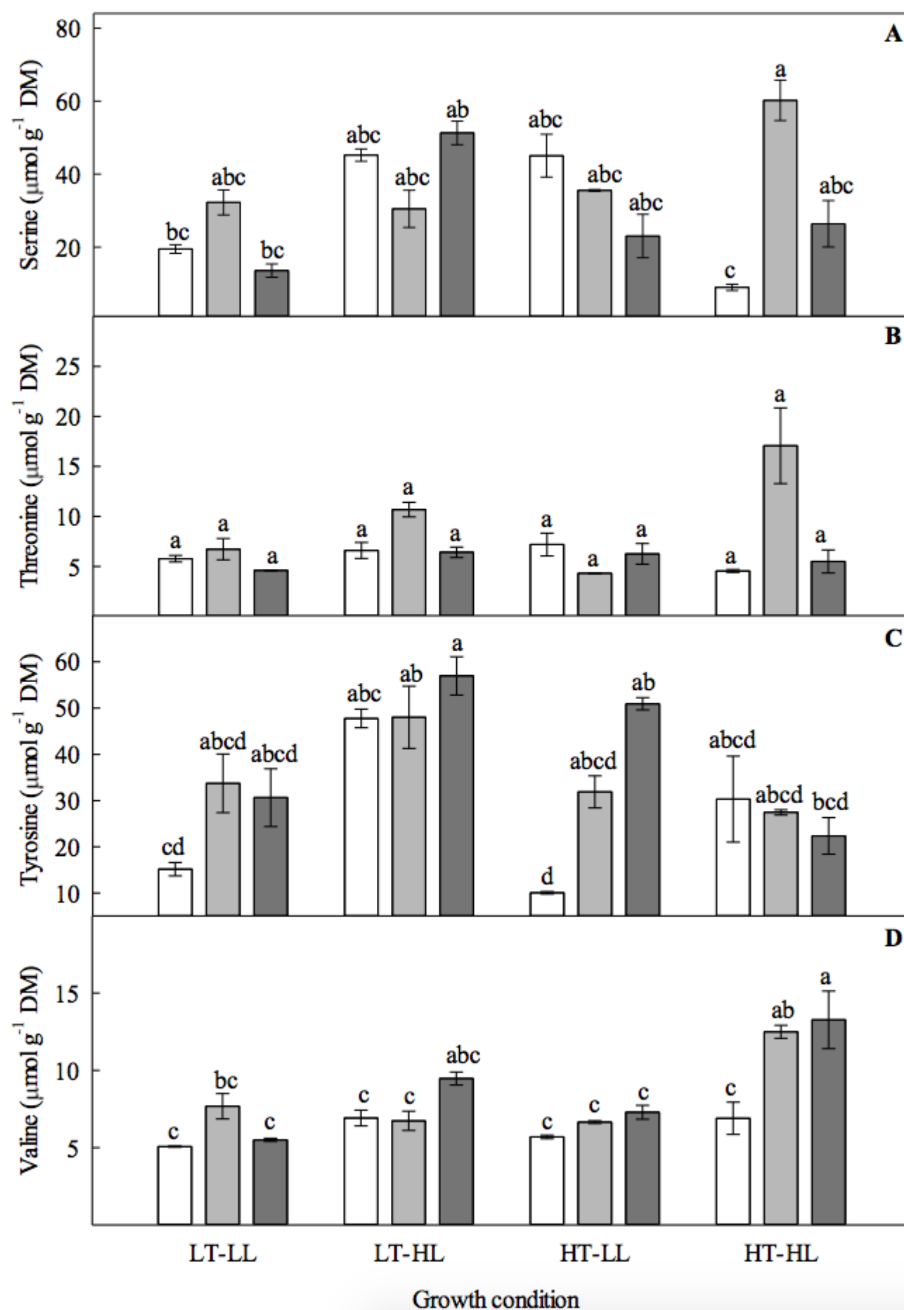

Fig. 3.12. Serine, threonine, tyrosine, and valine content of canola plants grown under two temperatures, two light intensities, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) serine; (B) threonine; (C) tyrosine; (D) valine. Otherwise, as per Fig. 3.1.

3.3.12. Pearson's correlation coefficients

Pearson's correlation analysis showed numerous significant relationships between growth and physiological parameters; these relationships, in regards to methane emissions and ethylene evolution, are shown below (Tables 3.12a,b and 3.13). Methane was positively correlated with ethylene evolution; similarly, both methane and ethylene were positively correlated with height, leaf area ratio (LAR), moisture content, chlorophyll content, and carotenoids, whereas they were negatively correlated with stem diameter, all aspects of plant biomass, leaf mass per area (LMA), net CO_2 assimilation (A_N), and photochemical quenching (qP). Methane was positively correlated with leaf number, stomatal conductance (g_s), and maximum quantum yield of PSII (F_v/F_m).

There were also several interesting relationships between methane, ethylene, and several amino acids (Table 3.13). Methane and ethylene were both positively correlated with methionine content, and negatively correlated with lysine and valine. Methane was also negatively correlated with glycine, isoleucine, and proline (Table 3.13).

Table 3.12a. Pearson's correlation coefficients (upper values) and *P*-values (lower values) for relationship between methane emissions, ethylene evolution, and growth and physiological parameters of canola plants, grown as described in Table 3.1. Significant correlations are indicated in bold.

Factor	Methane	Ethylene
Ethylene	**0.743** **0.000**	-
Stem height	**0.773** **0.000**	**0.752** **0.000**
Stem diameter	**-0.422** **0.010**	**-0.525** **0.001**
Leaf number	**0.409** **0.000**	0.218 0.202
Total mass	**-0.551** **0.000**	**-0.565** **0.000**
LMA	**-0.628** **0.000**	**-0.636** **0.000**
LAR	**0.639** **0.000**	**0.754** **0.000**
A_N	**-0.328** **0.049**	**-0.396** **0.017**
g_s	**0.408** **0.013**	-0.003 0.986
F_v/F_m	**0.381** **0.022**	0.306 0.070
qP	**-0.709** **0.000**	**-0.538** **0.00**

Table 3.12b. Pearson's correlation coefficients (upper values) and *P*-values (lower values) for relationship between methane emissions, ethylene evolution, and growth and physiological parameters of canola plants, grown as described in Table 3.1. Significant correlations are indicated in bold.

Factor	Methane	Ethylene
Ethylene	**0.743** **0.000**	-
Total chlorophyll	**0.527** **0.001**	**0.577** **0.000**
Carotenoids	**0.423** **0.010**	**0.452** **0.006**
NBI	**0.654** **0.000**	**0.535** **0.001**
Moisture	**0.770** **0.000**	**0.680** **0.000**
Flavonoids	**-0.759** **0.000**	**-0.528** **0.001**

Table 3.13. Pearson's correlation coefficients (upper values) and *P*-values (lower values) for relationship between methane emissions, ethylene evolution, and amino acid content of canola plants, grown as described in Table 3.1. Significant correlations are indicated in bold.

Factor	**Methane**	**Ethylene**
Ethylene	**0.743** **0.000**	-
Glycine	**-0.429** **0.009**	-0.309 0.066
Isoleucine	**-0.507** **0.002**	-0.306 0.069
Lysine	**-0.550** **0.001**	**-0.457** **0.005**
Methionine	**0.441** **0.007**	**0.349** **0.037**
Proline	**-0.339** **0.043**	-0.236 0.167
Valine	**-0.543** **0.001**	**-0.549** **0.001**

3.4. DISCUSSION

This study revealed that higher light decreased both ethylene and methane emissions (Fig. 3.1), and plants under these conditions had a larger biomass and thicker leaves (Figs. 3.2 – 3.4), as expected due to increased light for photosynthetic processes (Fan *et al.*, 2013). While it was previously hypothesized that visible light would not play an influential role on aerobic methane emissions (Bruhn *et al.*, 2014), light is crucial to plant development, and this study confirms earlier work on the effects of visible light on CH_4 emissions (Martel & Qaderi, 2017). As discussed previously, increasing evidence in support of aerobic methane from plants has made the phenomenon undeniable, regardless of the remaining uncertainty surrounding the pathway of production. Emissions are largely regulated by environmental factors and differ among species (Bruhn *et al.*, 2009; Vigano *et al.*, 2010; Wang *et al.*, 2011a,b; Qaderi & Reid, 2011, 2014; Liu *et al.*, 2015; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al.*, 2017; Martel & Qaderi, 2017).

In contrast to earlier work on six different species, including canola (Qaderi & Reid 2009, 2011), temperature was found to have no significant effect on methane emissions (Table 3.1), but since it is known there is a species-specific (Wang *et al.*, 2008; Martel & Qaderi, 2017) and cultivar-specific response (Abdulamajeed & Qaderi, 2017), this is not unreasonable. The cultivar of canola used in this study is a genetically modified cultivar from BrettYoung Seeds, modified for clubroot resistance. This cultivar may also be more resilient to temperature changes than wild-type cultivars, and may therefore be less responsive to temperature fluctuations. When temperature and light were combined, their interaction was significant on both methane and ethylene, and the highest methane and

ethylene production was found in canola grown under lower temperatures with lower light intensity; since these produced small plants indicative of stress (Wang & Frei, 2011), and stressed plants are known to release more methane (Qaderi & Reid, 2011), this make sense. Methane and ethylene content were both negatively correlated with plant biomass and positively correlated with stem height and diameter, indicating that the smaller the plant, the greater the emissions. Since lower light intensity provides less light for photosynthetic processes, resulting in smaller plants, natural light conditions may act as a mitigating factor. In confirmation of earlier work on two species, methane was positively correlated with moisture content (Table 3.12b; Qaderi & Reid, 2014; Martel & Qaderi, 2017).

From a growth and physiological perspective, temperature had much fewer significant effects than light intensity; temperature decreased stem mass, with significant effects on all gas exchange parameters, but had no significant effects on other parameters. This could indicate that light intensity has a higher influence than temperature on this cultivar of canola; previous work on different cultivars of canola has found that higher temperatures resulted in shorter plants with smaller leaves (Qaderi *et al*., 2006; Slauenwhite & Qaderi, 2013), and decreased chlorophyll fluorescence and overall biomass (Qaderi *et al*., 2006, 2010; Slauenwhite & Qaderi, 2013). Furthermore, numerous studies have shown a reduced seed yield when canola was subjected to temperature stress further in development (Gan *et al*., 2004; Aksouh-Harradj *et al*., 2006; Faraji *et al*., 2009), but that temperature stress in the seedling and vegetative stages have less-pronounced effects from which canola plants can recover (Gan *et al*., 2004). The few negative effects of temperature in this study could indicate that this particular cultivar of canola is robust and resistant to temperature stress,

or that canola seedlings can recover from temperature stress in early developmental stages (Gan *et al*., 2004).

Light intensity, on the other hand, had numerous effects on canola plants, and significantly influenced the majority of growth and physiological parameters (Tables 3.1-3.11). In young tomato plants, growth under higher light intensity of 450 µmol photons m^{-2} s^{-1} led to larger plants with a higher net photosynthesis, and increased leaf thickness through increased palisade mesophyll growth and a greater number of cellular layers to protect the plants from increased irradiance (Fan *et al*., 2013). In this experiment, the canola plants grown under higher light intensity of 600 µmol photons m^{-2} s^{-1} had a similarly increased leaf thickness, but had reduced net CO_2 assimilation and stomatal conductance (Fig. 3.5); this could indicate that either the high light level, or the distance between the lighting equipment and the plants, chosen for this experiment was too much for this plant, and may have resulted in damage to the photosynthetic apparatus (Takahashi & Murata, 2008; Fan *et al*., 2013). Indeed, visible damage was apparent on the leaves of plants grown under the high light treatments, and plants grown under higher light had reduced chlorophyll content (Table 3.7). These negative effects of light may in part explain the effects of light on photosynthesis; methane was negatively correlated with net CO_2 assimilation and positively correlated with stomatal conductance, and methane therefore increased under periods of reduced A_N, but increased with increasing g_s (Table 3.12a).

From a metabolic perspective, the factors used in this study had many significant effects. Temperature increased the amount of five out of 13 measured amino acids, and decreased two (Tables 3.9 – 3.11). These results agree with earlier studies (Wolf *et al.,* 1982; Ribeiro

et al., 2014, 2015; Glaubitz *et al*., 2015) that reported the increase of several to all measured amino acids, but more amino acids were found unaffected in this study. As expected, proline increased dramatically in response to temperature (Table 3.1; Claussen, 2005). In contrast, the amount of most amino acids were found to decrease in response to temperature in potato (Hancock *et al*., 2014), but were largely unaffected in soybean (Wolf *et al*.,1982). Contrasting results between studies could be indicative of species-dependent responses or the effects of diurnal metabolic cycles (Florez-Sarasa *et al*., 2012).

As expected, most amino acids increased in response to high light, with nine amino acids increasing and only three decreasing, including methionine (Tables 3.9 – 3.11). The increased amino acids (Gly, Ile, Leu, Lys, Pro, Ser, Thr, Tyr, and Val) are in accordance with earlier work on Arabidopsis (Hemm *et al*., 2004; Florez-Sarasa *et al*., 2012; Jänkänpää *et al.,* 2012), and buckwheat (Peng *et al*., 2015). When temperature and light were combined, the effects appeared additive on several amino acids, including Gly, Ile, Leu, Pro, and Val, with much larger content in plants grown under higher temperatures with high light in comparison to other factors (Fig. 3.9 – 3.12). However, since this is the first study examining the effects of temperature and light intensity on amino acid content, this needs further exploration.

Chemical application resulted in a variety of effects on methane, ethylene, and biochemical parameters, but had few effects on growth and physiology. Kinetin increased eight out of 13 amino acids, whereas silver nitrate increased only four and had no effect on seven of the measured amino acids. A previous study on *Xanthium* leaves showed that kinetin increased synthesis of amino acids, but they were not studied individually (Osborne,

1962). Methyl jasmonate, another ethylene promoter, has also been shown to increase amino acid content in plants such as grapevine (*Vitis vinifera* L.) (Garde-Cerdán *et al.*, 2016), but no prior studies have been found that examined the effects of silver nitrate application on amino acid metabolism.

In conclusion, this study showed a great influence of light intensity on emissions, growth and physiology, and plant biochemistry, and temperature had a lesser influence. This study represents the first experimental evidence that a number of amino acids may be involved in methane production. While methionine was the only amino acid that was positively correlated to methane and ethylene, five other amino acids (Gly, Ile, Lys, Pro, and Val) were negatively correlated with methane. Since the methionine pathway has a method for carbon recycling, with methyl groups being used for a variety of downstream purposes, this free methyl group may be released as methane under periods of stress. While this would confirm earlier results on the relationship between methionine and methane (Lenhart *et al.*, 2015), it would also indicate a more complicated relationship between methane and amino acids, and suggest that methyl groups from other amino acids may lead to methane production as well. The negative correlation with the other amino acids suggests that methyl groups may be cleaved from these compounds, decreasing the amino acid content and increasing release of methane, which would also agree with studies that suggest the relationship between reactive oxygen species (ROS) and cleavage of methyl groups (McLeod *et al.*, 2008; Wang *et al.*, 2011b; Wishkerman *et al.*, 2011). Additionally, the positive correlation between ethylene and methane indicates that there may be a relationship between the two compounds, but since correlation does not equal causation,

this requires further examination. This could be experimentally determined by using a stable isotope of ethylene.

Light is essential to plant growth, and is often manipulated for maximum plant growth and agricultural yield (Gautam *et al*., 2015), and the implications of this study are therefore important. Since higher light intensity decreased emissions, and light intensity is greatest at the equator (Fu, 2003), with the sun's rays perpendicular to the Earth's surface, plant migration away from the equator and towards the poles (Jaakola and Hohtola, 2010; Qaderi *et al*., 2015) may decrease the light intensity to which plants are exposed, thereby increasing emissions. Furthermore, since methane is a potent greenhouse gas that has a higher warming potential than CO_2, a mechanistic understanding of the method of methane production is a priority. While aerobic methane emissions from plants may account for only a small portion of the global methane budget, a thorough understanding of this phenomenon will allow for both a more accurate estimation of the global methane budget and an examination of potential mitigation methods.

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4.0. EFFECTS OF BLUE LIGHT ON METHANE EMISSIONS FROM PLANTS

4.1. INTRODUCTION

Light is one of the most important environmental factors that determine plant growth and success (Estell *et al*., 2016; Fazal *et al*., 2016; Huché-Thélier *et al*., 2016); light is crucial not only for photosynthesis, but also for fine-tuned regulation of plant development (Huché-Thélier *et al*., 2016). Light includes three different aspects – light quality, quantity, and duration, each of which has a number of effects on plant growth, development and biochemistry, including biomass accumulation and the biosynthesis of primary and secondary metabolites (Ahmad *et al*., 2016). Even small changes in light quality can influence both physiological and biochemical parameters, and thus also have a significant influence on plant morphology and function (Zheng & van Labeke, 2017).

From incoming light, plants absorb approximately 90% of red and blue light, making them sensitive to any alteration in light environment (Darko *et al*., 2014; Lee *et al*., 2014). All plants have photoreceptors, including phytochromes for red (R) and far-red (FR) light along with croptochromes and phototropins for blue light (Hogewoning *et al*., 2010), which can detect aspects of light through a process called photomorphogenesis (Kopsell *et al*., 2015). Blue light is a high-energy wavelength that stimulates a number of plant responses (Jeong *et al*., 2014). Along with ultraviolet and red light, blue light is involved in photomorphogenic responses, including seedling development, vegetative growth, flowering, and branching, and a certain level of blue light is required for normal plant development (Huché-Thélier *et al*., 2016). Furthermore, light is a critical component of cellular differentiation, including expansion of hypocotyls, chloroplast development, leaf

expansion, and initiation of flowering (Hemm *et al*., 2004). While blue-deficient light suppresses plant biomass accumulation, optimal levels can stimulate greater mass due to increased photosynthetic efficiency (Johkan *et al*., 2010), but many responses to blue light act in species-, and even cultivar-dependent manners (Son & Oh, 2013; Estell *et al*., 2016).

Blue light can result in a variety of morphological effects. In high or absolute amounts, blue light decreases growth rate as well as both fresh and dry mass in tomato (Hernández & Kabuta, 2016), cucumber (Hernández & Kabuta, 2016), and buckwheat (Lee *et al*., 2014); however, light containing optimal irradiance of blue light leads to increased biomass in cucumber (Hogewoning *et al*., 2010) and tomato (Ma *et al*., 2014). Optimal levels also led to increased fruit yield and quality in tomato (Ma *et al*., 2014). Contrary to these findings, low levels of blue light decrease biomass in both sunflower and chrysanthemum (Martel & Qaderi, 2017), which supports the species-dependent differences.

At supraoptimal levels blue light stimulates stem elongation and leaf expansion (Hernández & Kabuta, 2016). At optimal levels, however, plant height and length of hypocotyls and epicotyls decrease with increasing blue irradiance in cucumber (Hernández & Kabuta, 2016), buckwheat (Lee *et al*., 2014), grapevine (Poudel *et al*., 2008), chrysanthemum (Jeong *et al*., 2014), lettuce (Son & Oh, 2013), and a number of specialty crops (see Kopsell *et al*., 2015). Decreased stem elongation leads to more compact, nutrient-dense plants, which can be a desirable trait (Huché-Thélier *et al*., 2016). Blue light at any level stimulates leaf expansion through an increased proton efflux on epidermal cells (Ma *et al*., 2010; Hernández & Kabuta, 2016), which results in greater leaf area in plants, such as chrysanthemum (Zheng & van Labeke, 2017). This may also be one of the reasons

why blue light results in increased leaf thickness, through an increase in palisade parenchyma and upper epidermis, leading to a subsequent increase in mesophyll cells and the formation of leaf intercellular spaces (Schuerger *et al*., 1997; Macedo *et al*., 2011). Indeed, leaf thinning due to shade conditions has been partially attributed to a decrease in blue light under these growth conditions (Schuerger *et al*., 1997).

From a biochemical perspective, blue light also induces the accumulation of chlorophyll (Poudel *et al*., 2008; Hogewoning *et al*., 2010; Hernández & Kubota, 2016; Martel & Qaderi 2017), except in absolute concentrations (Hernández & Kubota, 2016), and there is overwhelming evidence in support of the blue light-induced increase in biosynthesis of flavonoids (Jung *et al*., 2013; Son & Oh, 2013; Ouzounis *et al*., 2014; Ouzounis *et al*., 2015; Fazal *et al*., 2016; Huché-Thélier *et al*., 2016; Ouzounis *et al*., 2016; Martel & Qaderi, 2017; Zheng & van Labeke, 2017), carotenoids (Ohashi-Kaneko et al., 2007; Lefsrud et al., 2008; Li and Kubota 2009; Kopsell et al., 2014; Sebastian & Prasad, 2014; Zheng & van Labeke, 2017), anthocyanins (Sebastian & Prasad, 2014), and antioxidants (Johkan *et al*., 2010; Nascimento *et al*., 2013; Son & Oh, 2013). Bioaccumulation of these compounds occurs due to increased expression of related genes (Jung *et al*., 2013) and results in increased tolerance to both biotic and abiotic stress (Ma *et al*., 2014; Hoffmann *et al*., 2015).

The positive effect of blue light on plant metabolism is also commonly observed in the nutritional value. Rice (Sebastian & Prasad, 2014) and broccoli (Kopsell & Sams, 2013) plants grown under blue light had increased essential nutrients, including potassium, sulfur, phosphorous, magnesium, and calcium, along with an increased content of organic acids

and protein thiols in rice (Sebastian & Prasad, 2014). Blue light leads to increased primary metabolism, including increased content of sugars as well as amino acids and organic acids (Florez-Sarasa *et al*., 2012; Jänkänpää *et al*., 2012; Jung *et al*., 2013). In a study on tomato, 14 free amino acids (Ala, Thr, Ser, Gly, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, Arg, and Pro) increased in plants treated with blue light, whereas glutamic and aspartic acids were unaffected. GABA, a non-protein amino acid derived from glutamic acid, also increased in response to blue light (Dhakal & Baek, 2014). Similarly, increases in all measured amino acids were found in rice (Jung *et al*., 2013), cucumber, broccoli, basil, tomato (Huché-Thélier *et al*., 2016), and chrysanthemum, with a drastic increase in proline (Zheng & van Labeke, 2017). Blue light is also known to increase total protein content (Voskresenskaya, 1972; Sebastian & Prasad, 2014).

As discussed in previous chapters, aerobic methane emissions from plants are a small but significant contributor to the global methane budget (Keppler *et al*., 2006), though the extent of this remains under debate. Methane emissions are influenced by environmental factors (McLeod *et al*., 2008; Vigano *et al*., 2008; Bruhn *et al*., 2009; Qaderi and Reid, 2009; Wang *et al*., 2009; Qaderi & Reid, 2011; Wang *et al*., 2011a, b; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al*., 2017; Martel & Qaderi, 2017), including visible light (Martel & Qaderi, 2017). Studies have shown that L-methionine (Met), an important amino acid, can act as a precursor of methane from plants (Lenhart *et al*., 2015), along with other precursors including pectin (Keppler *et al*., 2008; Bruhn *et al*., 2009). While the stable isotope study has confidently shown that Met plays a role in aerobic emissions, authors acknowledge this isn’t direct evidence that Met is a biological precursor, and it is possible

that it may simply get oxidized in the presence of ROS, resulting in methyl group liberation (Lenhart *et al*., 2015). It is therefore important to examine amino acids beyond L-Met to determine relationships between all methylated amino acids and methane emissions. Additionally, amino acids have effects on taste, and can have sweetness, bitterness, sourness, or saltiness, and can therefore affect the taste of food crops, including canola, giving a practical application to this study (Dhakal & Baek, 2014).

While it was originally hypothesized that visible light may play no role in methane emissions (Bruhn *et al*., 2014), recent work has suggested that blue light resulted in increased methane release from sunflower and chrysanthemum (Martel & Qaderi, 2017); however, this is the only published study thus far to examine visible light and light quality and their relationship to aerobic methane emissions. Additionally, while the effects of light intensity have received a significant amount of attention, research on the effects of light quality on plant physiology and biochemistry are less numerous (Hernández & Kubota, 2016).

Blue light was chosen for this study for a number of reasons. First, since plants absorb 90% of incoming blue light (Darko *et al*., 2014; Lee *et al*., 2014), small changes in blue light levels induce observable effects. Second, blue light is a high-energy wavelength that causes observable changes in the content of amino acids (Jung *et al*., 2013). Third, approximately half of sunlight is composed of visible light, making visible light important for plant development (Fu, 2003). Finally, blue light has been shown to induce methane emissions (Martel & Qaderi, 2017). The purposes of this study were to (i) observe the effects of blue light, at several different amounts, on aerobic methane emissions from

canola; (ii) determine whether blue light promoted observable responses of canola plants to application of ethylene promoter and inhibitor; (iii) quantify a number of amino acids to assess their potential as precursors of methane emissions from plants, and (iv) examine the effects of blue light on amino acid content of canola. It was hypothesized that, based on previous research, blue light would promote higher aerobic methane emissions and greater amino acid content. Additionally, it was hypothesized that, based on the results presented in this thesis thus far, that methane emissions would be related to multiple amino acids and that methane release would be the result of methyl group cleavage.

4.2. METHODS

4.2.1. Plant material and growth conditions

Seeds of canola (6056 CR, BrettYoung Seeds, Winnipeg, Manitoba) were germinated and grown as described in Chapter 3, section 3.2.1 (see page 81). Plants were left to acclimate for seven days until the emergence of the first set of true leaves, and were then randomly assigned to one of the four experimental conditions; control, with no supplemental blue light; low blue, with 0.4 μmol photons m^{-2} s^{-1} of supplemental blue; medium blue, with 4.0 μmol photons m^{-2} s^{-1} of supplemental blue light; or high blue, with 8.0 μmol photons m^{-2} s^{-1} of supplemental blue light. These levels of blue light were chosen because they represented the minimum, middle, and maximum levels of blue light that could be obtained using the experimental diodes. Blue light was supplied using light-emitting diodes (LED) units (SnapLite, model APS-2509, Quantum Devices, Barneveld, WI), with a peak wavelength at 450 nm. Within each chamber, three trays were placed, and plants were assigned to one of three chemical treatments as described in Chapter 3, section 3.2.1 (see

page 81; plants received either the control chemical treatment, with no application, application of an ethylene promoter (Kinetin, 10^{-4} M), or an ethylene inhibitor (10^{-4} M silver nitrate), with 100 µl of the chemical solution applied every other day. There was a total of 12 experimental conditions, with plants grown for 21 days under treatments with bi-weekly pot rotation to reduce positional effects. The experiment was replicated in full three times to ensure repeatability, with reversal of chambers each time.

4.2.2. Measurement of methane and ethylene emissions

Methane and ethylene emissions were measured as described in Chapter 2, section 2.2.2 (see page 38).

4.2.3. Plant growth and dry mass accumulation

Plant growth and biomass accumulation were determined, and from these growth indices were calculated, as described in Chapter 2, section 2.2.3 (see page 39).

4.2.4. Gas exchange

Gas exchange parameters were measured as described in chapter 3, section 3.2.4 (see page 82). The only difference in this method was that the light emitted from the diode was set to 300 µmol photon m^{-2} s^{-1} and was not changed, since only this light intensity was used for this experiment.

4.2.5. Chlorophyll fluorescence

Chlorophyll fluorescence was measured as described in Chapter 2, section 2.2.4 (see page 39).

4.2.6. Photosynthetic pigments

Photosynthetic pigments were determined as described in Chapter 2, section 2.2.5 (see page 40).

4.2.7. Moisture content

Moisture content of leaves were calculated as described in Chapter 2, section 2.2.6 (see page 40).

4.2.8. Epicuticular wax

Leaf surface waxes were determined as described in Chapter 3, section 3.2.8 (see page 83).

4.2.9. Flavonoids and nitrogen balance index

Flavonoids and nitrogen balance index (NBI) were measured as described in Chapter 2, section 2.2.7 (see page 41).

4.2.10. Metabolite profiling

Metabolite profiling was conducted as described in Chapter 3, section 3.2.10 (see page 84).

4.2.11. Data analysis

The overall effects of blue light intensity and chemical application, along with their interactions, were determined on the growth and physiological characteristics of canola grown under four different levels of blue light (control, 0 µmol photons m^{-2} s^{-1}; low, 0.4 µmol photons m^{-2} s^{-1}; medium, 4.0 µmol photons m^{-2} s^{-1}; or high, 8.0 µmol photons m^{-2} s^{-1}), and applied with either no chemical, with an ethylene promoter (kinetin, 10^{-4} M), or an

ethylene inhibitor (silver nitrate, 10^{-4} M), using ANOVA for split-plot design (SAS Institute, 2011). For this analysis, blue light intensity was treated as the main plot, and chemical treatment as the subplot, and growth chambers and trials as replications (Hinkelman and Kempthorne, 2008). A one-way ANOVA using Scheffé's multiple-comparison procedure at the 5% confidence level was used to determine the difference among treatments. Furthermore, Pearson's correlation coefficient at the 5% confidence interval was used to determine the relationships between parameters.

4.3. RESULTS

4.3.1. Methane and ethylene emissions

Ethylene emissions were significantly influenced by chemical application. Ethylene emissions were significantly decreased by both high levels of blue light and silver nitrate application, but not by low or medium levels of blue light, and this was not significant on the basis of one-way ANOVA (Table 4.1; Fig. 4.1). Methane, on the other hand, was significantly affected by blue light, but not by chemical application, with emissions increasing steadily with increasing blue light (Table 4.1; Fig. 4.1; see Appendix B1). Interactions between light and chemical application were not significant for ethylene or methane.

Table 4.1. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on methane and ethylene emissions of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Ethylene	Methane
Blue light (L)	3	2.7↓	27.1***↑
Main plot error	8	-	-
Chemical (C)	2	8.7** – ↓	0.5
L x C	6	1.3	2.9*
Subplot error	16	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

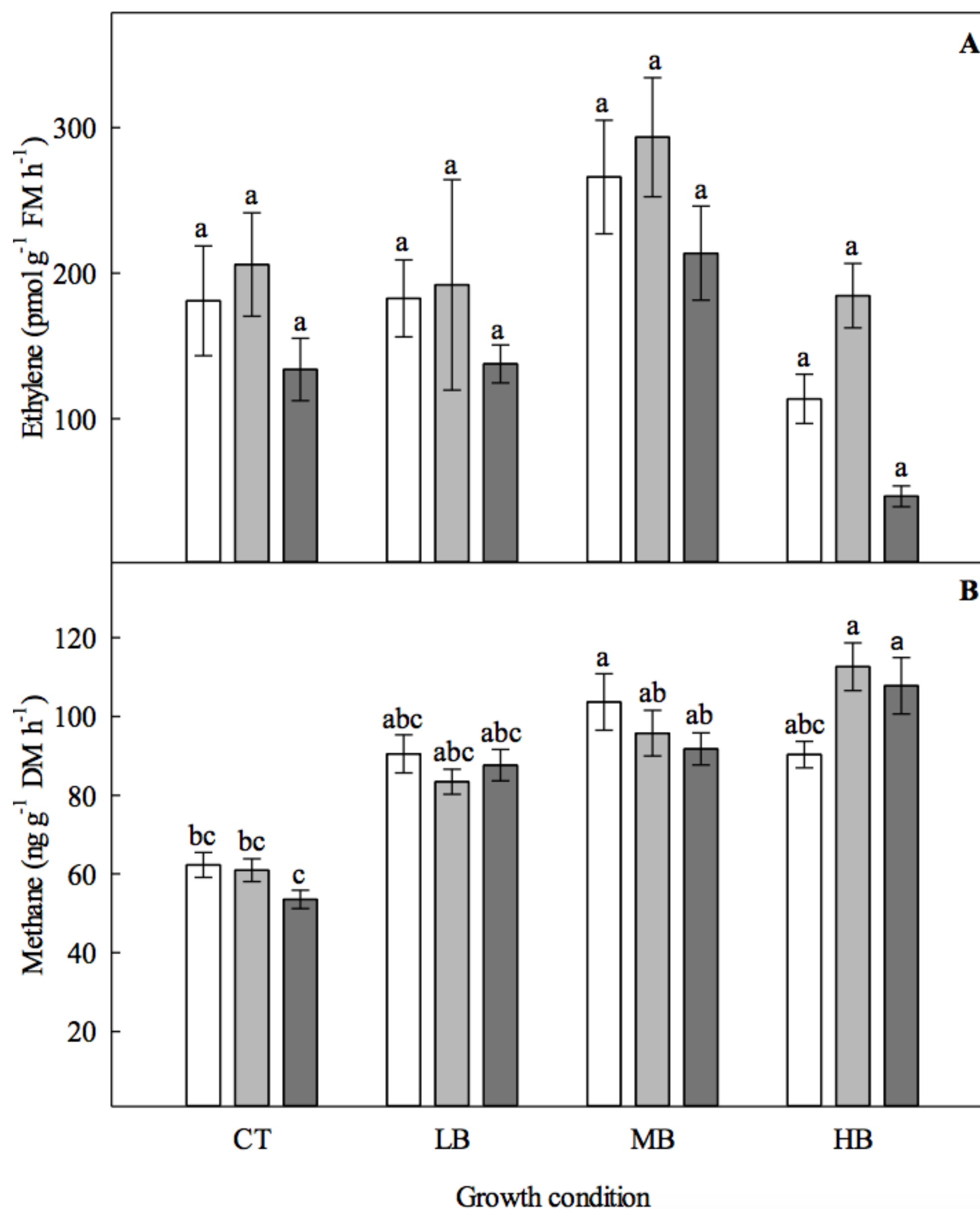

Fig. 4.1. Ethylene and methane emissions from canola plants grown under four different levels of blue light (CT, control; LB, low blue, 0.4 µmol photons m^{-2} s^{-1}; MB, medium blue, 4.0 µmol photons m^{-2} s^{-1}; HB, high blue, 8.0 µmol photons m^{-2} s^{-1}) and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) ethylene emissions; (B) methane emissions. Data are means ± SE (n = 3), and bars surmounted by different letters are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison test.

4.3.2. Growth and biomass accumulation

All aspects of plant growth were significantly affected by blue light, but not by chemical application. Stem height was increased by low and medium levels of blue light, but high levels of blue light yielded similar heights to control plants; however, this was not significant according to the one-way ANOVA (Fig. 4.2A). Stem diameter and leaf area were both decreased by increased blue light (Table 4.2; Fig. 4.2). Similarly, all aspects of plant biomass were significantly decreased by blue light, but only root mass was mildly affected by chemical application (Table 4.3; Fig. 4.3; see Appendix B1).

Growth indices including leaf mass per area (LMA), leaf mass ratio (LMR), and leaf area ratio (LAR) were significantly affected by blue light, and LMA, LMR, and S:R ratio were affected by chemical application (see Appendix B2). Similarly, LMA and LAR were significantly affected by the interactions between factors (Table 4.4). Low and medium levels of blue light decreased LMA but increased LAR. Application of silver nitrate significantly increased LMR (Table 4.4), but this was not significant on the basis of the one-way ANOVA (Fig. 4.4). On the basis of interaction between blue light and chemical application, plants that were grown under high levels of blue light with no chemical application had the highest LMR, and plants grown under low or medium blue light, irrespective of chemical treatment, had the lowest LMR. Conversely, plants grown under low or medium blue light, irrespective of chemical treatment, had the highest LAR, and plants grown under either no blue light or high blue light without chemical treatment had the lowest LAR (Fig. 4.4).

Table 4.2. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on growth and development of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Stem height	Leaf number	Leaf area	Stem diam.
Blue light (L)	3	1.1	5.0*–	21.3***↓	56.1****↓
Main plot error	8	-	-	-	-
Chemical (C)	2	1.5	0.8	2.1	1.9
L x C	6	9.3***	2.3	1.5	2.1
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

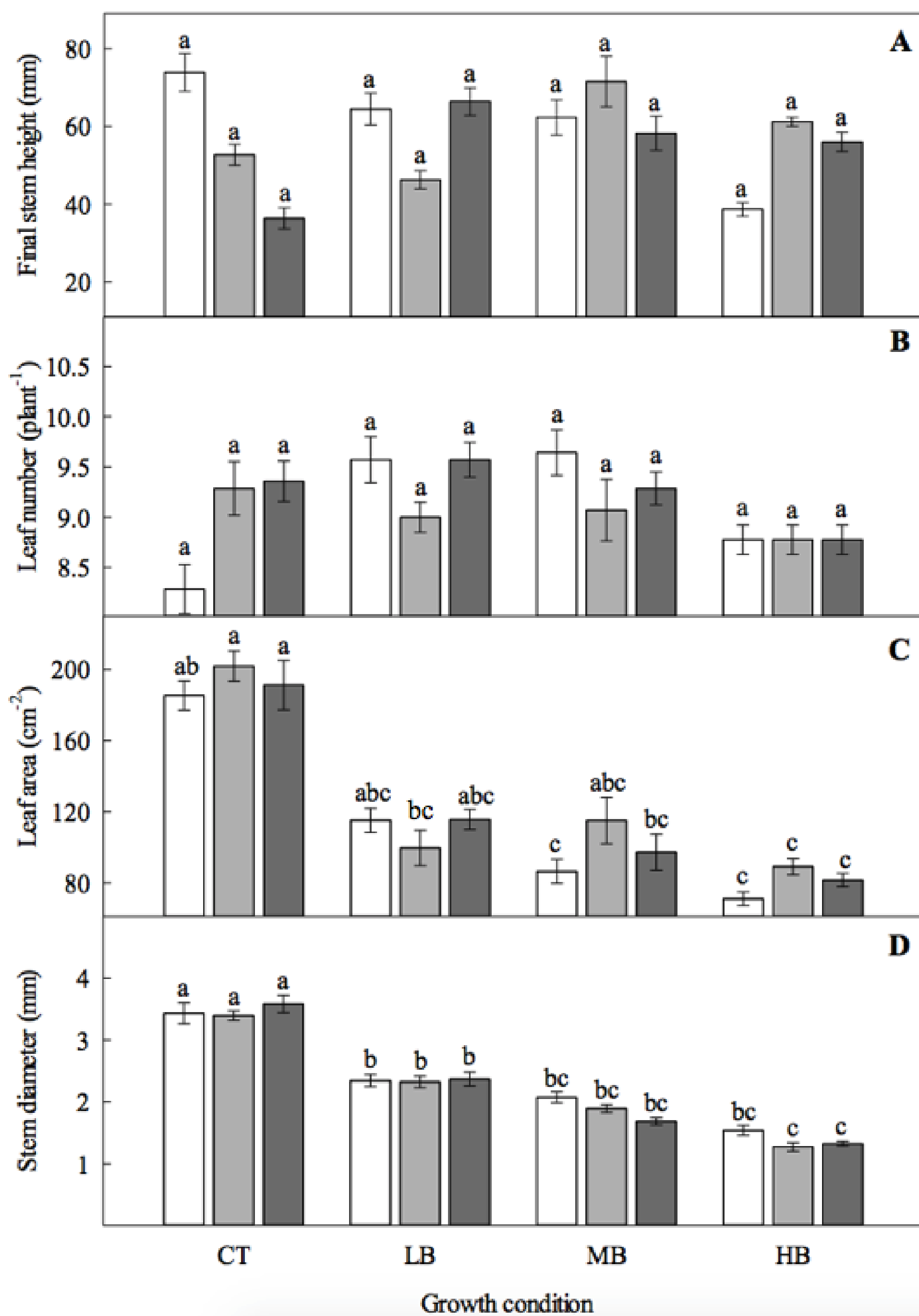

Fig. 4.2. Growth and development of canola plants grown under four blue light levels, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) final stem height; (B) leaf number; (C) leaf area; (D) stem diameter. Otherwise, as per Fig. 4.1.

Table 4.3. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on biomass accumulation of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Leaf mass	Stem mass	Root mass	Total mass
Blue light (L)	3	48.8****↓	22.7***↓	24.9***↓	61.9***↓
Main plot error	8	-	-	-	-
Chemical (C)	2	0.4	2.5	4.3*– –	0.3
L x C	6	2.5	2.1	2.3	2.3
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

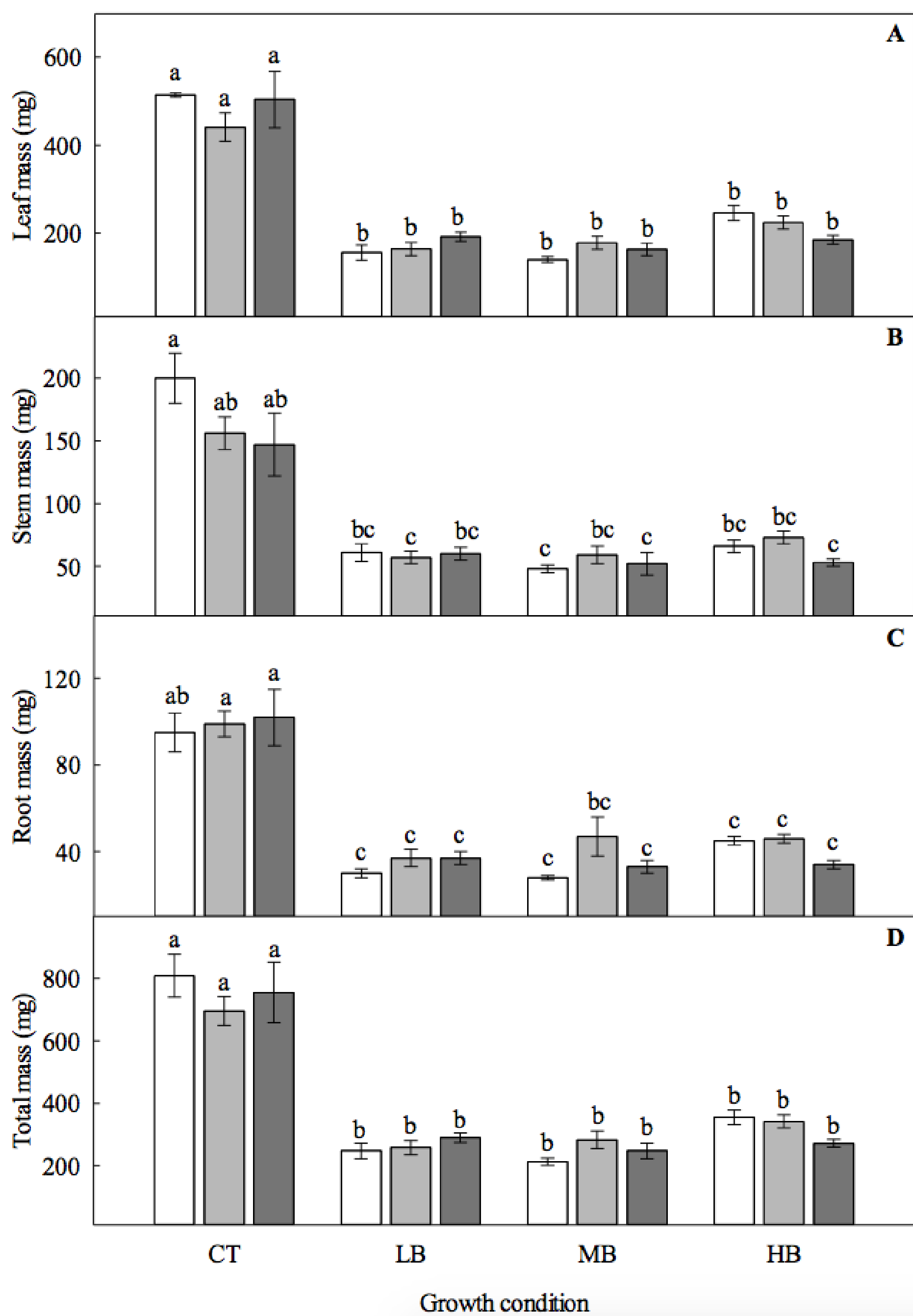

Fig. 4.3. Biomass accumulation of canola plants grown under four blue light levels, and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) leaf mass; (B) stem mass; (C) root mass; (D) total mass. Otherwise, as per Fig. 4.1.

Table 4.4. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on growth indices of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	LMA	LMR	LAR	S:R mass ratio
Blue light intensity (L)	3	9.7**↓	2.0	8.5**↑	0.5
Main plot error	8	-	-	-	-
Chemical (C)	2	6.6**– –	5.6*–↑	0.2	4.4*– –
L x C	6	10.2***	1.2	4.4**	1.3
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

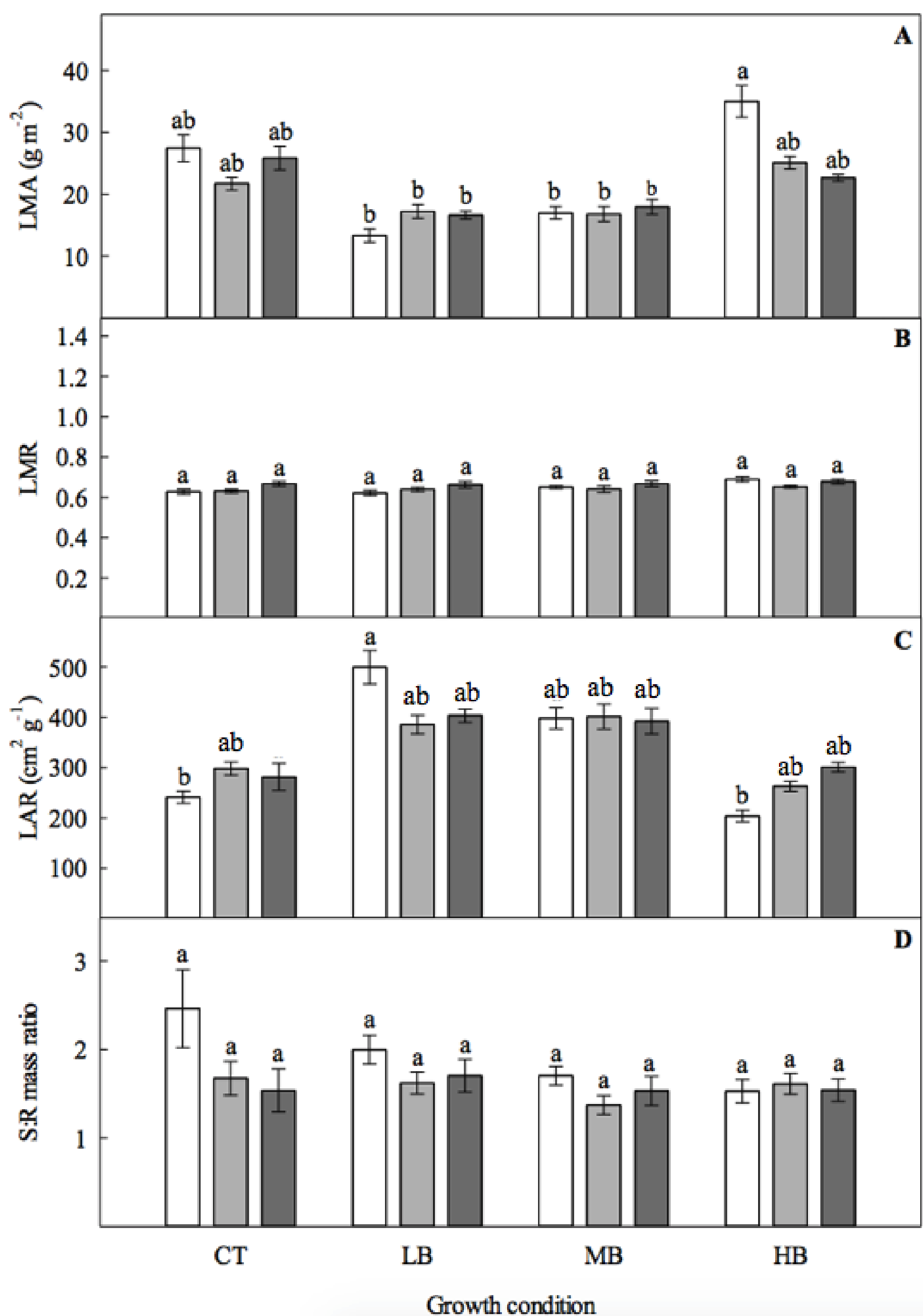

Fig. 4.4. Growth indices of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) LMA; (B) LMR; (C) LAR; (D) S:R mass ratio. Otherwise, as per Fig. 4.1.

4.3.3. Gas exchange

All aspects of gas exchange were significantly decreased by blue light and were affected the interaction between light and chemical application, but only net CO_2 assimilation (A_N) and stomatal conductance (g_s) were significantly affected by chemical application (Table 4.5; see Appendix B2). Both kinetin and silver nitrate decreased g_s, whereas only silver nitrate decreased A_N (Table 4.5). On the basis of the interactions, lowest gas exchange values were found under high blue light with either chemical application, and the highest values under the control condition (Fig. 4.5).

Table 4.5. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on gas exchange of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	A_N	*E*	g_s	WUE
Blue light (L)	3	141.1****↓	7.8**↓	41.8****↓	49.6****↓
Main plot error	8	-	-	-	-
Chemical (C)	2	12.9***–↓	1.8	8.7**– –	0.8
L x C	6	20.8****	12.6****	6.7**	3.2*
Subplot error	16	-	-	-	-

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

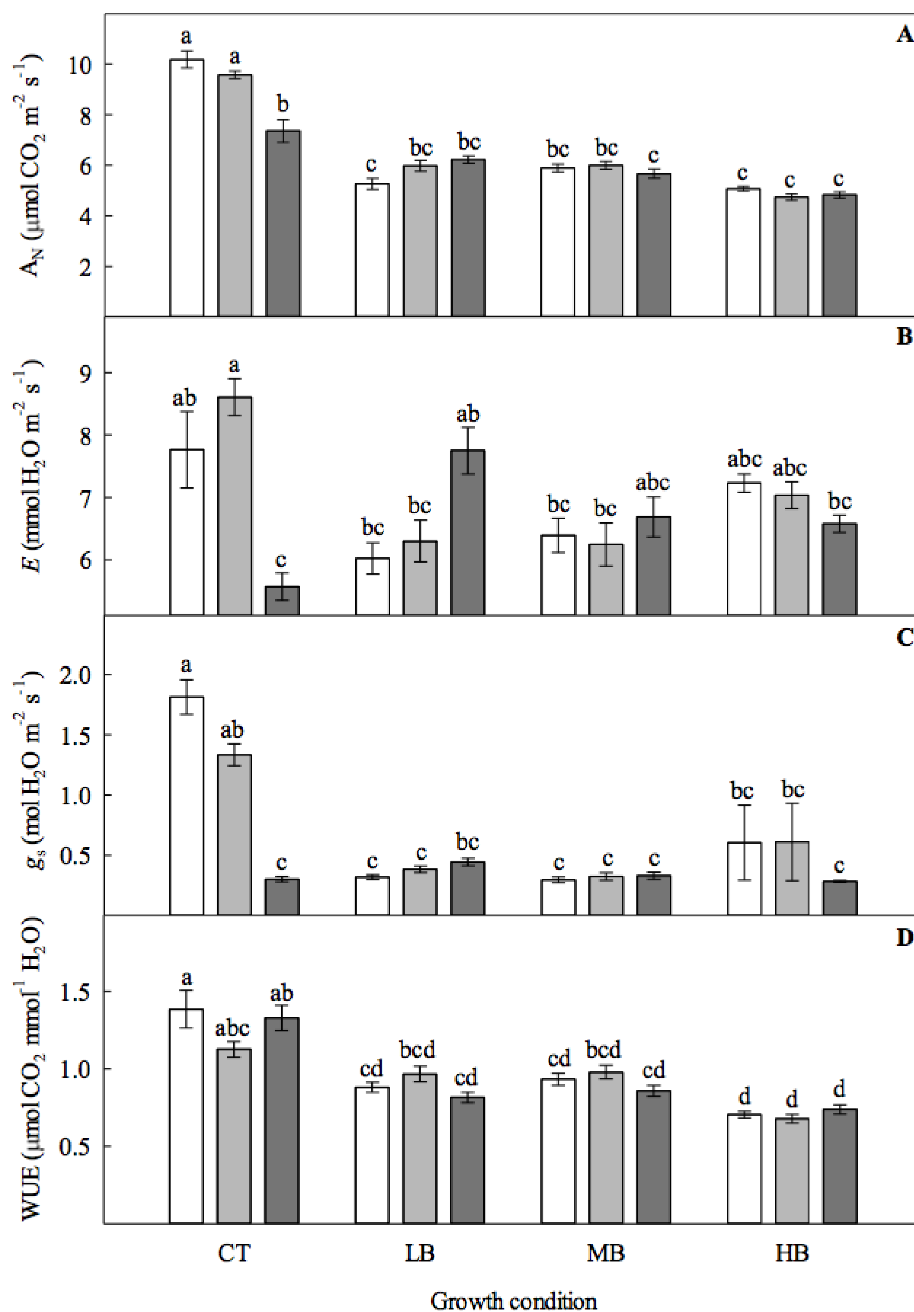

Fig. 4.5. Gas exchange of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) A_N, net CO_2 assimilation; (B) E, transpiration; (C) g_s, stomatal conductance; (D) WUE, water use efficiency. Otherwise, as per Fig. 4.1.

4.3.4. Chlorophyll fluorescence

Both the effective quantum yield of PSII (ϕPSII) and non-photochemical quenching (qNP) were significantly increased by blue light (Table 4.6; see Appendix B3) but unaffected by chemical application, and the other fluorescence parameters were unaffected by experimental conditions in split-plot analysis (Table 4.6). Both maximum quantum yield of PSII (F_v/F_m) and photochemical quenching (qP) had no significant differences according to the one-way ANOVA (Fig. 4.6).

Table 4.6. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on chlorophyll fluorescence of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	ϕPSII	F_v/F_m	qNP	qP
Blue light (L)	3	24.3***↑	1.0	8.5***↑	0.8
Main plot error	8	-	-	-	-
Chemical (C)	2	0.8	1.0	0.1	0.9
L x C	6	0.9	1.0	1.2	1.0
Subplot error	16	-	-	-	-

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

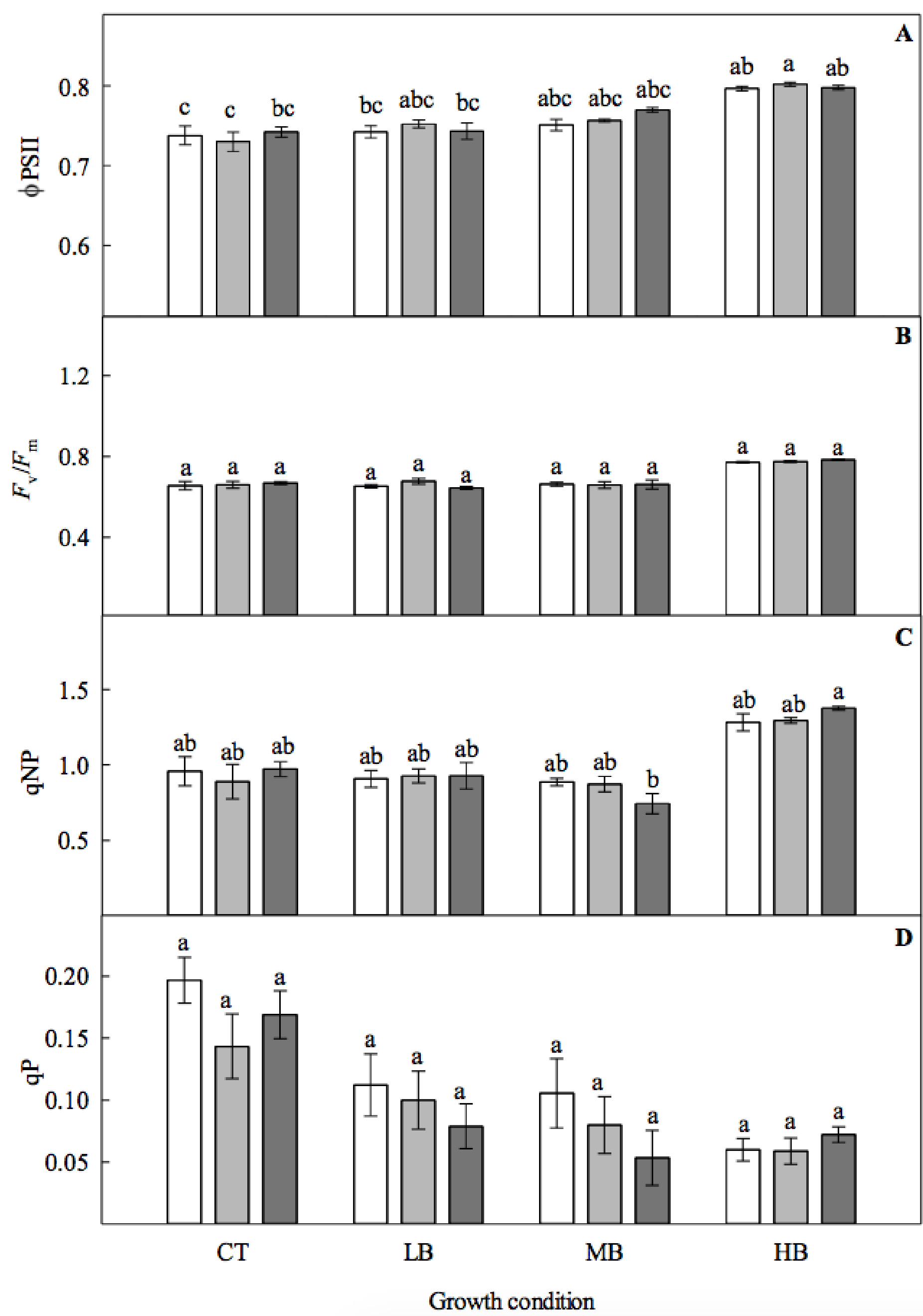

Fig. 4.6. Chlorophyll fluorescence of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray), or ethylene inhibitor (silver nitrate, medium gray bars). (A) ϕPSII, effective quantum yield of PSII; (B) F_v/F_m, maximum quantum yield of PSII; (C) qNP, non-photochemical quenching; (D) qP, photochemical quenching. Otherwise, as per Fig. 4.1.

4.3.5. Photosynthetic pigments

All photosynthetic pigments were significantly affected by both blue light and chemical application (Table 4.7; see Appendix B3). Chlorophyll (Chl) *a*, Chl *b*, total Chl, and carotenoids were significantly increased by blue light, but plants grown under different levels of blue light (low, medium, or high blue) did not differ from one another (Table 4.7; Fig. 4.7). Silver nitrate application increased Chl *a,* Chl *b*, total Chl, and carotenoid content, whereas kinetin decreased Chl *a* and Chl *b* in reference to plants treated with silver nitrate. Plants treated with kinetin did not differ from control plants (Table 4.7). The Chl *a*/*b* ratio, on the other hand, decreased with increasing blue light (Table 4.7).

Table 4.7. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on photosynthetic pigments of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*	Carotenoids
Blue light (L)	3	8.2***↑	22.4***↑	11.1***↑	13.2***↓	2.9
Main plot error	8	-	-	-	-	-
Chemical (C)	2	18.7****↓↑	7.6**–↑	16.5***–↑	6.0*↓–	8.4**– –
L x C	6	2.3	1.2	2.0	3.0*	2.3
Subplot error	16	-	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

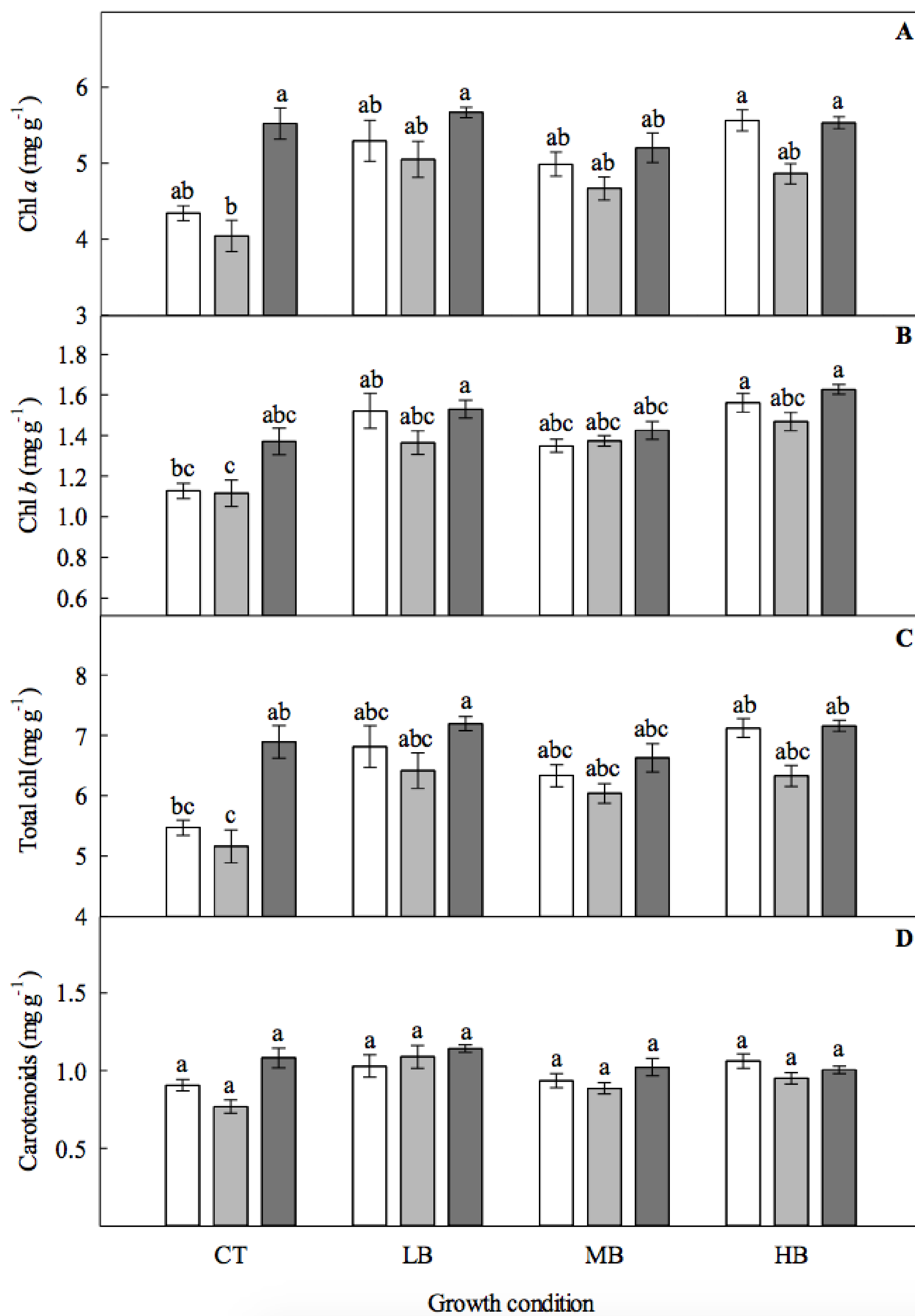

Fig. 4.7. Photosynthetic pigments of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) chlorophyll (Chl) *a*; (B) Chl *b*; (C) total Chl; (D) carotenoids. Otherwise, as per Fig. 4.1.

4.3.6. Moisture content

Moisture content was unaffected by blue light or chemical application, but was significantly affected by the interaction between the two. The highest moisture content was found in plants grown under medium blue light with no chemical application, and the lowest in plants grown under either medium or high blue light with silver nitrate application (Table 4.8; Fig. 4.8A).

4.3.7. Epicuticular wax

Epicuticular wax was significantly affected by blue light, chemical application, and the interaction between the two. Wax was drastically increased under high blue light, and was also increased by both chemical applications (Table 4.8; see Appendix B4). Wax content was highest in plants grown under high blue light with silver nitrate application and lowest in control plants with silver nitrate application (Fig. 4.8C).

4.3.8. Nitrogen balance index and flavonoids

Nitrogen balance index (NBI) and flavonoid content were significantly influenced by both blue light and the interaction between blue light and chemical application, but were unaffected by chemical application alone (Table 4.8). NBI was significantly increased by blue light whereas flavonoid content was significantly reduced by blue light treatment (Table 4.8; see Appendix B5). On the basis of interaction, NBI was lowest in plants grown under control lighting with either chemical application, and was highest in plants grown under high blue light with either chemical application. The highest flavonoid content was found in plants grown under control lighting with silver nitrate application, and the lowest

under medium blue light with no chemical application (Fig. 4.8 B, C).

Table 4.8. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on moisture content, epicuticular wax, nitrogen balance index, and flavonoids in (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Moisture	Wax	NBI	Flavonoids
Blue light (L)	3	1.2	112.7****↑	25.7***↑	23.9**↓
Main plot error	8	-	-	-	-
Chemical (C)	2	1.6	18.4****↑–	1.9	0.5
L x C	6	5.6**	12.5****	8.3***	5.2**
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

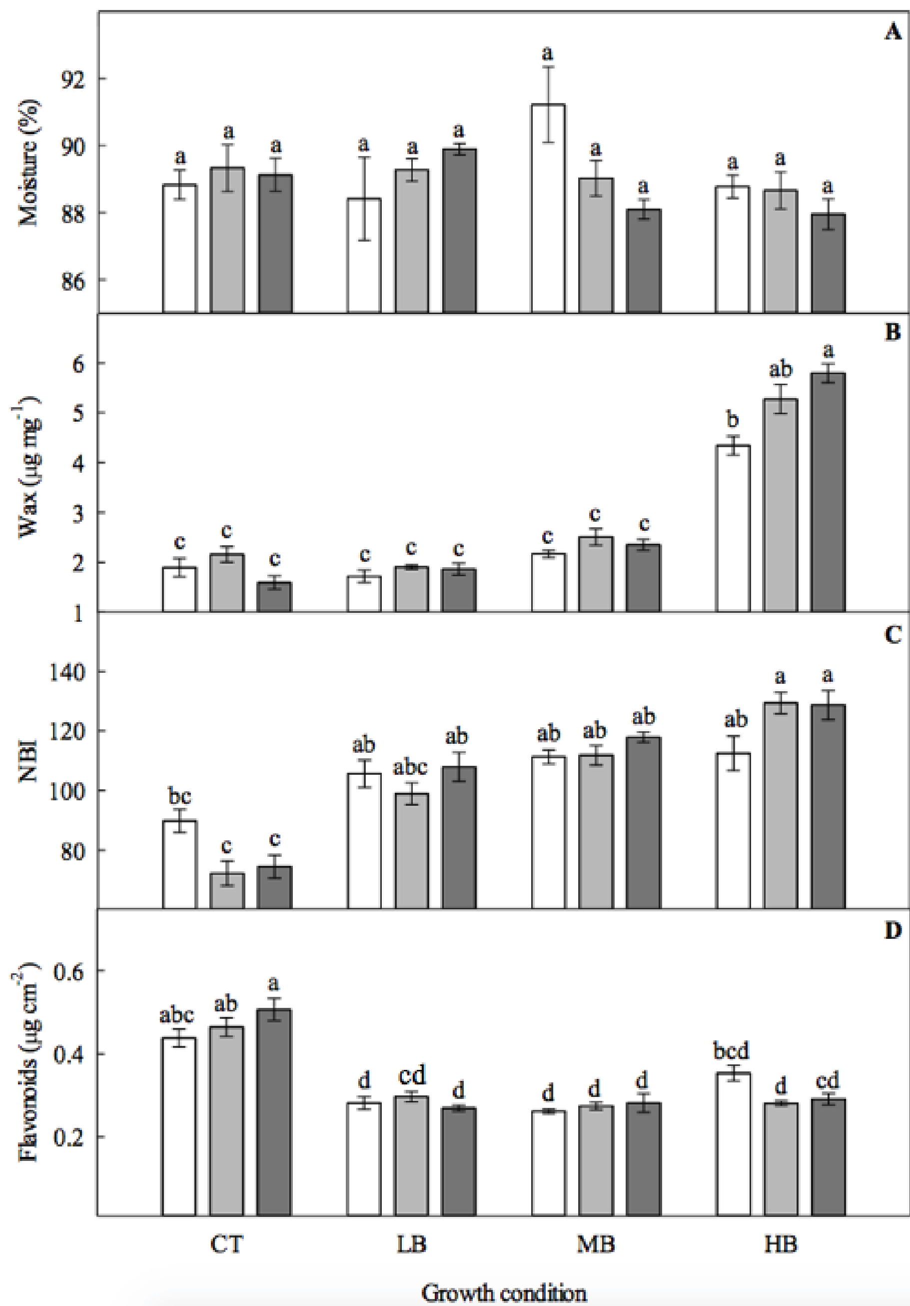

Fig. 4.8. Moisture content, epicuticular wax, nitrogen balance index, and flavonoids of canola plants grown under four blue light levels and applied with either no chemical (control, white), an ethylene promoter (kinetin, light gray), or ethylene inhibitor (silver nitrate, medium gray). (A) moisture content; (B) epicuticular wax; (C) NBI, nitrogen balance index; (D) flavonoids. Otherwise, as per Fig. 4.1.

4.3.9. Metabolite profiling

Apart from phenylalanine (Phe), all amino acids were significantly affected by blue light (Tables 4.9 – 4.11; see Appendix B5). Only six out of 13 amino acids, including alanine (Ala), leucine (Leu), lysine (Lys), methionine (Met), tyrosine (Tyr), and valine (Val), were significantly affected by chemical application, and eight out of the 13, including Ala, glycine (Gly), Leu, Lys, Met, threonine (Thr), and Val, were affected by the interaction between blue light and chemical application (Tables 4.9 – 4.11).

Ala was significantly decreased by low blue but increased by medium blue, with high blue plants comparable to the control; aspartic acid (Asp) was significantly increased by all levels of blue light; Gly was also increased by all levels of blue light, but high levels of blue light reduced content in comparison to low and medium levels; isoleucine (Ile), Thr, and Val were significantly increased by high blue only; Leu was increased by all levels of blue light, with high blue having a higher content than low or medium blue; Lys was decreased by all levels of blue light, but to a greater extent by medium and high blue; Met was increased by all levels of blue light, but drastically so by medium blue; proline (Pro) was drastically increased by high levels of blue; Ser was increased only by medium and high blue, with plants at high blue having a much greater content than those at medium blue; and Tyr was decreased by all levels of blue light, but more so by high blue (Tables 4.9 – 4.11).

In comparison to the control, kinetin application decreased leucine content, but increased Lys, Tyr, and slightly increased Val. Silver nitrate decreased Ala, and Leu, but increased Tyr and Val (Tables 4.9 – 4.11). On the basis of interactions between factors,

highest content of Ala occurred in plants grown at medium blue with kinetin, and lowest Ala under plants grown at low blue with silver nitrate (Fig. 4.9A). Highest Gly content occurred in plants grown at medium blue with silver nitrate, and lowest Gly content in plants grown without supplemental blue light, irrespective of chemical application (Fig. 4.9C). Highest Leu content occurred in plants grown at high blue, and lowest Leu under controlled lighting, both with no chemical application (Fig. 4.10B). Highest Lys content occurred in plants grown at no supplemental blue with silver nitrate application, and lowest Lys in plants grown at medium blue with kinetin application (Fig. 4.10C). Highest Met content occurred in plants grown at medium blue with silver nitrate application, and lowest Met in plants grown with no supplemental blue light with either no chemical or with kinetin application (Fig. 4.11A). Highest Thr content occurred in plants grown at high blue with silver nitrate application, and lowest Thr in plants grown at medium blue with silver nitrate application (Fig. 4.12B). Highest Tyr content occurred in plants grown at controlled lighting with silver nitrate application, and lowest Tyr in plants grown at high blue with kinetin application (Fig. 4.12C). Highest Val content occurred in plants grown at high blue with silver nitrate application, and lowest Val in plants grown without supplemental blue light and with no chemical application (Fig. 4.12D).

Table 4.9. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on content of amino acids alanine (Ala), aspartic acid (Asp), glycine (Gly), isoleucine (Ile), and leucine (Leu) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control; (±) may increase or decrease depending on the level of blue light.

Source of Variation	df	Ala	Asp	Gly	Ile	Leu
Blue light (L)	3	7.3**±	7.7**↑	111.1****↑	190.8****↑	88.9****↑
Main plot error	8	-	-	-	-	-
Chemical (C)	2	4.0*– –	1.0	1.5	0.6	33.7****↓↓
L x C	6	2.8*	1.2	3.8*	0.8	9.5***
Subplot error	16	-	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Table 4.10. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on content of amino acids lysine (Lys), methionine (Met), phenylalanine (Phe), and proline (Pro) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Lys	Met	Phe	Pro
Blue light (L)	3	499.3****↓	80.7****↑	1.2	73.2****↑
Main plot error	8	-	-	-	-
Chemical (C)	2	16.9****↑	10.3**–↑	0.9	1.6
L x C	6	10.4****	10.5****	2.0	2.3
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Table 4.11. Summary of split-plot ANOVA (*F* value) for effects of blue light and chemical application on content of amino acids serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val) of canola (*Brassica napus*) plants grown under experimental conditions for 21 days. Arrows indicate the direction of the significant effects; for chemical application, the first arrow indicates the effects of the ethylene promoter, kinetin, and the second arrow indicates the effects of the inhibitor, silver nitrate. (↑) increased; (↓) decreased; (–) not significantly different from the control.

Source of Variation	df	Ser	Thr	Tyr	Val
Blue light (L)	3	10.7***↑	14.8***↑	348.7****↓	9.0**↑
Main plot error	8	-	-	-	-
Chemical (C)	2	3.5	2.5	19.1****↑	13.7***– –
L x C	6	2.7	4.6**	7.8***	2.8*
Subplot error	16	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

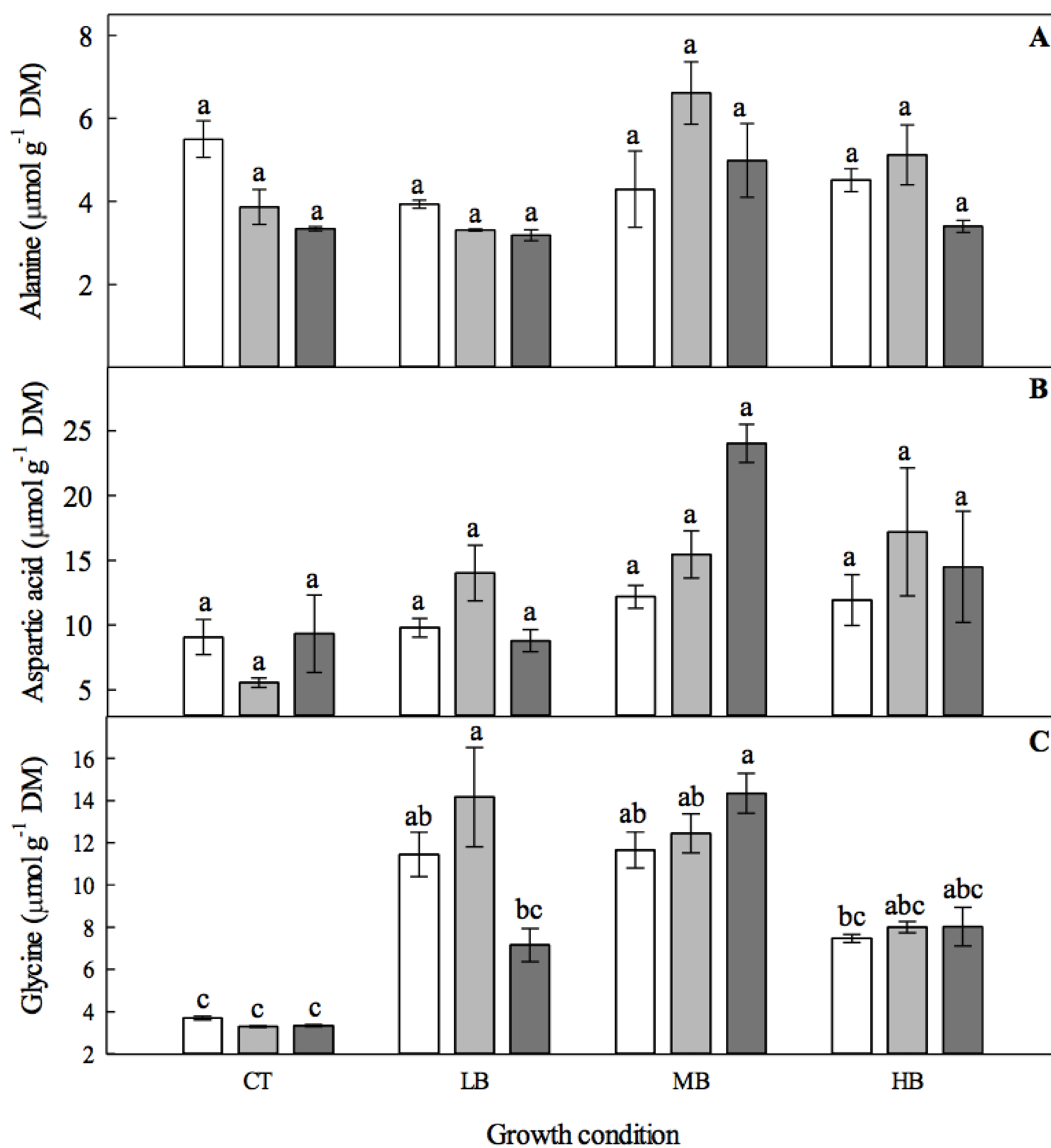

Fig. 4.9. Alanine, aspartic acid, and glycine content of canola plants grown four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) alanine; (B) aspartic acid; (C) glycine. Otherwise, as per Fig. 4.1.

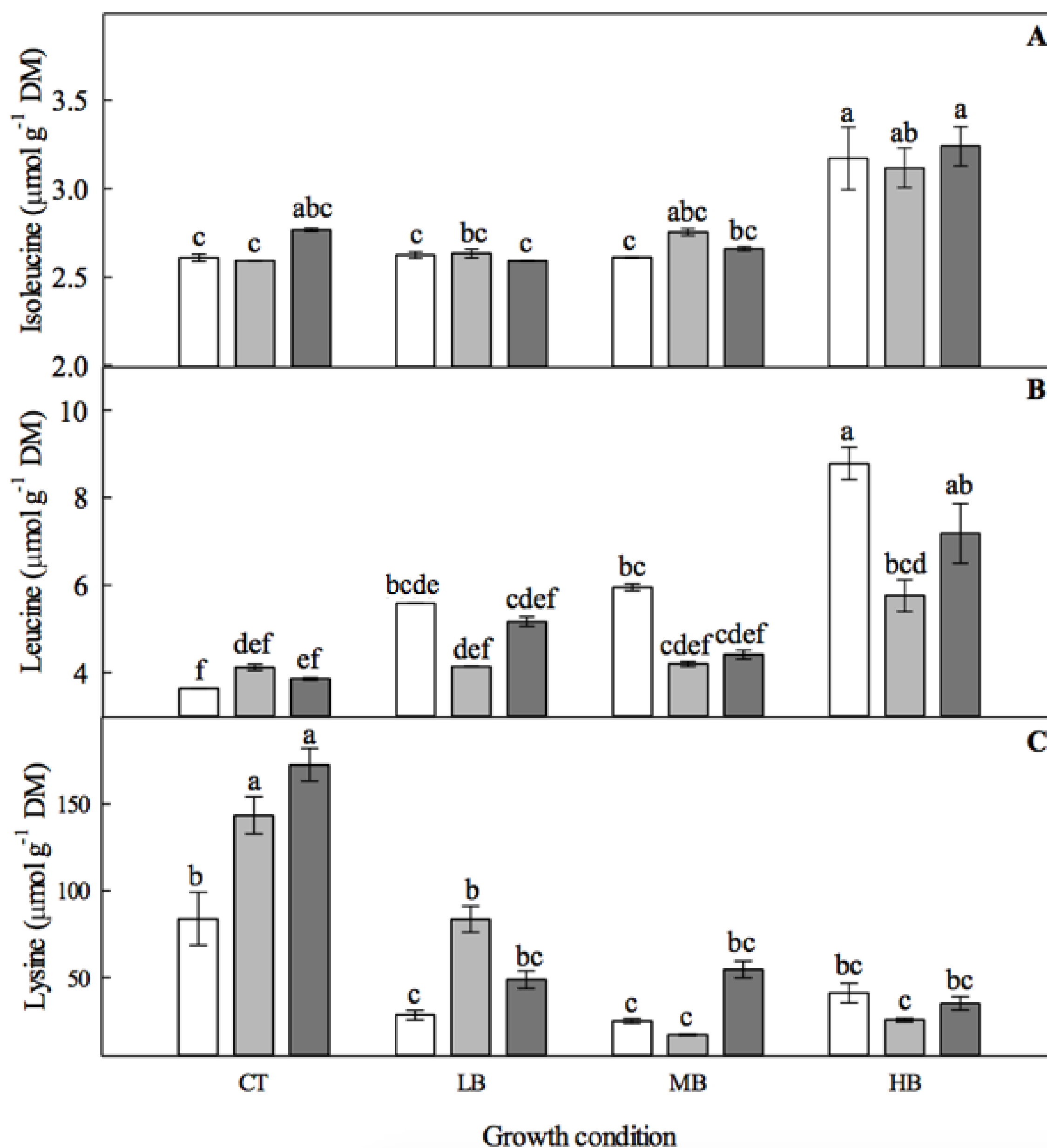

Fig. 4.10. Isoleucine, leucine, and lysine content of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) isoleucine; (B) leucine; (C) lysine. Otherwise, as per Fig. 4.1.

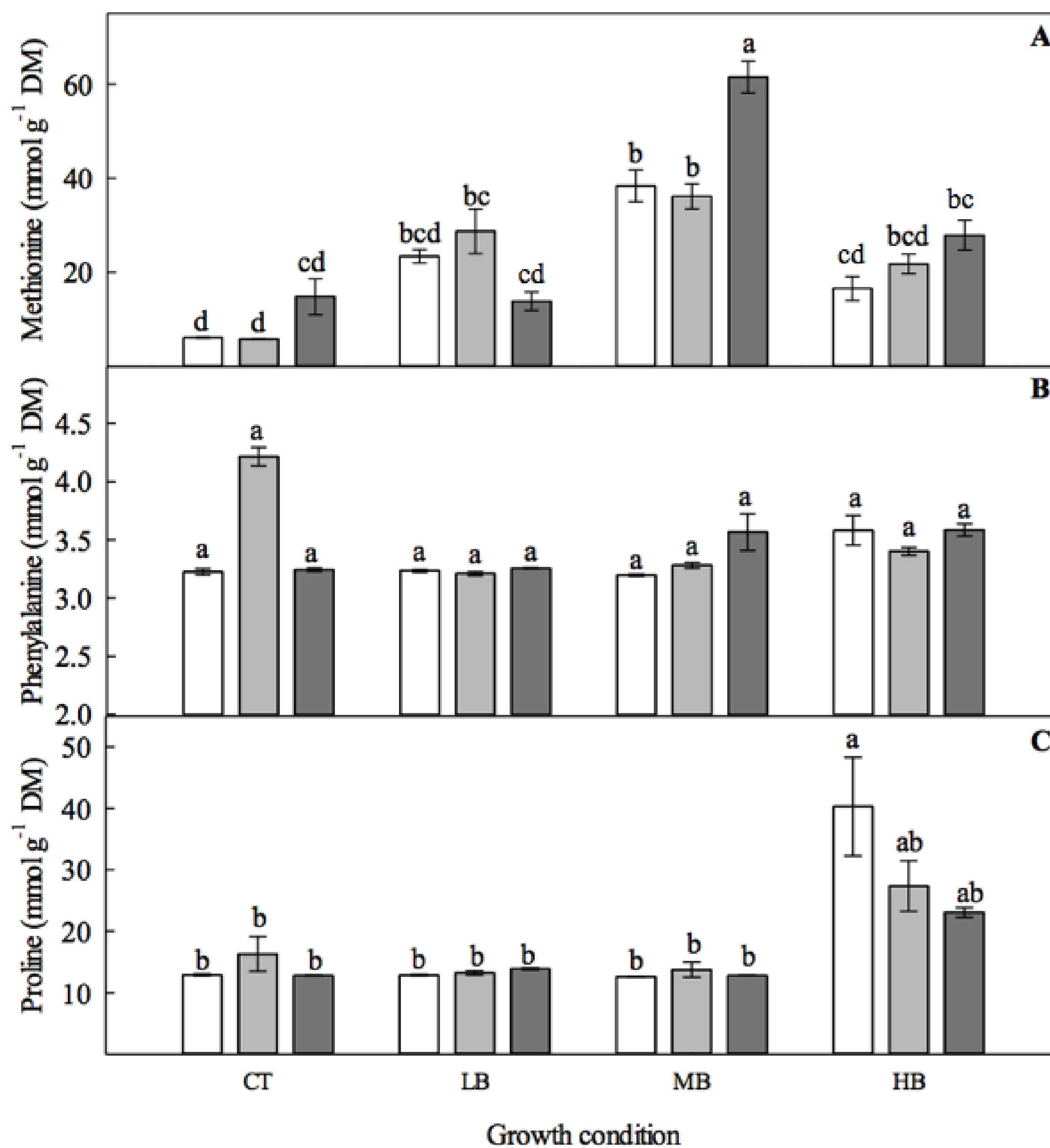

Fig. 4.11. Methionine, phenylalanine, and proline content of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) methionine; (B) phenylalanine; (C) proline. Otherwise, as per Fig. 4.1.

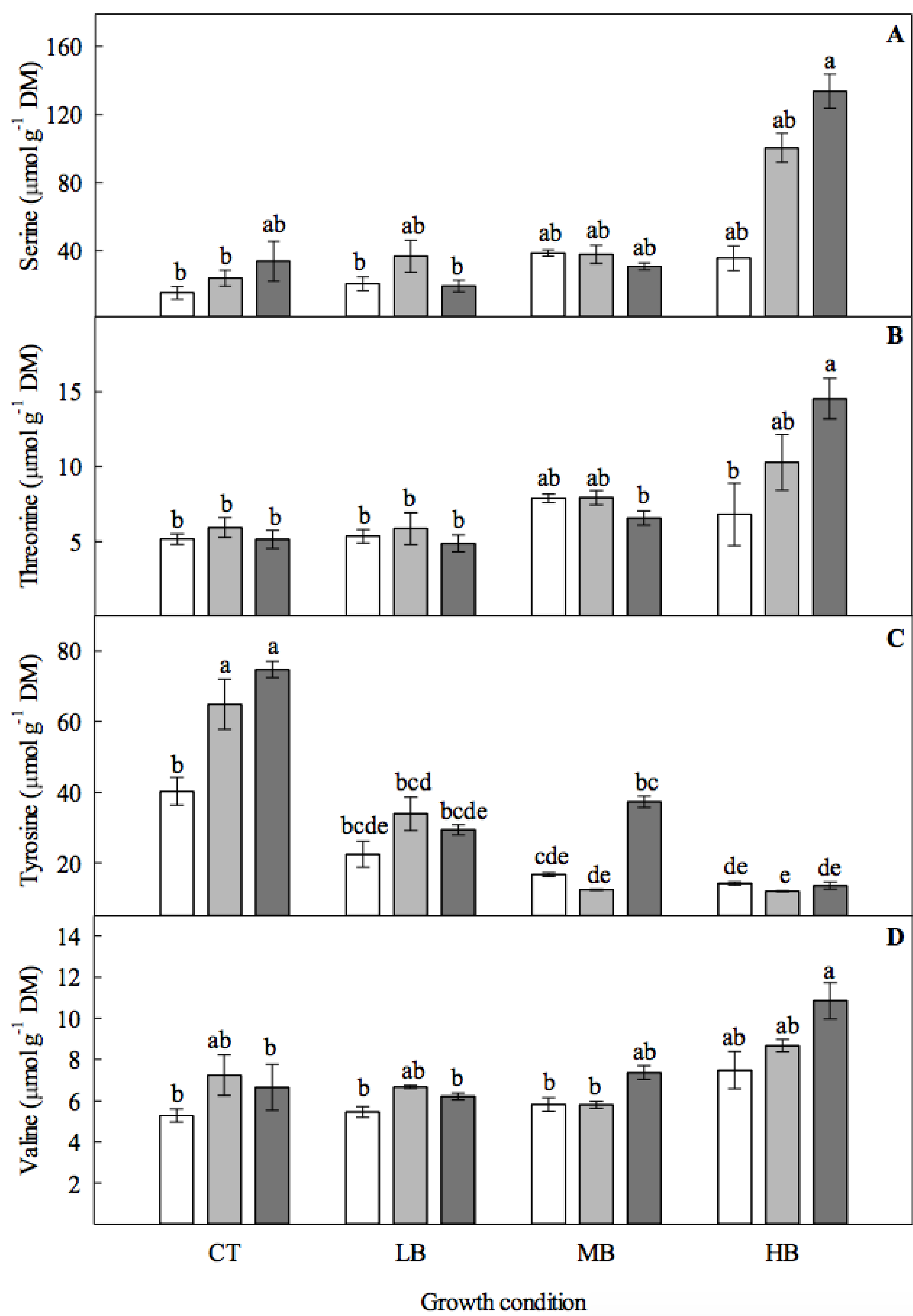

Fig. 4.12. Serine, threonine, tyrosine, and valine content of canola plants grown under four blue light levels and applied with either no chemical (control, open bars), an ethylene promoter (kinetin, light gray bars), or ethylene inhibitor (silver nitrate, medium gray bars). (A) serine; (B) threonine; (C) tyrosine; (D) valine. Otherwise, as per Fig. 4.1.

4.3.10. Pearson's correlation coefficients

Methane emissions were negatively correlated with stem diameter, total mass, net CO_2 assimilation (A_N), and stomatal conductance (g_s), but were positively correlated with chlorophyll *b*, effective quantum yield of PSII (ϕPSII), flavonoids, and epicuticular wax. Ethylene, on the other hand, was positively correlated with moisture content and negatively correlated with epicuticular wax. Ethylene and methane were not significantly correlated (Table 4.12). Methane was positively correlated with aspartic acid, glycine, isoleucine, leucine, methionine, serine, and threonine, but negatively correlated with lysine and tyrosine. Ethylene was negatively correlated with isoleucine but was not correlated with any other amino acids (Table 4.13).

Table 4.12. Pearson's correlation coefficients (upper value) and *P*-values (lower value) for relationship between methane emissions, ethylene evolution, and growth and physiological parameters of canola plants, grown under experimental conditions as described in Table 4.1. Significant correlations are indicated in bold.

Factor	Methane	Ethylene
Ethylene	0.141 0.413	-
Stem diameter	**-0.836** **0.000**	0.020 0.908
Leaf area	**-0.752** **0.000**	0.118 0.491
Total mass	**-0.710** **0.000**	-0.119 0.489
A_N	**-0.744** **0.000**	0.055 0.750
gs	**-0.482** **0.003**	-0.052 0.761
Chl *b*	**0.530** **0.001**	-0.286 0.091
Moisture	0.022 0.898	**0.546** **0.001**
ϕPSII	**0.579** **0.000**	-0.194 0.257
Flavonoids	**-0.780** **0.000**	0.191 0.263
Wax	**0.366** **0.028**	**-0.363** **0.030**

Table 4.13. Pearson's correlation coefficients (upper value) and *P*-values (lower value) for relationship between methane emissions, ethylene evolution, and amino acid content of canola plants, grown under experimental conditions as described in Table 4.1. Significant correlations are indicated in bold.

Factor	Methane	Ethylene
Ethylene	0.141 0.413	-
Aspartic acid	**0.405** **0.014**	0.187 0.275
Glycine	**0.531** **0.001**	0.295 0.081
Isoleucine	**0.440** **0.007**	**-0.342** **0.041**
Leucine	**0.550** **0.001**	-0.265 0.119
Lysine	**-0.790** **0.000**	-0.129 0.453
Methionine	**0.488** **0.003**	0.270 0.111
Serine	**0.638** **0.000**	-0.259 0.128
Threonine	**0.622** **0.000**	-0.199 0.245
Tyrosine	**-0.813** **0.000**	-0.043 0.804
Valine	**0.454** **0.005**	**-0.376** **0.024**

4.4. DISCUSSION

While earlier work on post-harvest fruits has found that blue light increased ethylene evolution (Gong *et al*., 2015; Ballester & Lafuente, 2017), this study revealed that high blue light decreased ethylene evolution, and increasing blue light significantly increased methane emissions (Table 4.1; Fig. 4.1). Contrary to work on sunflower and chrysanthemum, which found the greatest methane emissions at low blue (LB; Martel & Qaderi, 2017), highest methane emissions occurred from plants grown at high blue (HB; Table 4.1, Fig. 4.1), but the increase in methane in response to blue light is consistent among the three species examined thus far, and these may represent species-dependent differences. It is interesting to note that, with supplemental blue light, chemical application influenced ethylene evolution as expected, especially at high blue light, with kinetin application increasing ethylene and silver nitrate decreasing it (Fig. 4.1).

Growth with supplemental blue light significantly decreased plant growth and biomass parameters, with the exception of final height and leaf number (Tables 4-2 – 4.3; Fig. 4.2. – 4.3), but had no effect on growth indices (Table 4.4; Fig. 4.4). These results are consistent with prior research on lettuce (Johkan *et al*., 2010; Son & Oh, 2013), sunflower, and chrysanthemum (Martel & Qaderi, 2017), which may be in part due to the decrease in net CO_2 assimilation and stomatal conductance in plants grown with supplemental blue light (Table 4.5; Fig. 4.5). Red and blue wavelengths are most effective in photosynthesis (Sebastian & Prasad, 2014), and most studies have found an increase in photosynthetic processes in response to blue light (Hogewoning *et al*., 2010; Johkan *et al*., 2010; Matsuda *et al*., 2010; Zheng & van Labeke, 2017) in part due to increased stomatal number, width,

and pore aperture (Schuerger *et al*., 1997; Yorio *et al*., 2001; Matsuda *et al*., 2004; Hogewoning *et al*., 2010; Matsuda *et al*., 2010; Son & Oh, 2013; Hernández & Kubota, 2016), as found in cucumber (Hogewoning *et al*., 2010; Hernández & Kubota, 2016), rice (Matsuda *et al*., 2004), and spinach (Matsuda *et al*., 2010). These studies largely examined blue light ratios; in studies examining low levels of supplemental blue light, photosynthetic processes have decreased in both sunflower and chrysanthemum (Martel & Qaderi, 2017), and were unaffected in radish, lettuce, and spinach (Yorio *et al*., 2001). It is therefore evident that there is a species-specific response, and that responses depend largely on specific levels of supplemental blue as well as the other light used. Additionally, feedback down-regulation of photosynthesis is associated with carbohydrate accumulation in leaves (Hogewoning *et al*., 2010), and since plants were more compact when grown under blue light, they may have been more nutrient-dense as well.

The mild increase in both effective (ϕPSII) and maximum quantum yield of PSII F_v/F_m), along with the increase in qNP (Table 4.6; Fig. 4.6) suggests that the energy quenching mechanism was functioning properly and even better than control plants, indicating a higher protection against ROS formation (Sebastian & Prasad, 2014). Blue light also decreased photochemical quenching (qP), and this effect was greater with increasing levels of blue light; since qP allows for protection against absorbed radiation (Lazár, 2015) through closing PSII reaction centers (Maxwell & Johnson, 2000), it is possible that blue light is not sufficiently damaging to warrant closure of these centers. The decrease in flavonoid content observed in response to blue light, however, suggests a decreased antioxidant capacity and potential oxidative damage, but this contrasts earlier work that

has suggested increased antioxidant capacity with blue light exposure (Jung *et al*., 2013; Hoffmann *et al*., 2015; Zheng & van Labeke, 2017).

Earlier studies have also suggested an increase in leaf thickness in plants grown with supplemental blue light, through an increase in palisade mesophyll, upper epidermis, and leaf intercellular spaces (Schuerger *et al*., 1997; Macedo *et al*., 2011), but the lack of significant effect of blue light on growth indices, including leaf mass per area (LMA), leaf mass ratio (LMR), and leaf area ratio (LAR), suggest that leaf thickness was not altered in this study (Table 4.4; Fig. 4.4). The lack of blue light effect on leaf thickness suggests that aerobic methane emissions from this species is not the result of the methane stores in leaf air spaces, though it has been hypothesized that leaf thickness and structure may play a role in species-dependent emission differences (Watanabe *et al*., 2012).

Epicuticular wax has been shown to act as a precursor of methane under both oxic and anoxic conditions (Bruhn *et al*., 2014), and wax content significantly increased at high blue conditions (Table 4.8; Fig. 4.8). Growth at low blue or medium blue did not alter epicuticular wax, and the epicuticular wax of these plants was comparable to the control. Although wax content could have contributed to the emissions observed under HB (Bruhn *et al*., 2014), it would not have made a significant contribution to emissions at low blue or medium blue, and other parameters, such as plant metabolites, must have contributed to the increased emissions with supplemental blue light. It is likely that methyl group cleavage from epicuticle wax contributed a baseline amount to methane emissions.

High-energy blue wavelengths trigger blue light-specific photoreceptors, which then activate a cascade of metabolic responses (Kopsell & Sams, 2013), and this is evident in

the current literature examining the response of primary metabolites to blue light. Broccoli (*Brassica oleracea* var. italica) plants grown with supplemental blue light had increased nutrient accumulation (Kopsell & Sams, 2013), which is indicative of increased metabolic processes. Rice plants grown with supplemental blue light for short durations (7 days) had increased levels of amino acids, including Ser, Val, Ala, Thr, Asp, Asg, Val, Arg, Glu, Pro, and Tyr, with increased organic acids and fatty acids in comparison to plants grown at other wavelengths of light (Jung *et al*., 2013). Similarly, tomato fruits exposed to blue light for seven days had higher content of all free amino acids (Thr, Ser, Gly, Val, Met, Ile, Leu, Tyr, Phe, Lys, histidine (His), arginine (Arg), and Pro), except Asp and glutamic acid (Glu), in comparison to fruits kept in darkness or exposed to red light (Dhakal & Baek, 2014). Plant primary metabolites have also been shown to increase in cucumber, broccoli, basil, and tomato (Huché-Thélier *et al*., 2016), but the effects of light quality on primary metabolism depends both on the species and the specific quantities and ratios of the light used in the studies (Park *et al*., 2013). Because of this, some studies can differ in their results simply because they used a different percentage or quantity of blue light.

In this experiment, out of 13 measured amino acids, 12 of them, including all amino acids except for phenylalanine (Phe), were significantly influenced by blue light (Tables 4.9 – 4.11). Amino acids that were significantly increased by blue light included alanine (Ala), aspartic acid (Asp), glycine (Gly), Isoleucine (Ile), Leucine (Leu), methionine (Met), proline (Pro), serine (Ser), threonine (Thr), and valine (Val), whereas amino acids that were decreased by blue light include lysine (Lys) and tyrosine (Tyr) (Tables 4.9 – 4.11; Figs. 4.9 – 4.12). Aspartic acid is an amino acid that leads to the downstream biosynthesis of amino

acids Lys, Thr, Met, and Ile (Dhakal & Baek, 2014), and out of these, Thr, Met, and Ile were significantly increased (Figs. 4.9 – 4.12). This indicates a greater funneling of compounds into metabolic pathways (Kopsell & Sams, 2013). Interestingly, the content of Ala, Gly, and Met was highest at medium blue, but decreased slightly at high blue, such that low blue and high blue conditions were comparable (Figs. 4.10 – 4.11). Several amino acids, including Ile, Pro, Ser, Thr, and Val, increased only at high blue conditions (Figs. 4.10 – 4.12), which reiterates the importance of both light levels and light ratios in metabolomics studies. Increased amino acid content with blue light, which also resulted in decreased biomass, indicates more nutrient-dense plants (Ouzounis *et al*., 2015); however, in terms of crop yield, smaller plants would require a greater number of individual plants as well as increased cropland to produce the same amount of food, and this therefore has important implications for agriculture.

In this study, methane was negatively correlated with stem diameter, leaf number, and total mass, indicating that methane emissions are increased from smaller plants. Emissions were also negatively correlated with net CO_2 assimilation (A_N) and stomatal conductance (Table 4.12), indicating that emissions increased with decreased stomatal conductance. Contrary to prior research, moisture content was not correlated with methane emissions (Watanabe *et al*., 2012; Qaderi & Reid, 2014; Martel & Qaderi, 2017), but they were positively correlated with wax and negatively correlated with flavonoids, both of which may suggest plants that were adapted to stress conditions (Table 4.12). From a biochemical perspective, several amino acids were positively correlated with methane emissions, including Asp, Gly, Ile, Leu, Met, Ser, Thr, and Val, whereas Lys and Tyr were negatively

correlated (Table 4.13). Since most amino acids were greatly increased by blue light, and the only two that decreased were Lys and Tyr, this is not unreasonable. In contrast to expectations, methane was not significantly correlated with ethylene emissions (Table 4.12), indicating that plant biomass and metabolism has a greater influence on aerobic methane emissions, and methane and endogenous ethylene may not be related.

It is also worth noting that among the amino acids that increased in response to blue light, six of them, including Ala, Val, Leu, Ile, Met, and Thr, have methyl groups that could be liberated by reactive oxygen species (ROS), and methyl group liberation has been a proposed source of aerobic methane emissions (Keppler *et al*., 2008; Bruhn *et al*., 2009; Messenger *et al*., 2009; Wang *et al*., 2009; Martel & Qaderi, 2017). Since blue light has been shown to increase the production of ROS compounds in chrysanthemum (Zheng & van Labeke, 2017), this could explain the results of this experiment. Indeed, all of these except Ala are positively correlated with methane emissions in this study (Table 4.13). Decreased flavonoid content could indicate either a lack of oxidative damage, or a decreased capacity to cope with accumulation of ROS compounds, and therefore the liberation of amino acid methyl groups through ROS production cannot be ruled out, and in fact provides a reasonable hypothesis.

In conclusion, this study confirms the influence of blue light on methane emissions, as blue light greatly increased methane emissions, but only high blue decreased ethylene; this suggests that methane and ethylene may not actually be related, as found in Chapter 3. It is expected that methane increased in response to blue light because blue light (450 nm) is a high-energy wavelength that induces the accumulation of a number of compounds

(Kopsell & Sams, 2013). This could have increased the number of available methyl groups for cleavage and release of methane. This study is the first to examine the relationship between methane and blue light-induced changes in plant morphology and metabolism. Blue light decreased plant growth, biomass, gas exchange, photochemical quenching, and flavonoids, but increased wax, and most measured amino acids; this could indicate that higher levels of blue light overload the photoreceptors and act as a stress factor. The observed increase in methane emissions may be in part due to the plants being smaller, more nutrient-dense, and more metabolically active. The positive relationship between methylated amino acids and methane indicates that aerobic methane emissions may be the result of methyl group cleavage. While a clear relationship has been shown between amino acids, blue light, and aerobic methane emissions, the exact biochemical mechanism of methane production remains unknown, though this research may support the hypothesis that there is not one specific pathway. Instead, cleavage of methyl groups from a variety of metabolically important compounds, including pectin, epicuticular wax, and amino acids such as L-Methionine may be the origin of methane emissions. While aerobic methane emissions account for only a small portion of the global methane budget, understanding what facilitates methane production may lead to both better quantification of emissions and the possibility for mitigation of emissions.

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5.0. EFFECTS OF EXOGENOUS ETHYLENE ON METHANE EMISSIONS FROM PLANTS

5.1. INTRODUCTION

As discussed extensively in this thesis, the discovery in 2006 (Keppler *et al*., 2006) that plants release aerobic methane emissions brought significant controversy to the scientific community, but subsequent research has confirmed the phenomenon and examined the effects of environmental factors as well as sought out potential precursors. One of the suggested precursors is L-Methionine (Met), an essential amino acid produced by plants that cannot be synthesized by humans (Amir, 2008). Methionine leads to the production of ethylene, a simple gaseous plant hormone with the formula C_2H_4, with two carbons double-bonded. It is synthesized by the activity of 1-aminocyclopropane carboxylic acid synthase (ACS), from the third and fourth carbons of methionine, following which the sulphur-bound methyl group is recycled back into Met through the Yang cycle (Khan, 2004; Amir, 2008; Hopkins & Hüner, 2009).

Ethylene has been shown to influence a number of plant growth and physiological processes, including biomass accumulation, stomatal movement, and the rate of photosynthesis (Heredia & Cisneros-Zevallos, 2009; Iqbal *et al*., 2012). It also has a regulatory role in seed germination, vegetative growth, fruit ripening, and leaf senescence, and is an important regulating factor for responses to both biotic and abiotic stress (Iqbal *et al*., 2013; Habben *et al*., 2014). Since C_2H_4 receptors are located in the membrane of both the endoplasmic reticulum and the Golgi apparatus, ethylene can diffuse through plant cell walls to have intracellular interactions (Iqbal *et al*., 2013).

While all plants produce and respond to C_2H_4, there are a number of factors, including tissue type, timing of treatment (Hurr *et al.*, 2013; Amoah & Terry, 2016), plant growth stage, species, and the levels of C_2H_4 released (Amoah & Terry, 2016), which influence the effects of C_2H_4 on plants. Some plants have a reduced number of intracellular receptors, leading to reduced sensitivity of these plants to treatment with exogenous C_2H_4 (Iqbal *et al.*, 2012; Amoah & Terry, 2016). For example, some studies have shown that C_2H_4 increases photosynthesis (Grewal *et al.*, 1993; Khan *et al.*, 2005) while others have shown that C_2H_4 decreases it (Kays & Pallas, 1980; Rajala & Peltonen-Sainio, 2001; Khan *et al.*, 2005). This is partially dependent on C_2H_4 concentration, as low levels of ethylene have been shown to stimulate gas exchange processes whereas higher concentrations have proven inhibitory (Khan, 2004). Differential responses may also be the result of plant sensitivity to C_2H_4, as C_2H_4-insensitive plants have been shown to have reduced gas exchange in comparison to C_2H_4-sensitive plants (Iqbal *et al.*, 2012).

The majority of studies examining exogenous C_2H_4 look at postharvest fruit (El-Kereamy *et al.*, 2003; Chidley *et al.*, 2013; Amoah & Terry, 2016), with very few examining exogenous C_2H_4 on plants, and none found that examine application of exogenous C_2H_4 to vegetative tissue. Furthermore, most studies examining C_2H_4 use application of an ethylene-stimulating compound as opposed to applying pure ethylene gas to plants or fruits. In fruit, ethylene is directly involved in controlling enzyme biosynthesis, pigment production, and starch degradation (Chidley *et al.*, 2013). In postharvest sweet potato (*Ipomoea batatas* L.), exogenous ethylene treatment led to decreased fruit mass as a result of increased water loss, but also altered nutritional content due to accelerated

monosaccharide catabolism and phenolic accumulation (Amoah & Terry, 2016). In postharvest mango (*Mangifera indica*, cv. Alphonso), ethylene induced the biosynthesis of a number of plant volatiles which led to faster fruit ripening and decreased shelf life (Chidley *et al*., 2013).

In postharvest cucumber (*Cucumis sativus* L.) stored in exogenous ethylene levels of 10 $\mu L\ L^{-1}$, cucumber fruit had increased chlorophyll degradation, decreased cellular integrity, and increased cell wall solubility (Hurr *et al*., 2009). Subsequent studies by this lab showed that programmed cell death also resulted, which was evident through increased nuclease activity (Hurr *et al*., 2010), production of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), and increased electrolyte leakage (Hurr *et al*., 2013). Chlorophyll degradation, evident in all of their studies on cucumber, occurred in part due to cellular decompartmentalization as well as increased macromolecule catabolism (Hurr *et al*., 2013). They discovered that, for cucumber, the critical threshold was 2-4 days of ethylene exposure, following which programmed cell death increased, resulting in a rapid reduction of fruit quality (Hurr *et al*., 2013). The sensitivity of cucumber fruit to exogenous ethylene may be in part due to the inability of this fruit to shift the redox status, which allowed for the accumulation of ROS compounds and a reduction in cellular integrity, and led to postharvest losses (Hurr *et al*., 2013).

Increased sensitivity to ethylene may not be only species-dependent, as certain environmental conditions lead to ethylene production that is beyond what plants can tolerate. This is referred to as stress ethylene, and has been shown in plants grown under nitrogen-deficient conditions, leading to over-production of ethylene and a subsequent

decrease in photosynthesis and plant growth (Iqbal *et al*., 2011). It is therefore evident that ethylene has the potential to decrease critical plant parameters, resulting in plant stress. In support of this, a transgenic canola (*Brassica napus*) was created that expressed the bacterial ACC deaminase gene, which led to ethylene deficiency and subsequent reduction in seed size (Walton *et al*., 2012). In canola-quality Indian mustard (*Brassica juncea*), application of ethephon, an ethylene-releasing compound, lead to increased growth, gas exchange, and nitrogen accumulation, whereas addition of ethephon to wild-type canola impeded proper seed development (Khan *et al*., 2008; Walton *et al*., 2012). There is therefore an optimal level of ethylene required for normal plant functioning, but levels beyond this can have negative effects and result in damage and plant stress.

At optimal levels, exogenous ethylene can induce the accumulation of flavonoids and promote photosynthesis due to increased chlorophyll content in leaves (Kazan *et al*., 2015). In sunflower (*Helianthus annuus*) and peas (*Pisum sativum*), both chlorophyll content and gas exchange are highly sensitive to ethylene; levels as low as 0.25 $\mu L\ L^{-1}$ induced chlorophyll degradation and a 33% reduction in photosynthesis (Kays and Pallas, 1980). In contrast, soybean plants exposed to 10 $\mu L\ L^{-1}$ had decreased net CO_2 assimilation as well as decreased the effective quantum yield of PSII (ϕPSII) following as little as 4 h of exposure (Wullschleger *et al*., 1992). Photochemical quenching (qP) was also decreased, whereas qNP was increased. At higher concentrations, ethylene can disrupt the capacity of mesophyll tissue to assimilate CO_2 in high concentrations, but may not have an effect on Rubisco activity (Wullschleger *et al*., 1992).

It is evident that ethylene has a significant influence on plants as well as postharvest fruits, but the effects of exogenous ethylene on plant metabolism remains poorly understood. The purposes of this study were to (i) examine the effects of exogenous ethylene application, through direct exposure to ethylene gas, on aerobic methane emissions; (ii) observe the effects of exogenous ethylene exposure, for different durations, on plant growth and physiology; and (iii) determine the effects of exogenous ethylene on amino acid content in canola (*Brassica napus*) to examine the relationship between amino acid content and aerobic methane release in this experiment. It was hypothesized that exogenous ethylene application would increase methane emissions, in part due to the stress caused by high levels of ethylene, and that exogenous ethylene would influence plant growth, physiology, and content of amino acids. Based on results from earlier studies in this thesis, it was also expected that aerobic methane emissions would be related to multiple acids, reinforcing the hypothesis that methane results from methyl group cleavage.

5.2. METHODS

5.2.1. Plant material and growth conditions

Seeds of canola (*Brassica napus* L. cv. 6056 CR, BrettYoung Seeds, Winnipeg, Manitoba) were germinated and planted as described in Chapter 2, section 2.2.1 (see page 36). Following transplantation, pots were transferred to a growth chamber (model PGR15, Conviron, Controlled Environments LTD., Winnipeg, Manitoba, Canada), set to 22/18°C on a 16 h photoperiod, with the PPFD and light supply as described in Chapter 2, section 2.2.1 (see page 36). Plants were left to acclimate for 7 days, to allow for the emergence of the first true leaves, and were then randomly assigned to one of three experimental

conditions; no exogenous ethylene (control), low levels of exogenous ethylene (exposure for one hour), and high levels of exogenous ethylene (exposure for two hours), for a total of 3 experimental conditions. For exogenous ethylene treatment, plants were placed in Plexiglas boxes (25 cm tall x 25 cm wide x 45 cm deep), and a 15 s burst of ethylene gas (Air Liquide, Halifax, Nova Scotia, Canada), the smallest possible amount to get a consistent peak on the GC-FID, was applied before the boxes were closed, resulting in air concentrations of approximately 36 μmol ml^{-1}, or 36 mmol L^{-1}. To prevent moisture buildup, moisture grabbers (Concrobium, Siamons Internation, Toronto, Ontario, Canada) were placed inside each Plexiglas box. Plants were grown under experimental conditions for 7, 14, or 21 days. Due to small plant size under exogenous ethylene treatments and the limited number of plants that could fit in each Plexiglas box, plants grown for 7 days and 14 days were used only for measurement of measurements of methane emissions, ethylene evolution, and metabolite profiling. These parameters therefore had nine experimental growth conditions, whereas all other measured parameters had three experimental growth conditions (control, 1 h, or 2 h, grown for 21 days). Experiments were conducted three times, with at least three measurements per trial to ensure statistical robustness, and plants were placed in different Plexiglas boxes and rotated daily. Control plants were placed in a different chamber of the same model to account for ethylene release from plants following daily treatments. To account for chamber differences, plants were rotated between chambers every other day, just prior to the daily ethylene treatment.

5.2.2. Measurement of methane and ethylene emissions

Measurement of methane and ethylene emissions were performed as described in Chapter 2, section 2.2.2 (see page 38). Leaves were cleanly excised at least one full hour following exogenous ethylene treatment.

5.2.3. Plant growth and dry mass accumulation

Plant growth and biomass accumulation were determined, and growth indices calculated from these values, as described in Chapter 2, section 2.2.3 (see page 39).

5.2.4. Gas exchange

Gas exchange was measured as described in Chapter 4, section 4.2.4 (see page 146).

5.2.5. Chlorophyll fluorescence

Chlorophyll fluorescence was measured as described in Chapter 2, section 2.2.4 (see page 39).

5.2.6. Photosynthetic pigments

Photosynthetic pigments were determined as described in Chapter 2, section 2.2.5 (see page 40).

5.2.7. Moisture content

Moisture content was calculated as described in Chapter 2, section 2.2.6 (see page 41).

5.2.8. Flavonoids and nitrogen balance index

Flavonoids and nitrogen balance index (NBI) were measured as described in Chapter 2, section 2.2.7 (see page 41).

5.2.9. Metabolite profiling

Metabolite profiling was conducted as described in chapter 3, section 3.2.10 (see page 84).

5.2.10. Data analysis

The effects of time (7 days, 14 days, or 21 days) and exogenous ethylene exposure (36 mmol L^{-1} for 0 h (control), 1 h (low), or 2 h (high)) were determined for methane, ethylene, and amino acids using ANOVA for split-plot design (SAS Institute, 2011). For this analysis, time was the main plot and exogenous ethylene the subplot, with growth chambers and trials as the replications (Hinkelman and Kempthorne, 2008). To determine differences among treatments for all measured parameters, including growth and physiological parameters, a one-way ANOVA using Scheffé's multiple-comparison procedure at the 5% confidence interval was used. Since growth and physiological parameters were only measured at 21 days, with three experimental conditions, results are expressed only in graph form because C_2H_4 exposure length was the only factor. Additionally, relationships between parameters were determined using Pearson's correlation coefficients (SAS Institute, 2011).

5.3. RESULTS

5.3.1. Methane and ethylene emissions

Methane and ethylene emissions were significantly affected by both time and exogenous ethylene treatment (Table 5.1). Ethylene emissions increased over time, and increased substantially with each exogenous C_2H_4 treatment; however, plants grown for 14 days and plants grown for 21 days had similar ethylene emissions. The highest ethylene emissions occurred after 2 h of daily exogenous exposure for 14 and 21 days, and the lowest occurred from the control plants (Fig. 5.1A). Methane emissions, on the other hand, decreased from 7 days to 14 days, but then increased slightly at 21 days, with increased emissions with exogenous C_2H_4 exposure. The highest emissions occurred at 7 d or 21 d from plants with 2 h exogenous C_2H_4 exposure, and the lowest in control plants at 14 d (Fig. 5.2B).

Table 5.1. Summary of split-plot ANOVA (*F* value) for effects of time and exogenous ethylene (C_2H_4) on methane and ethylene emissions of canola (*Brassica napus* cv. 6056 CR). Plants were grown for 7, 14, or 21 days and were applied with one of three ethylene treatments (control, no exogenous ethylene; low, 1 h daily treatment of 36 mmol L^{-1} C_2H_4; high, 2 h daily treatment of 36 mmol L^{-1} C_2H_4).

Source of Variation	df	Ethylene	Methane
Time (T)	2	799.4****	74.3****
Main plot error	6	-	-
Ethylene (E)	2	2359.1****	49.3****
T x E	4	348.6****	3.2*
Subplot error	12	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

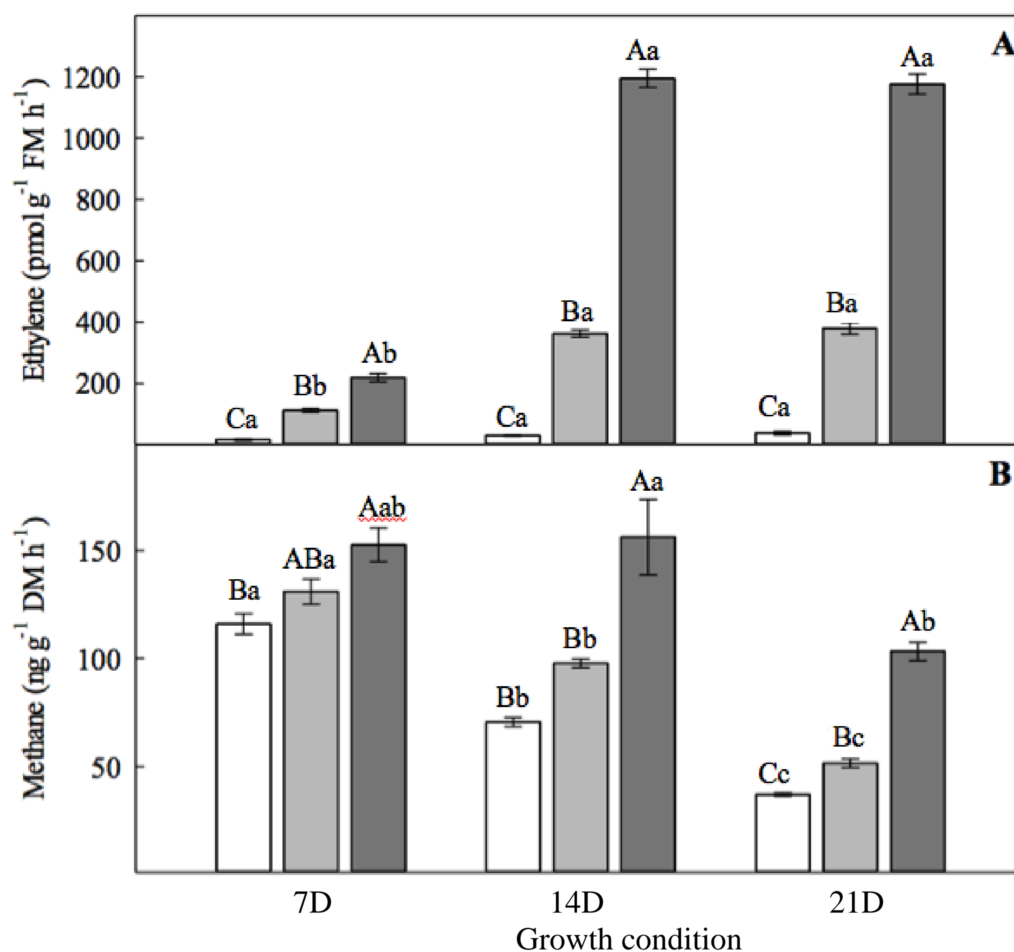

Fig. 5.1. Ethylene and methane emissions from canola plants grown for three time periods (7D, 7 days; 14D, 14 days; 21D, 21 days), and treated with either no exogenous ethylene (control, open bars), 1 h of daily treatment with 36 mmol L^{-1} exogenous C_2H_4 (low, light gray bars), or 2 h of daily treatment with 36 mmol L^{-1} exogenous C_2H_4 (high, medium gray bars). (A) ethylene emissions; (B) methane emissions. Data are means ± SE (n = 3), and bars surmounted by different letters are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison test. Upper case, within each growth time; lower-case letters, within each ethylene treatment duration.

5.3.2. Growth and biomass accumulation

All aspects of plant growth were decreased by exogenous ethylene. Plants treated for 2 h had reduced stem height, stem diameter, leaf area, and leaf number in comparison to those treated for 2 h (Fig. 5.2). Leaf number and growth rate decreased with exogenous C_2H_4 exposure regardless of exposure length (Fig. 5.2C, E). Dry mass accumulation of all plant parts also decreased with exogenous C_2H_4, to a greater extent with 2 h of exposure. Plants exposed to 2 h daily exogenous C_2H_4 exposure were significantly smaller than those exposed to 1 h of treatment, and they were both smaller than control plants (Fig. 5.3A – D). Growth indices LMR and LAR increased in response to exogenous ethylene; for LMR, exposure time had no effect, whereas with LAR, plants exposed to exogenous C_2H_4 for 2 h had a much higher LAR than those exposed for 1 h (Fig. 5.3F – G). On the other hand, LMA and S:R mass ratio decreased with exogenous C_2H_4, with 2 h treatment decreasing LMA more than 1 h of treatment, whereas S:R mass ratio did not differ between 1 h and 2 h of treatment (Fig. 5.3E, H).

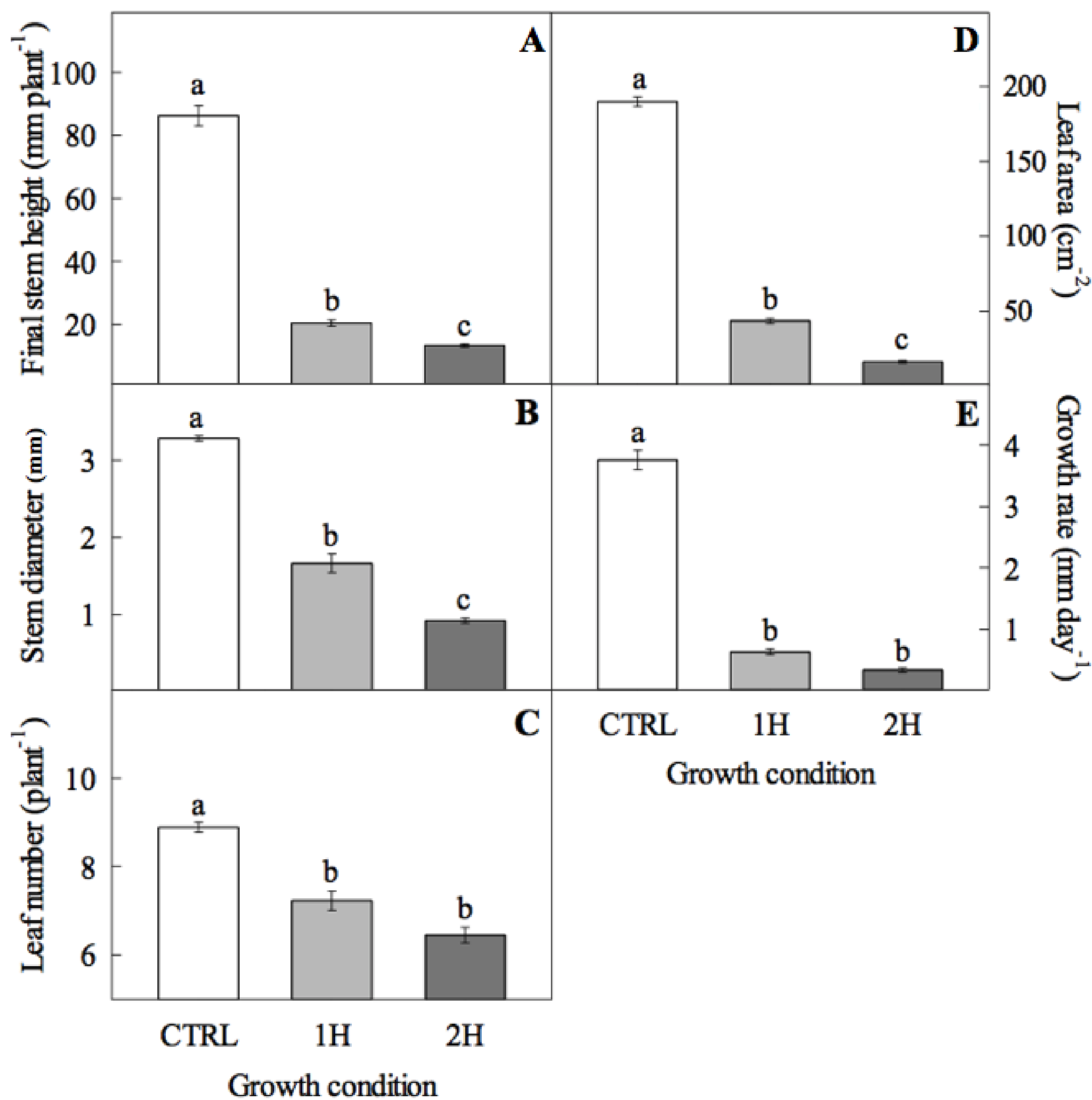

Fig. 5.2. Growth and development of canola (*Brassica napus*) plants grown for 21 days and either treated with no exogenous ethylene (CTRL), 1 hour of daily treatment with 36 mmol L^{-1} exogenous ethylene (1H), or 2 hours of daily treatment with 36 mmol L^{-1} exogenous ethylene (2H). (A) plant height; (B) stem diameter; (C) leaf number; (D) leaf area; (E) growth rate. Otherwise, as per Fig. 5.1.

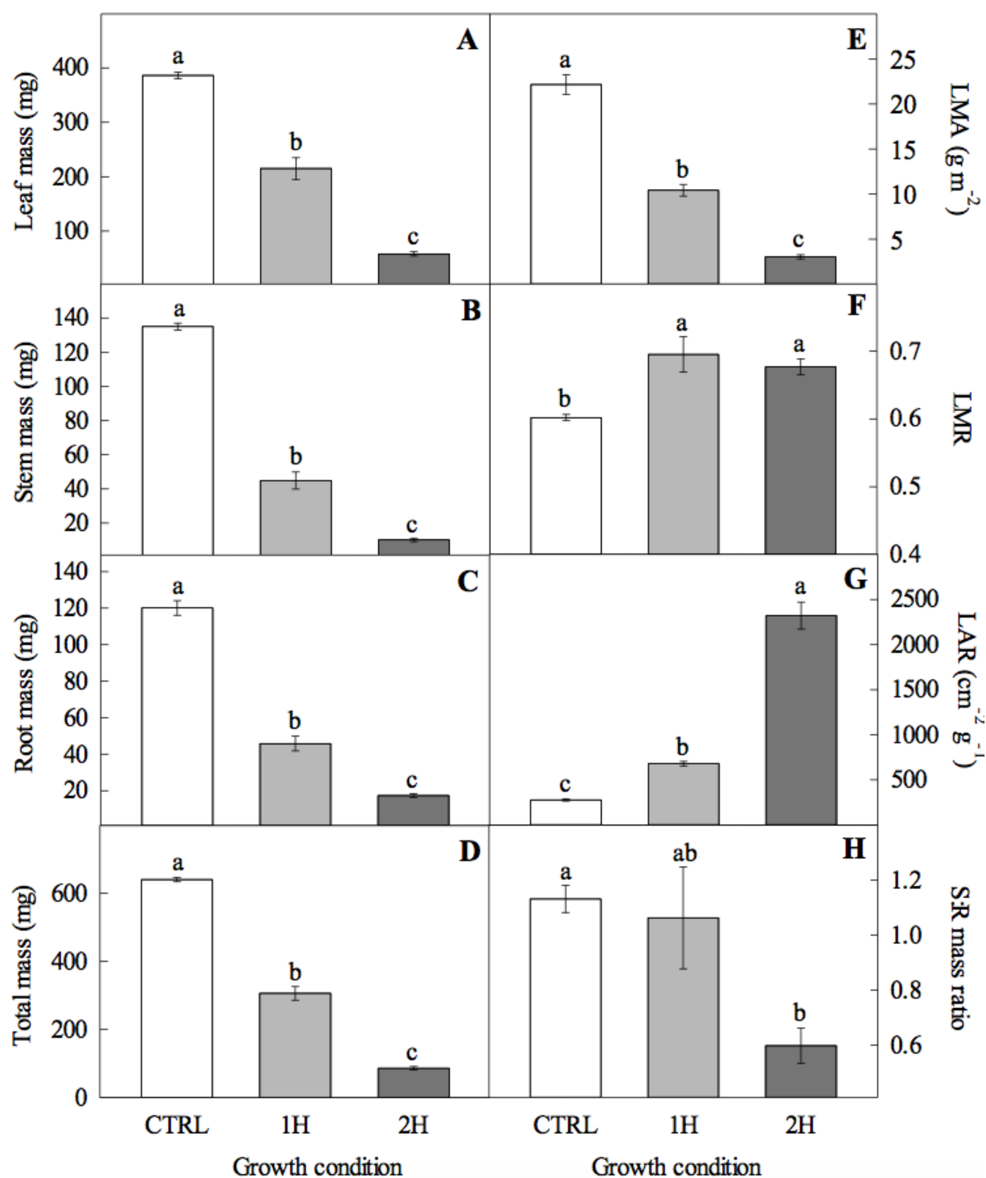

Fig. 5.3. Biomass accumulation and growth indices of canola (*Brassica napus*) plants grown for 21 days and either treated with no exogenous ethylene (CTRL), 1 hour of daily treatment with 36 mmol L^{-1} exogenous ethylene (1H), or 2 hours of daily treatment with 36 mmol L^{-1} exogenous ethylene. (A) leaf mass; (B) stem mass; (C) root mass; (D) total mass; (E) LMA, leaf mass per area; (F) LMR, leaf mass ratio; (G) LAR, leaf area ratio; (H) S:R mass ratio, shoot to root mass ratio. Otherwise, as per Fig. 5.1.

5.3.3. Gas exchange

With the exception of stomatal conductance (g_s), all aspects of gas exchange were significantly decreased by exogenous ethylene treatment. Net CO_2 assimilation (A_N) decreased more in plants treated with 2 h of exogenous C_2H_4 than plants treated with 1 h. Transpiration (*E*) decreased only in plants treated with 2 h of exogenous C_2H_4, whereas plants treated with 1 h did not significantly differ from the control. Water use efficiency (WUE) decreased with exogenous ethylene treatment, regardless of treatment duration (Fig. 5.4A – D).

5.3.4. Chlorophyll fluorescence

All aspects of chlorophyll fluorescence decreased with exogenous C_2H_4 treatment, and effective quantum yield of photosystem II (ϕPSII), non-photochemical quenching (qNP), and photochemical quenching (qP) decreased to a greater extent with 2 h of exogenous ethylene treatment. In contrast, maximum quantum yield of PSII (F_v/F_m) was decreased by exogenous C_2H_4 irrespective of treatment duration (Fig. 5.4E – H).

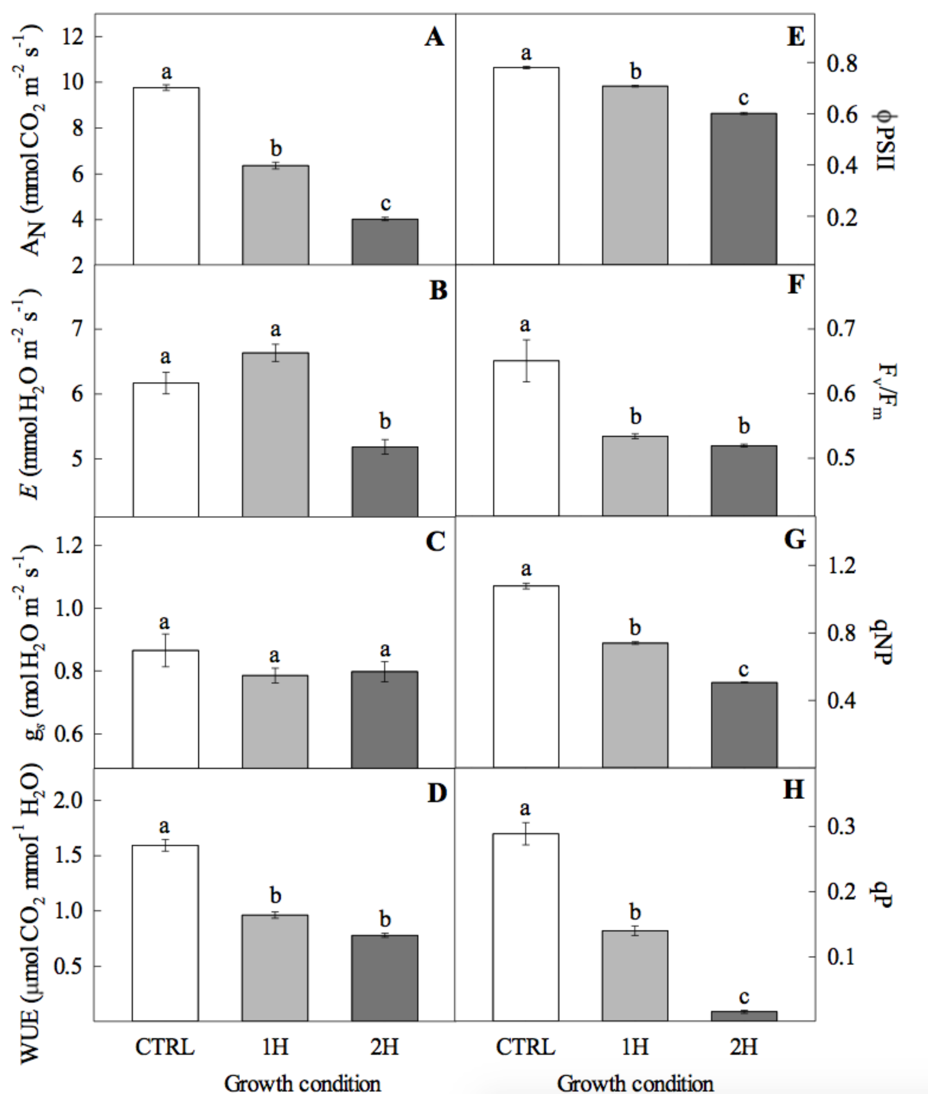

Fig. 5.4. Gas exchange and chlorophyll fluorescence of canola (*Brassica napus*) plants grown for 21 days and either treated with no exogenous C_2H_4 (CTRL), 1 hour of daily treatment with 36 mmol L^{-1} exogenous C_2H_4 (1H), or 2 hours (2H) of daily treatment with 36 mmol L^{-1} exogenous C_2H_4. (A) A_N, net CO_2 assimilation; (B) E, transpiration; (C) g_s, stomatal conductance; (D) WUE, water use efficiency; (E) ϕPSII, effective quantum yield of photosystem II (PSII); (F) F_v/F_m, maximum quantum yield of PSII; (G) qNP, non-photochemical quenching; and (H) qP, photochemical quenching. Otherwise, as per Fig. 5.1.

5.3.5. Photosynthetic pigments

All photosynthetic pigments decreased with exogenous C_2H_4 treatment, and these parameters decreased to a greater extent with 2 h of exogenous C_2H_4 treatment in comparison to 1 h of treatment. The chlorophyll *a*/*b* ratio, on the other hand, was lowest in plants treated with 1 h of daily exogenous ethylene, whereas plants treated with 2 h exogenous C_2H_4 did not differ significantly from either the 1 h treated plants or the controls (Fig. 5.5 A – E).

5.3.6. Moisture

Moisture content was significantly decreased only by 2 h of daily C_2H_4 treatment, but plants treated for 1 h did not differ from the control (Fig. 5.5F).

5.3.7. Flavonoids and nitrogen balance index

Nitrogen balance index (NBI) decreased, whereas flavonoids increased in response to exogenous C_2H_4 treatment. Plants treated for 2 h daily had significantly higher NBI, but lower flavonoid content than plants treated for 1 h daily (Fig. 5.5G – H).

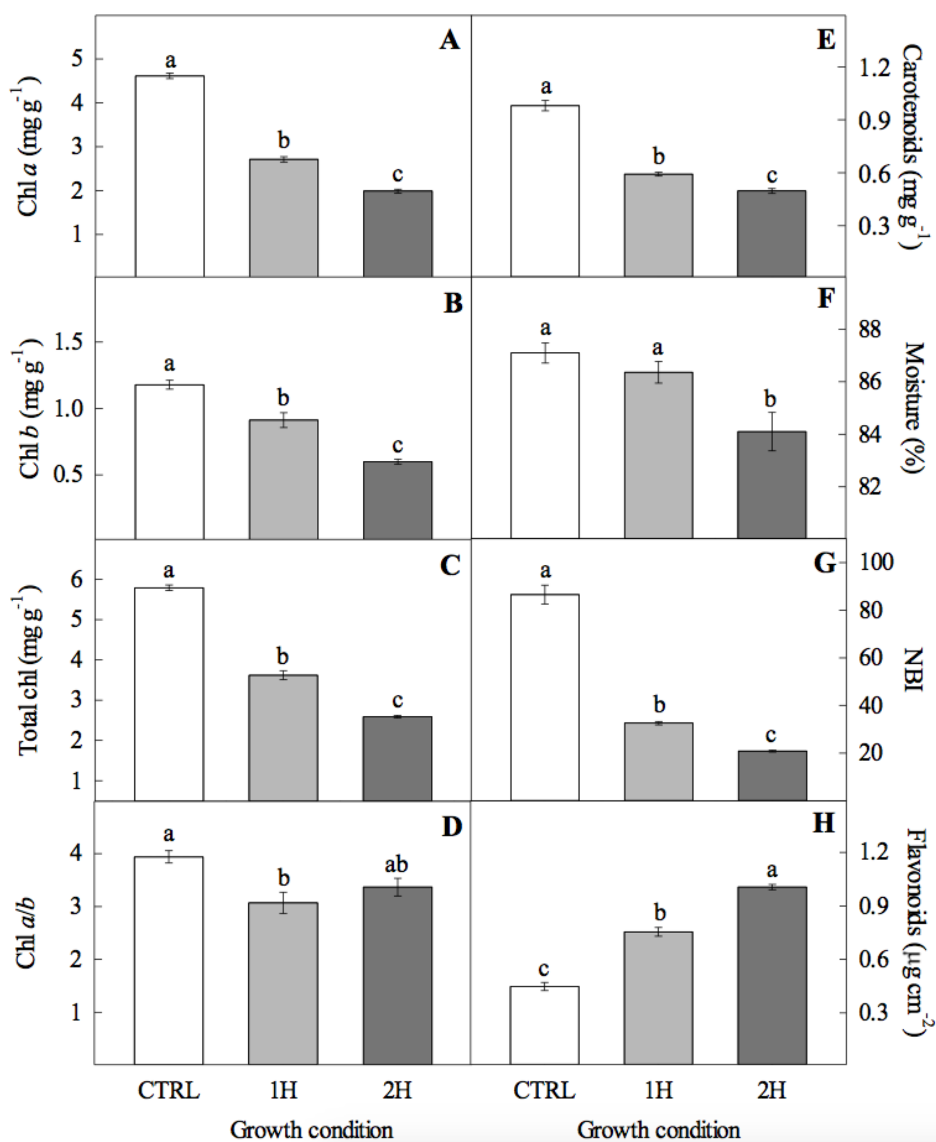

Fig. 5.5. Photosynthetic pigments, moisture content, nitrogen balance index (NBI) and flavonoid content of canola (*Brassica napus*) plants grown for 21 days and either treated with no exogenous ethylene (CTRL), 1 hour of daily treatment with 36 mmol L^{-1} exogenous ethylene (1H), or 2 hours (2H) of daily treatment with 36 mmol L^{-1} exogenous ethylene. (A) Chlorophyll (Chl) *a*; (B) Chl *b*; (C) total chlorophyll; (D) chlorophyll *a*/*b* ratio; (E) carotenoids; (F) moisture content; (G) NBI, nitrogen balance index; (H) flavonoids. Otherwise, as per Fig. 5.1.

5.3.8. Metabolite profiling

Amino acids alanine (Ala), aspartic acid (Asp), glycine (Gly), isoleucine (Ile), leucine (Leu), methionine (Met), threonine (Thr), tyrosine (Tyr), and valine (Val) were affected by both time (T) and exogenous ethylene treatment (E), along with the interaction between the two (Tables 5.2 – 5.4). Lysine (Lys) was affected only by ethylene and the T x E interaction, serine (Ser) was only affected by ethylene treatment, and proline was only affected by time and the T x E interaction (Tables 5.3 – 5.4).

Out of the 13 measured amino acids, seven increased over time (Asp, Ile, Leu, Met, Pro, Thr, and Val), two decreased over time (Ala and Gly), and four were unaffected by time (Lys, Phe, Ser, and Tyr; Figs. 5.6 – 5.8). In regard to exogenous ethylene, two amino acids increased with ethylene treatment (Ala, Gly), nine decreased (Asp, Ile, Leu, Lys, Met, Ser, Thr, Tyr, Val), and two were unaffected (Phe, Pro; Figs. 5.6 – 5.8). The interactions between T x E differed among amino acids. For Asp, Leu, Lys, Met, and Tyr, the highest amino acid content occurred in the control plants grown for 21 days, and the lowest in plants exposed to 2 h of exogenous C_2H_4 (Figs. 5.6B, 5.7B,C, 5.8A, 5.9C). For Ile, the highest amino acid content occurred in the control plants grown for 14 days, and the lowest in control plants grown for 7 days (Fig. 5.7A). For Ala, Pro, and Val, the highest content occurred in plants grown for 14 days and exposed to 1 h of exogenous C_2H_4, and the lowest in plants grown for 14 days and exposed to 2 h exogenous C_2H_4 for Ala (Fig. 5.6A), 7 days at any treatment length for Pro (Fig. 5.8C), and 7 days with 2 h exogenous C_2H_4 for Val (Fig. 5.9D). For Ser and Thr, the highest content occurred in the control plants grown for 14 days, and the lowest occurred in plants grown for 14 days with 1 h of exogenous C_2H_4

for Ser (Fig. 5.9A), and 21 days for 1 h of exogenous C_2H_4 for Thr (Fig. 5.9B). For Gly and Phe, the highest content occurred in plants grown for 7 days and exposed to 1 h exogenous C_2H_4, whereas the lowest content occurred in plants grown for 14 days with 2 h of exogenous C_2H_4 treatment (Figs. 5.6C, 5.8B).

Table 5.2. Summary of split-plot ANOVA (*F* value) for effects of time and exogenous ethylene on content of amino acids alanine (Ala), aspartic acid (Asp), glycine (Gly), isoleucine (Ile), and leucine (Leu) of canola (*Brassica napus* cv. 6056 CR). Plants were grown for 7, 14, or 21 days and were applied with one of three ethylene treatments (control, no exogenous ethylene; low, 1 h daily treatment of 36 mmol L^{-1}; high, 2 h daily treatment of 36 mmol L^{-1}).

Source of Variation	df	Ala	Asp	Gly	Ile	Leu
Time (T)	2	90.5****	79.0****	3.23	6.5*	8.9*
Main plot error	6	-	-	-	-	-
Ethylene (E)	2	43.1****	92.8****	16.2***	5.1*	9.5**
T x E	4	75.9****	56.8****	19.4****	4.2*	9.5**
Subplot error	12	-	-	-	-	-

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Table 5.3. Summary of split-plot ANOVA (*F* value) for effects of time and exogenous ethylene on content of amino acids lysine (Lys), methionine (Met), phenylalanine (Phe), and proline (Pro) of canola (*Brassica napus* cv. 6056 CR). Plants were grown under experimental conditions as described in Table 5.2.

Source of Variation	df	Lys	Met	Phe	Pro
Time (T)	2	2.7	14.1**	0.2	230.4****
Main plot error	6	-	-	-	-
Ethylene (E)	2	40.2****	15.9***	2.3	0.6
T x E	4	5.2*	7.0**	2.2	15.9****
Subplot error	12	-	-	-	-

**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Table 5.4. Summary of split-plot ANOVA (*F* value) for effects of time and exogenous ethylene on content of amino acids serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val) of canola (*Brassica napus* cv. 6056 CR). Plants were grown under experimental conditions as described in Table 5.2.

Source of Variation	df	Ser	Thr	Tyr	Val
Time (T)	2	0.3	4.3	15.6**	65.5****
Main plot error	6	-	-	-	-
Ethylene (E)	2	7.5**	11.3**	10.4**	27.9****
T x E	4	1.0	5.2*	6.9**	13.8***
Subplot error	12	-	-	-	-

**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

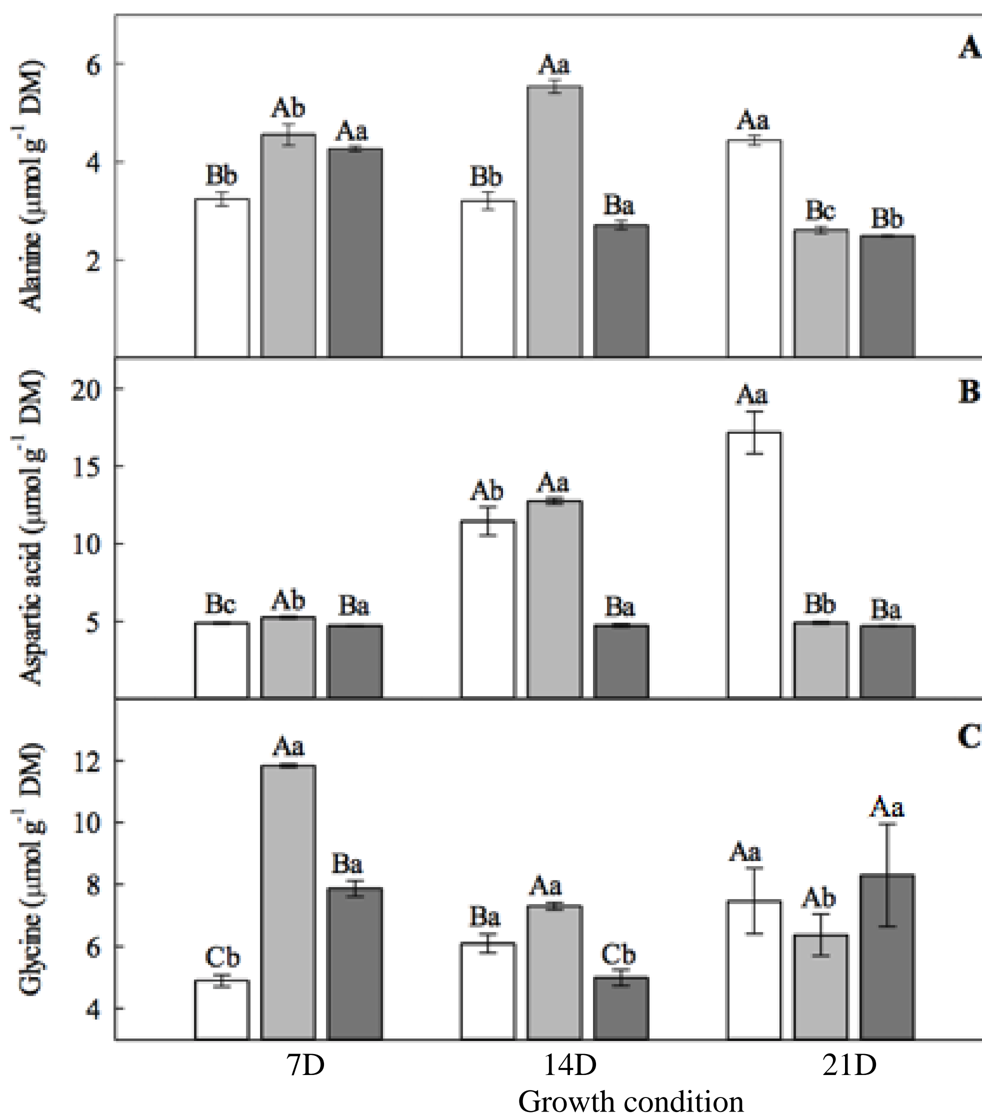

Fig. 5.6. Alanine, aspartic acid, and glycine content of canola plants grown for three time periods (7D, 7 days; 14D, 14 days; 21D, 21 days), and treated with either no exogenous ethylene (control, open bars), 1 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (low, light gray bars), or 2 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (high, medium gray bars). (A) alanine; (B) aspartic acid; (C) glycine. Upper case, within each growth time; lower-case letters, within each ethylene treatment duration. Otherwise, as per Fig. 5.1.

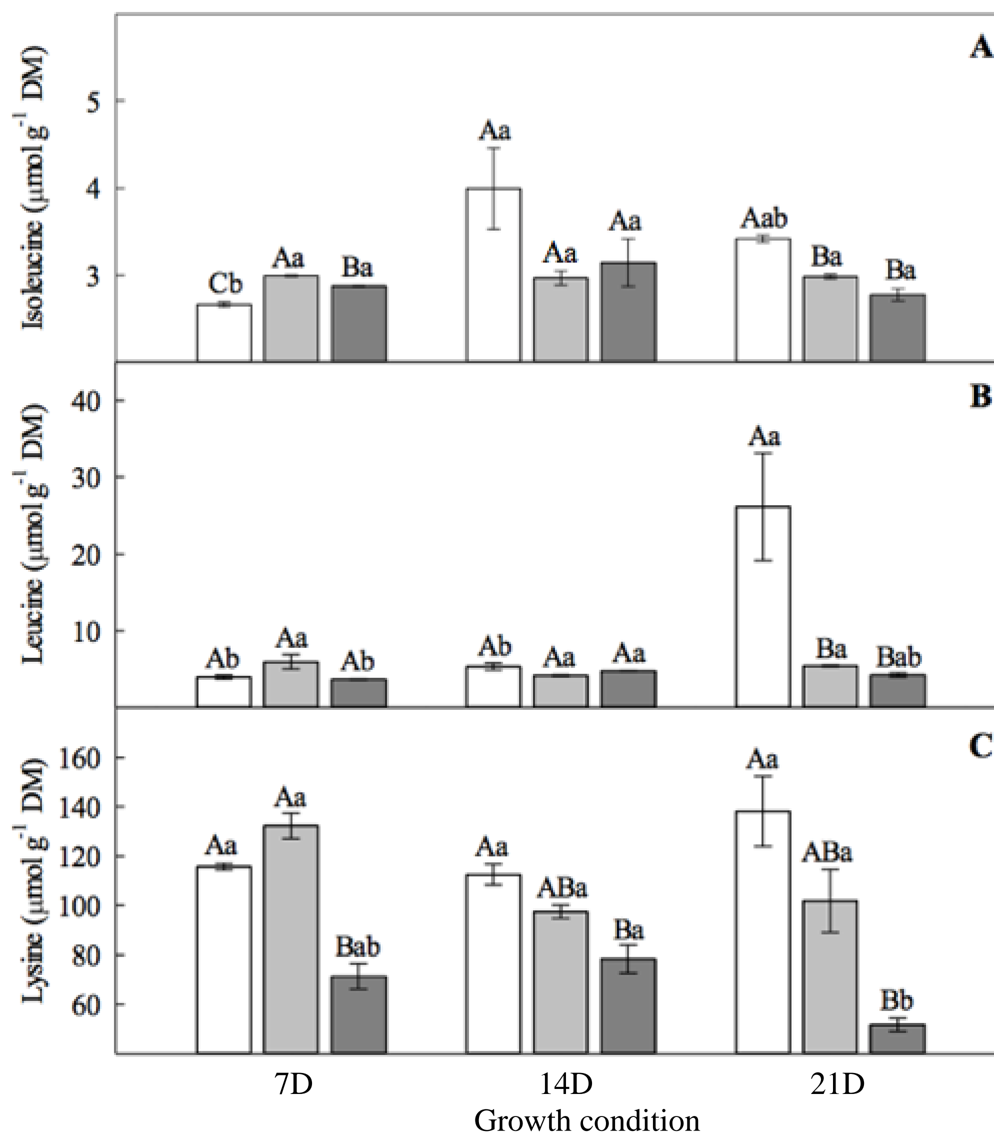

Fig. 5.7. Isoleucine, leucine, and lysine content of canola plants grown for three time periods (7D, 7 days; 14D, 14 days; 21D, 21 days), and treated with either no exogenous ethylene (control, open bars), 1 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (low, light gray bars), or 2 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (high, medium gray bars). (A) isoleucine; (B) leucine; (C) lysine. Upper case, within each growth time; lower-case letters, within each ethylene treatment duration. Otherwise, as per Fig. 5.1.

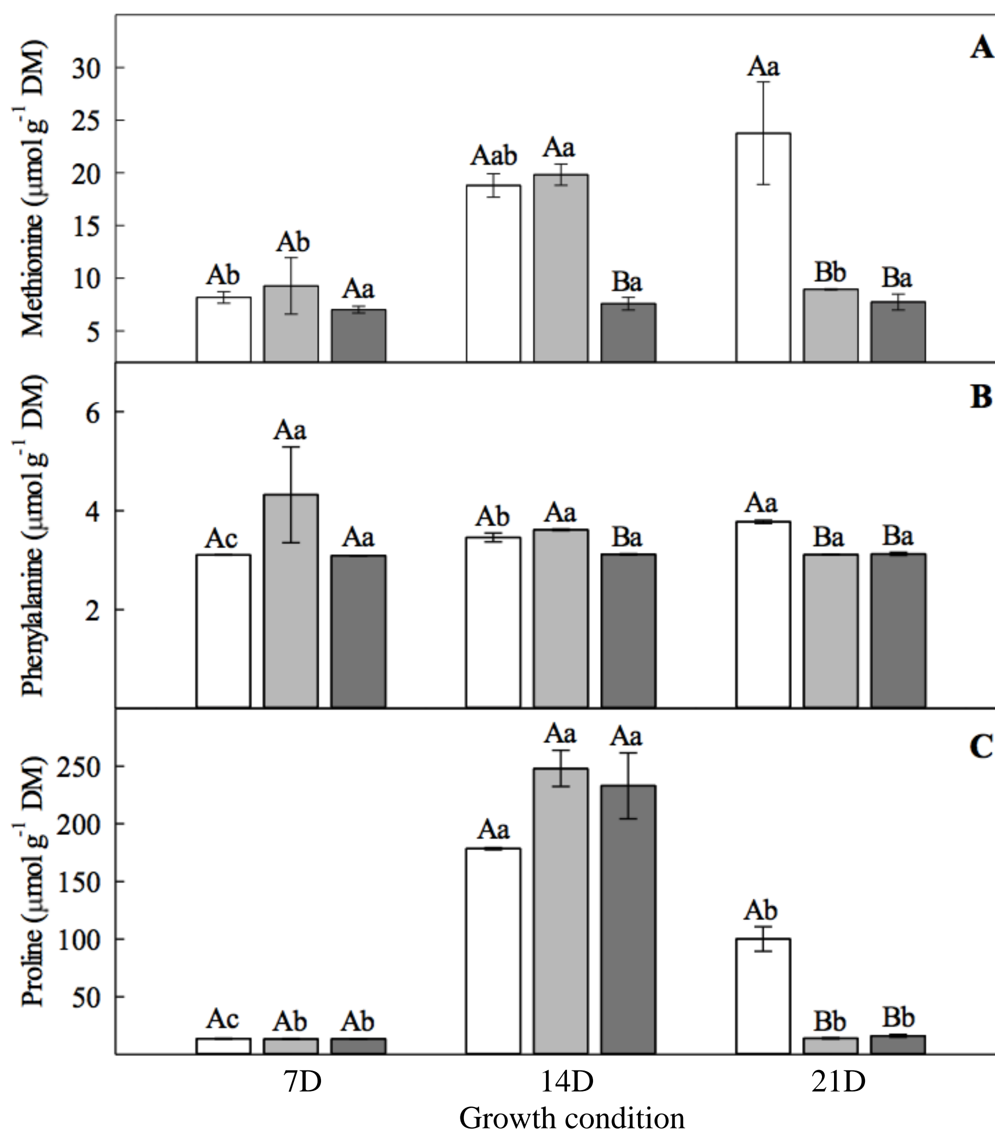

Fig. 5.8. Methionine, phenylalanine, and proline content of canola plants grown for three time periods (7D, 7 days; 14D, 14 days; 21D, 21 days), and treated with either no exogenous ethylene (control, open bars), 1 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (low, light gray bars), or 2 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (high, medium gray bars). (A) methionine; (B) phenylalanine; (C) proline. Upper case, within each growth time; lower-case letters, within each ethylene treatment duration. Otherwise, as per Fig. 5.1.

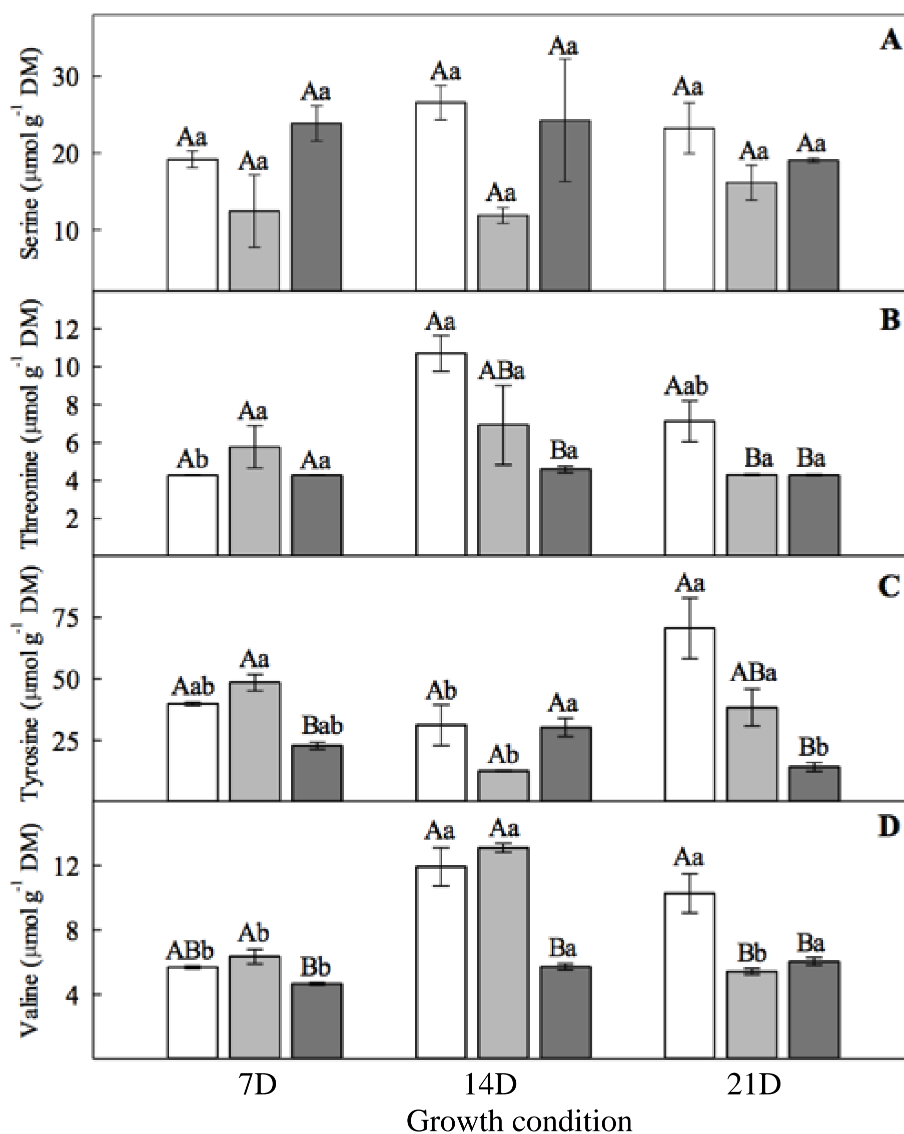

Fig. 5.9. Serine, threonine, tyrosine, and valine content of canola plants grown for three time periods (7D, 7 days; 14D, 14 days; 21D, 21 days), and treated with either no exogenous ethylene (control, open bars), 1 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (low, light gray bars), or 2 h of daily treatment with 36 mmol L^{-1} exogenous ethylene (high, medium gray bars). (A) serine; (B) threonine; (C) tyrosine; (D) valine. Upper case, within each ethylene treatment duration; lower-case letters, each growth time. Otherwise, as per Fig. 5.1.

5.3.9. Pearson's correlation coefficients

In this study, both methane and ethylene have shown significant relationships with a number of measured parameters, including a positive correlation between methane and ethylene. Both aerobic methane emissions and ethylene evolution were negatively correlated with all plant growth and biomass parameters, gas exchange, effective quantum yield of PSII (ϕPSII), chlorophyll and carotenoid content, and NBI (Table 5.5). Furthermore, methane and ethylene were both negatively correlated with Ala, Asp, Ile, Met, and Tyr, and ethylene was additionally negatively correlated with Pro (Table 5.6).

Table 5.5. Pearson's correlation coefficients (upper value) and *P*-values (lower value) for relationship between methane emissions, ethylene evolution, and growth and physiological parameters of canola plants grown for 21 and treated with either 0 h, 1 h, or 2 h of 36 mmol L^{-1} exogenous ethylene application. Only significant relationships are shown.

Factor	Methane	Ethylene
Ethylene	**0.901**	-
	0.001	
Moisture content	**-0.860**	**-0.956**
	0.003	**0.000**
Stem height	**-0.732**	**-0.787**
	0.025	**0.012**
Stem diameter	**-0.839**	**-0.895**
	0.005	**0.001**
Leaf area	**-0.767**	**-0.821**
	0.016	**0.007**
Total biomass	**-0.869**	**-0.881**
	0.002	**0.002**
A_N	**-0.873**	**-0.938**
	0.002	**0.000**
E	**-0.686**	**-0.726**
	0.041	**0.027**
WUE	**-0.779**	**-0.841**
	0.013	**0.004**
ϕPSII	**-0.909**	**-0.992**
	0.001	**0.000**
Total Chl	**-0.835**	**-0.904**
	0.003	**0.001**
Carotenoids	**-0.779**	**-0.840**
	0.013	**0.005**
NBI	**-0.772**	**-0.832**
	0.015	**0.005**

Table 5.6. Pearson's correlation coefficients (upper values) and *P*-values (lower values) for relationship between methane emissions, ethylene evolution, and amino acid content of canola plants grown for 21 days and treated with either 0 h, 1 h, or 2 h of 36 mmol L^{-1} exogenous ethylene application. Only significant relationships are shown.

Factor	Methane	Ethylene
Ethylene	**0.901**	
	0.001	
Alanine	**-0.711**	**-0.760**
	0.032	**0.017**
Aspartic acid	**-0.679**	**-0.730**
	0.044	**0.026**
Isoleucine	**-0.837**	**-0.881**
	0.005	**0.002**
Methionine	**-0.630**	**-0.686**
	0.049	**0.041**
Proline	-0.659	**-0.695**
	0.054	**0.037**
Tyrosine	**-0.764**	**-0.856**
	0.017	**0.003**

5.4. DISCUSSION

The purpose of this study was to examine the effects of time and exogenous ethylene application on growth, physiological, and metabolic parameters of canola (*Brassica napus*). Plants were grown for 7, 14, or 21 days and applied with either 0 h, 1 h, or 2 h of daily treatment with 36 mmol L^{-1} of exogenous ethylene (C_2H_4) gas. Only methane, ethylene, and amino acid content were measured for plants grown for 7 and 14 days, due to the small size of plants and limited available leafy mass for measurements. Plants grown for 21 days were used for all measurements. These results showed that plants treated with exogenous ethylene were smaller, with decreased photosynthetic processes and chlorophyll content, and is clearly indicative of plant stress. The levels of exogenous C_2H_4 used in this study were therefore sufficient to result in stressful conditions, and were not conductive to plant growth and success (Hurr *et al*., 2009, 2010, 2013; Chidley *et al*., 2013; Amoah & Terry, 2016). While this extreme level of plant stress was not intended, it was the smallest amount of ethylene that could be obtained using the experimental apparatus.

In response to exogenous C_2H_4 treatment, ethylene evolution drastically increased, especially from plants treated for 2 h daily. Ethylene emissions increased from 7d to 14d, but were relatively unchanged from 14d to 21d, suggesting that plants may have acclimated during this time period (Fig. 5.1A). Methane emissions, on the other hand, decreased over time, but increased with exogenous ethylene treatment, and this was most evident in the plants treated for 2 h (Fig. 5.1B). These results are in agreement with earlier work that also showed a decrease in methane emissions over time in both sunflower (*Helianthus annuus*) and chrysanthemum (*Chrysanthemum coronoarium*) (Martel & Qaderi, 2017).

Additionally, prior research has shown that methane emissions increased from stressed plants (McLeod *et al*., 2008; Vigano *et al*., 2008; Bruhn *et al*., 2009; Qaderi & Reid, 2009; Wang *et al*., 2009; Wang *et al*., 2011a, b; Qaderi & Reid, 2011; Bruhn *et al*., 2014; Abdulamajeed & Qaderi, 2017; Abdulamajeed *et al*., 2017; Martel & Qaderi, 2017), which is further confirmed in this study. Since methane and ethylene emissions were positively correlated with each other, this may represent a relationship between the two; however, this may be misleading. Application of ethylene gas increased ethylene emissions from plants, likely due to ethylene stored in leaf intercellular spaces; similarly, the stress conditions increased methane emission, which was likely indirectly caused by ethylene as opposed to a direct relationship.

It has been reported that visible effects of exogenous ethylene appear in plants with concentrations as low as 1.0 $\mu L\ L^{-1}$ of ethylene gas (Lee & LaRue, 1992), whereas in this study, values of 36 mmol L^{-1} were used, and it is therefore expected that this extreme value of ethylene caused stress conditions within the plants. Such an extreme value of ethylene was used in this study because it was the lowest possible concentration that could be obtained using pure ethylene gas, and was achieved by opening the ethylene cylinders for 15 s. It must therefore be acknowledged that, while the plants had obvious visible changes, these changes are not reflective of the expected changes to plant growth and physiology in response to natural levels of ethylene, and the conditions used in this experiment were a stress condition for the plants.

Decreased growth, chlorophyll content, and gas exchange observed in this study are in agreement with earlier studies on soybean (*Glycine max*, Wullschleger *et al*., 1992),

sunflower and peas (*Pisum sativum*, Kays and Pallas, 1980), and the decrease in effective and maximum quantum yield of PSII (Fig. 5.4E, F) is in agreement with work done on sunflower and peas (Kays and Pallas, 1980). Ethylene promotes stomatal closure through mediated ROS production in guard cells of the stomata (Kazan *et al*., 2015); in stress conditions, the mediation of this response may have decreased, leading to increased ROS production. In conjunction with the decreased efficiency at capturing light and dissipating it as heat, shown through decreased chlorophyll fluorescence parameters, it is expected that canola plants treated with exogenous ethylene would have had increased production of reactive oxygen species (ROS). While flavonoid content almost doubled between the control plants and the plants treated for 2 h (Kazan *et al*., 2015), it is unlikely that this was sufficient to scavenge the over-production of H_2O_2 and other ROS compounds (Hurr *et al*., 2013). This would have increased methane production through increased methyl group cleavage of methane precursors.

In sunflower and pea, exogenous ethylene resulted in increased proteolysis, chlorophyll degradation, and loss of thylakoid integrity (Wullschleger *et al*., 1992), and it might therefore have disrupted some portion of the electron transport pathway. Since previous studies have shown that disruption of the electron transport chain, through disruption of cytochrome c oxidase (Wishkerman *et al*., 2011), increased aerobic methane emissions, this is relevant, and may have contributed to the observed increase in methane emissions in this study.

From a metabolic standpoint, no studies were found that examined the effect of exogenous ethylene on the production of primary metabolites, including amino acids. It is

known that ethylene influences plant metabolism, as exogenous ethylene, through application of the ethylene-releasing compound 2-chloroethylphosphonic acid, increased expression of genes related to anthocyanin biosynthesis, leading to an overall increase in anthocyanins in grape berries (*Vitis vinifera* L., El-Kereamy *et al*., 2003). Also, a study of several postharvest fruits exposed to exogenous ethylene showed an increase in phenol production (Heredia & Cisneros-Zevallos, 2009). In another study, ethylene promoted the accumulation of flavonoids (Kazan *et al*., 2015). In mango, ethylene induced gene transcription as well as the production of a number of metabolites, as shown by the rapid decay of post-harvest mango fruit in response to exogenous ethylene (Chidley *et al*., 2013). Based on these results, it is expected that exogenous ethylene would have influenced primary metabolism as well, since primary metabolites are precursors for secondary metabolites, including phenols.

In this study, it was found that about half of the quantified amino acids increased over time (Asp, Ile, Leu, Met, Pro, Thr, and Val), whereas only Ala and Gly decreased over time, and the remaining four were unaffected. It is expected that amino acids would increase over time as the plants grew larger, and this was especially evident in control plants. Exogenous ethylene, on the other hand, increased only two amino acids (Ala and Gly), and these were the same two that decreased over time. Only Phe and Pro were unaffected by ethylene, and the rest were decreased by exogenous ethylene treatment, with the lowest content observed in plants treated for 2 h daily, at one of the growth times, for every amino acid except serine (Figs. 5.6 – 5.8.). Since exogenous ethylene has been shown to increase macromolecule catabolism in a number of plants (Hurr *et al*., 2013; Amoah &

Terry, 2016), the decrease in amino acid content could have been the result of similar processes (Häusler *et al*., 2014; Hildebrandt *et al*., 2015). The catabolism of these compounds likely resulted in methyl group release, contributing to the observed aerobic methane emissions.

Amino acid catabolism in plants in the vegetative stage is generally representative of plant cells under stressed or senescent conditions. During senescence, proteins are degraded into amino acids, and resources are reallocated from leaves to other plant parts to promote early flowering and seed development. Senescence eventually leads to autophagy, a self-degradative process to sacrifice leaves in times of severe stress (Hildebrandt *et al*., 2015). Plants grown under exogenous ethylene had a reduced leaf number, with these plants showing visible symptoms of necrosis, and it is possible they were in the process of autophagy at the termination of the experiment. If plants were left to grow longer, it is hypothesized that autophagy of lower leaves would have occurred along with early flowering. Since exogenous ethylene has been shown to induce programmed cell death, this is reasonable (Hurr *et al*., 2010).

On the basis of Pearson's correlation coefficients, methane and ethylene were both shown to be related to a number of physiological and biochemical parameters, with all having negative correlations (Tables 5.5 – 5.6). As plant size, chlorophyll content, gas exchange, moisture content, and amino acid content decreased, both methane and ethylene increased. These results are in support of smaller, stressed plants emitting more methane in comparison to healthy, non-stressed plants. Since methionine was not the only amino acid to be negatively correlated with methane, these results also support the hypothesis that

methane release is the result of methyl group cleavage from a number of metabolites by ROS compounds (Messenger *et al*., 2009). Since ROS, including H_2O_2, were expected to increase in this study based on obtained results, and amino acids were decreased, it is expected that ROS compounds enhanced protein degradation and amino acid catabolism to induce leaf autophagy. In order to confirm this, future studies on aerobic methane emissions should measure ROS. Since these plants were under a severe stress condition, as the ethylene quantity in this study was much larger than the quantities shown in prior studies to induce a stress response, it is expected that leaves were in the process of being sacrificed so the plant could survive long enough to reproduce.

In conclusion, this study showed a drastic increase of ethylene evolution and a significant increase in aerobic methane emissions in response to exogenous ethylene treatment. Plants treated with ethylene had decreased growth, biomass, gas exchange, chlorophyll fluorescence, chlorophyll content, and decreased content of nine of the 13 measured amino acids. The results obtained in this study suggest that production of ROS increased, leading to decreased cellular integrity, increased amino acid catabolism, and possibly the induction of autophagy. In combination, these responses led to increased methane emissions. These results suggest that aerobic methane release is the result of methyl group cleavage. Since precursors, such as methionine (Lenhart *et al*., 2015), chlorophyll (Fraser *et al*., 2015), epicuticular wax (Bruhn *et al*., 2014), pectin (Bruhn *et al*., 2012), and ascorbic acid (Althoff *et al*., 2010), have been proposed, it is possible that the methane emissions in this study are additive of methyl groups from many different sources. Irrespective of the small contribution of aerobic methane emissions to the global methane

budget, environmental stress conditions will increase in the future, leading to an increased contribution of this source to the global budget. It is therefore important to understand its mechanism of production in order to potentially alleviate aerobic methane release. Since increased antioxidant capacity could potentially minimize emissions through increased ROS scavenging, and antioxidants are nutritionally desirable, this method of mitigation deserves examination in the future.

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6.0. GENERAL CONCLUSIONS

The four original objectives of this thesis were to (i) investigate the potential of ethylene, a product of the methionine pathway, to act as a precursor of methane; (ii) assess whether the primary metabolite L-methionine is a unique precursor; (iii) determine whether there is a unique biochemical pathway in the formation of aerobic methane from plants; and (iv) examine the effect of environmental factors on these parameters.

Application of ethylene promoters and inhibitors had very little effect on aerobic methane emissions, leading to the belief that ethylene promotion or inhibition would not lead to direct effects on aerobic methane emissions. The high-energy double bond present in ethylene makes it unlikely that ethylene is cleaved directly to produce two molecules of methane. To further assess this, one promoter and one inhibitor were applied to plants grown under a number of environmental conditions that were known to stimulate methane release. As expected, increasing light intensity decreased methane emissions, whereas increasing blue light increased emissions, and the ethylene promoter and inhibitor only exhibited observable effects when plants were grown under the highest level of blue light. Higher temperatures, however, had no effect on methane emissions; since a genetically engineered cultivar of canola was used in this study, it is possible that this cultivar is resistant to temperature stress and has a higher optimal temperature. Since there has been no prior work done on the resistance of this cultivar to other stresses, this cannot be confirmed. Throughout these experiments, methane emissions were shown to have relationships with multiple amino acids, suggesting that L-methionine may not be a unique precursor.

In the final experiment, exogenous ethylene treatment significantly increased methane emissions from plants, and ultimately led to conditions of extreme stress for canola plants. Chlorophyll degradation, decreased plant size, reduced chlorophyll fluorescence, and a reduction in amino acid content supported the hypothesis that reactive oxygen species (ROS) were being produced in abundance. This likely led to the catabolism of amino acids along with many other plant compounds, leading to the cleavage and subsequent liberation of methyl groups. This thesis supports the prior hypothesis that aerobic methane emissions are the by-product of ROS, such as hydrogen peroxide (H_2O_2), reacting with a number of plant compounds, and rejects the hypothesis that there is one unique precursor to aerobic methane emissions from plants. Since carbon is a valuable resource for plants, release of carbon molecules via aerobic methane is unlikely to be adaptive; instead, this process may represent a maladaptive process that occurs as a result of increased ROS in the plant, when the plant is ill-equipped to scavenge all the radicals. This typically occurs in plant stress, which would explain why stressed plants release more methane.

Based on these findings, it is expected that aerobic methane emissions will increase in the future. Increased stress conditions, including both biotic and abiotic stresses, will occur in the future as a result of climate change. These stress conditions often induce the formation of smaller, stressed plants with increased ROS production. Increased production of epicuticular wax, increased amino acid catabolism, disruption of the electron transport pathway, chlorophyll degradation, and a number of other processes will result in methyl group cleavage from many different compounds, resulting in a loss of resources for the

plant. Increased amino acid catabolism may decrease the nutritional value of plants, and the production of smaller plants in times of stress may result in agricultural losses.

The findings of this thesis are useful and applicable in many ways. Primarily, evidence presented here suggests that there is no novel pathway for the production of methane emissions, but that plants are instead losing carbon resources through ROS-mediated cleavage of methyl groups. Since high light intensity may overload the photosystem, blue light is a high-energy wavelength, and exogenous ethylene leads to oxidative stress and autophagy, all of these conditions may promote the evolution of ROS compounds and subsequent release of methane. Since ROS compounds are scavenged by antioxidant compounds, such as flavonoids and anthocyanins, and antioxidants are desirable characteristics for plant nutrition, selective breeding and genetic engineering to enhance plant antioxidants will yield multiple benefits. Secondly, knowledge of how methane emissions are released from plants may lead to a more accurate quantification of the global methane budget. Aerobic methane emissions from plants will continue to rise in the future due to increased climatic extremes, and research into the mitigation of this source is important. Finally, since canola is such a large contributor to the Canadian economy, further studies on the responses of this oilseed crop to climate change factors should be encouraged.

APPENDIX A: SUPPLEMENTARY INFORMATION, CHAPTER 3

Table A1. Effects of temperature, light intensity, and chemical application on methane, ethylene, plant growth, and biomass accumulation of canola (*Brassica napus*) plants (n=12). Plants were grown under two temperature regimes (22/18°C or 28/24°C), two light intensities (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}), and three chemical applications (control, 100 µl 10^{-4} M kinetin, 100 µl 10^{-4} M silver nitrate every other day). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. Methane (ng g^{-1} dry mass (DM) h^{-1}); ethylene (pmol g^{-1} fresh mass (FM) h^{-1}); stem height (mm); stem diameter (mm); leaf number ($plant^{-1}$); leaf area (cm^{-2}); dry mass (mg).

Properties	Parameters	Temperature		Light intensity		Chemical application		
		Lower	Higher	Lower	Higher	Control	Kinetin	Silver nitrate
Emissions	Methane	55.5 ± 2.9 A	48.1 ± 2.3 A	69.2 ± 1.9 A	35.0 ± 1.3 B	51.2 ± 3.1 A	53.8 ± 3.4 A	50.9 ± 2.8 A
	Ethylene	95.5 ± 0.2 A	105.4 ± 0.2 A	166.4 ± 0.4 A	54.4 ± 0.4 B	128.3 ± 0.5 A	111.2 ± 0.3 A	93.7 ± 0.5 A
Plant growth	Stem height	24.5 ± 1.4 A	26.3 ± 1.5 A	34.2 ± 1.1 A	16.6 ± 0.4 B	26.6 ± 2.3 A	24.6 ± 1.6 A	25.0 ± 1.5 A
	Stem diameter	3.5 ± 0.1 A	3.0 ± 0.1 A	2.9 ± 0.1 B	3.7 ± 0.1 A	3.3 ± 0.1 A	3.4 ± 0.1 A	3.2 ± 0.1 A
	Leaf number	8.8 ± 0.1 A	8.4 ± 0.2 A	9.0 ± 0.1 A	8.2 ± 0.2 B	8.6 ± 0.2 A	8.5 ± 0.2 A	8.7 ± 0.2 A
	Leaf area	89.5 ± 3.3 A	90.1 ± 3.3 A	90.2 ± 3.6 A	89.4 ± 3.0 A	94.1 ± 3.8 A	89.0 ± 4.0 A	86.4 ± 4.2 A
	Growth rate	0.9 ± 0.1 A	1.0 ± 0.1 A	1.4 ± 0.1 A	0.5 ± 0.0 B	1.0 ± 0.1 A	0.9 ± 0.1 A	0.9 ± 0.1 A
Dry mass	Leaf mass	304.2 ± 22.1 A	301.1 ± 19.7 A	213.0 ± 9.0 B	392.4 ± 22.1 A	314.5 ± 24.7 A	294.2 ± 23.8 A	299.3 ± 28.4 A
	Stem mass	66.7 ± 3.8 A	54.3 ± 2.8 B	50.5 ± 2.8 B	70.5 ± 3.5 A	65.7 ± 4.2 A	56.4 ± 3.4 A	59.4 ± 4.8 A
	Root mass	99.9 ± 14.5 A	71.6 ± 5.5 B	44.7 ± 2.2 B	126.8 ± A	76.5 ± 10.9 A	82.4 ± 12.0 A	98.3 ± 17.1 A
	Total mass	470.8 ± 38.7 A	427.0 ± 25.9 A	308.1 ± 12.9 B	589.7 ± 35.7 A	456.7 ± 50.1 A	433.1 ± 117 A	457.0 ± 48.0 A

Table A2. Effects of temperature, light intensity, and chemical application on growth indices and gas exchange of canola (*Brassica napus*) plants (n=12). Plants were grown under two temperature regimes (22/18°C or 28/24°C), two light intensities (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}), and three chemical applications (control, 100 µl 10^{-4} M kinetin, 100 µl 10^{-4} M silver nitrate every other day). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. LMA, leaf mass per area (g m^{-2}); LMR, leaf mass ratio; LAR, leaf area ratio (cm^{-2} g^{-1}); shoot to root (S:R) mass ratio; A_N, net CO_2 assimilation (µmol CO_2 m^{-2} s^{-1}); *E*, transpiration, mmol H_2O m^{-2} s^{-1}); g_s, stomatal conductance (mol H_2O m^{-2} s^{-1}); WUE, water use efficiency (µmol CO_2 $mmol^{-1}$ H_2O).

Properties	Parameters	Temperature		Light intensity		Chemical application		
		Lower	Higher	Lower	Higher	Control	Kinetin	Silver nitrate
Growth index	LMA	33.1 ± 1.7 A	34.9 ± 2.4 A	24.1 ± 0.7 B	44.0 ± 2.1 A	34.3 ± 2.6 A	33.1 ± 2.2 A	34.7 ± 2.7 A
	LMR	0.67 ± 0.01 B	0.70 ± 0.01 A	0.69 ± 0.00 A	0.67 ± 0.01 A	0.69 ± 0.01 A	0.68 ± 0.01 A	0.67 ± 0.01 A
	LAR	220.6 ± 8.3 B	248.3 ± 14.7 A	300.2 ± 8.9 A	168.7 ± 7.0 B	240.0 ± 16.9 A	234.6 ± 13.0 A	228.7 ± 14.3 A
	S:R mass ratio	1.0 ± 0.07 A	0.94 ± 0.08 B	1.18 ± 0.05 A	1.18 ± 0.05 B	1.22 ± 0.13 A	0.88 ± 0.06 A	0.81 ± 0.05 A
Gas exchange	A_N	5.7 ± 0.3 A	4.6 ± 0.1 B	4.5 ± 0.2 B	5.8 ± 0.2 A	5.6 ± 0.2 A	5.3 ± 0.3 A	4.5 ± 0.2 B
	E	3.4 ± 0.1 A	2.2 ± 0.1 B	2.6 ± 0.1 B	2.9 ± 0.2 A	3.1 ± 0.2 A	2.8 ± 0.1 A	2.5 ± 0.2 B
	g_s	0.23 ± 0.01 A	0.11 ± 0.01 B	0.19 ± 0.02 A	0.15 ± 0.02 B	0.19 ± 0.02 A	0.17 ±0.01 AB	0.15 ± 0.02 B
	WUE	1.8 ± 0.1 B	2.3 ± 0.1 A	2.0 ± 0.1 A	2.1 ± 0.1 B	2.0 ± 0.1 AB	1.9 ± 0.1 B	2.2 ± 0.2 A

Table A3. Effects of temperature, light intensity, and chemical application on chlorophyll fluorescence and photosynthetic pigments of canola (*Brassica napus*) plants (n=12). Plants were grown under two temperature regimes (22/18°C or 28/24°C), two light intensities (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}), and three chemical applications (control, 100 µl 10^{-4} M kinetin, 100 µl 10^{-4} M silver nitrate every other day). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. ϕPSII, effective quantum yield of PSII; F_v/F_m, maximum quantum yield of PSII; qNP, non-photochemical quenching; qP, photochemical quenching; chl (chlorophyll) *a* (mg g^{-1}), chl *b* (mg g^{-1}), total chl (mg g^{-1}), carotenoids (mg g^{-1}).

Properties	Parameters	Temperature		Light intensity		Chemical application		
		Lower	Higher	Lower	Higher	Control	Kinetin	Silver nitrate
Chl. fluorescence	ϕPSII	0.74 ± 0.01 A	0.75 ± 0.01 A	0.73 ± 0.01 B	0.76 ± 0.01 A	0.73 ± 0.01 A	0.75 ± 0.01 A	0.75 ± 0.01 A
	F_v/F_m	0.67 ± 0.01 A	0.67 ± 0.01 A	0.69 ± 0.01 A	0.65 ± 0.00 B	0.67 ± 0.01 A	0.67 ± 0.01 A	0.67 ± 0.01 A
	qNP	1.14 ± 0.05 A	0.97 ± 0.04 B	0.93 ± 0.03 B	1.18 ± 0.05 A	1.10 ± 0.05 A	1.08 ± 0.06 A	0.99 ± 0.05 A
	qP	0.24 ± 0.02 A	0.29 ± 0.03 A	0.15 ± 0.01 B	0.38 ± 0.02 A	0.29 ± 0.03 A	0.29 ± 0.03 A	0.24 ± 0.02 A
Pigments	Chl *a*	2.7 ± 0.1 A	2.8 ± 0.1 A	3.4 ± 0.1 A	2.2 ± 0.1 B	2.7 ± 0.2 A	2.9 ± 0.2 A	2.8 ± 0.1 A
	Chl *b*	0.8 ± 0.0 A	0.8 ± 0.0 A	1.0 ± 0.0 A	0.6 ± 0.0 B	0.8 ± 0.0 A	0.8 ± 0.0 A	0.8 ± 0.0 A
	Total Chl	3.5 ± 0.2 A	3.6 ± 0.2 A	4.3 ± 0.2 A	2.8 ± 0.1 B	3.5 ± 0.2 A	3.7 ± 0.2 A	3.6 ± 0.2 A
	Chl *a*/*b*	3.6 ± 0.0 A	3.6 ± 0.1 A	3.5 ± 0.1 A	3.7 ± 0.1 A	3.5 ± 0.1 A	3.7 ± 0.1 A	3.5 ± 0.1 A
	Carotenoids	0.6 ± 0.0 A	0.6 ± 0.0 A	0.7 ± 0.0 A	0.5 ± 0.0 B	0.6 ± 0.0 A	0.6 ± 0.0 A	0.6 ± 0.0 A

Table A4. Effects of temperature, light intensity, and chemical application on nutrient balance, moisture content, and protective compounds of canola (*Brassica napus*) plants (n=12). Plants were grown under two temperature regimes (22/18°C or 28/24°C), two light intensities (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}), and three chemical applications (control, 100 µl 10^{-4} M kinetin, 100 µl 10^{-4} M silver nitrate every other day). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. NBI, nitrogen balance index; moisture (%); flavonoids (µg cm^{-2}); epicuticle wax (µg mg^{-1}).

Properties	Parameters	Temperature		Light intensity		Chemical application		
		Lower	Higher	Lower	Higher	Control	Kinetin	Silver nitrate
Nitrogen balance	NBI	64.9 ± 4.1 B	78.3 ± 3.8 A	93.1 ± 2.8 A	50.0 ± 2.9 B	75.0 ± 5.6 A	68.1 ± 4.8 A	71.6 ± 4.5 A
Moisture	Moisture %	86.4 ± 0.3 A	86.5 ± 0.3 A	88.6 ± 0.2 A	84.3 ± 0.3 B	86.6 ± 0.4 A	86.3 ± 0.4 A	86.5 ± 0.4 A
Protective compounds	Flavonoids	0.70 ± 0.0 A	0.59 ± 0.0 B	0.36 ± 0.0 B	0.94 ± 0.0 A	0.61 ± 0.1 A	0.7 ± 0.1 A	0.6 ± 0.0 A
	Wax	0.03 ± 0.00 B	0.04 ± 0.00 A	0.03 ± 0.00 B	0.04 ± 0.00 A	0.03 ± 0.00 A	0.03 ± 0.00 A	0.03 ± 0.00 A

Table A5. Effects of temperature, light intensity, and chemical application on amino acid content (µmol g^{-1} DM) of canola (*Brassica napus*) plants (n=9). Plants were grown under two temperature regimes (22/18°C or 28/24°C), two light intensities (300 µmol photon m^{-2} s^{-1} or 600 µmol photon m^{-2} s^{-1}), and three chemical applications (control, 100 µl 10^{-4} M kinetin, 100 µl 10^{-4} M silver nitrate every other day). Data are means ± SE (n = 9 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. Amino acid content measured in µmol g^{-1} of dry mass (DM).

Amino acid	Temperature		Light intensity		Chemical application		
	Lower	Higher	Lower	Higher	Control	Kinetin	Silver nitrate
Alanine	3.54 ± 0.21 A	2.84 ± 0.12 B	3.29 ± 0.23 A	3.09 ± 0.13 B	3.81 ± 0.26 A	2.93 ± 0.10 B	2.83 ± 0.04 B
Aspartic acid	6.86 ± 0.63 B	8.86 ± 0.83 A	8.25 ± 0.89 A	7.47 ± 0.63 B	6.04 ± 0.32 B	8.75 ± 0.68 A	8.78 ± 1.00 A
Glycine	8.36 ± 0.55 A	9.31 ± 0.69 A	7.76 ± 0.42 B	9.98 ± 0.69 A	9.31 ± 0.60 A	8.93 ± 0.60 A	8.31 ± 0.69 A
Isoleucine	2.81 ± 0.07 B	3.84 ± 0.26 A	2.97 ± 0.07 B	3.68 ± 0.29 A	3.36 ± 0.26 A	3.46 ± 0.27 A	3.15 ± 0.13 A
Leucine	40.99 ± 7.89 B	56.07 ± 5.92 A	46.6 ± 4.91 A	50.4 ± 8.91 A	60.27 ± 5.68 A	42.49 ± 7.91 B	42.84 ± 7.30 B
Lysine	89.18 ± 12.14 A	81.22 ± 9.38 A	64.27 ± 11.37 B	106.13 ± 7.52 A	65.40 ± 11.58 B	79.79 ± 10.42 B	110.41 ± 7.75 A
Methionine	15.20 ± 2.49 A	14.83 ± 1.25 A	18.40 ± 2.14 A	11.64 ± 1.36 B	15.92 ± 3.06 A	14.26 ± 0.95 A	14.86 ± 1.29 A
Phenylalanine	3.22 ± 0.02 A	3.28 ± 0.04 A	3.27 ± 0.04 A	3.24 ± 0.02 A	3.19 ± 0.01 B	3.35 ± 0.04 A	3.21 ± 0.02 B
Proline	27.41 ± 2.92 B	123.91 ± 19.13 A	60.41 ± 16.84 B	90.91 ± 18.37 A	65.57 ± 19.98 B	104.97 ± 21.18 A	56.45 ± 8.58 B
Serine	32.05 ± 3.37 A	33.20 ± 4.77 A	28.15 ± 2.87 B	37.09 ± 4.84 A	29.68 ± 4.06 AB	39.62 ± 4.15 A	28.57 ± 3.80 B
Threonine	6.80 ± 0.51 A	7.47 ± 1.45 A	5.81 ± 0.37 B	8.47 ± 1.43 A	6.03 ± 0.35 AB	9.70 ± 1.69 A	5.69 ± 0.33 B
Tyrosine	38.71 ± 3.76 A	28.84 ± 3.32 B	28.74 ± 3.51 B	38.81 ± 3.57 A	25.82 ± 3.98 B	35.26 ± 2.56 A	40.23 ± 3.80 A
Valine	6.90 ± 0.39 B	8.72 ± 0.79 A	6.32 ± 0.27 B	9.30 ± 0.73 A	6.15 ± 0.28 B	8.39 ± 0.62 A	8.88 ± 0.79 A

APPENDIX B: SUPPLEMENTARY INFORMATION, CHAPTER 4

Table B1. Effects of blue light and chemical application on growth and physiological parameters of canola (*Brassica napus*) plants grown under four different levels of blue light (control, 0.4 µmol photon m^{-2} s^{-1}, 4.0 µmol photon m^{-2} s^{-1}, or 8 µmol photon m^{-2} s^{-1}) and applied with either no chemical, an ethylene promoter (10^{-4} M kinetin) or ethylene inhibitor (10^{-4} M silver nitrate). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. Methane (ng g^{-1} dry mass (DM) h^{-1}); ethylene (pmol g^{-1} fresh mass (FM) h^{-1}); stem height (mm); stem diameter (mm); leaf number (plant^{-1}); leaf area (cm^{-2}); dry mass (mg).

Properties	Parameters	Blue light				Chemical application		
		Control	Low	Medium	High	Control	Kinetin	Silver nitrate
Emissions	Methane	58.9 ± 1.7 C	87.3 ± 2.3 B	97.2 ± 3.4 AB	103.6 ± 3.7 A	86.8 ± 3.3 A	84.4 ± 3.0 A	81.5 ± 3.1 A
	Ethylene	186 ± 23 AB	1701 ± 26 AB	277 ± 28 A	115 ± 14 B	235 ± 26 A	213 ± 27 A	143 ± 13 B
Growth	Stem height	54.3 ± 3.1 B	59.0 ± 2.4 A	64.0 ± 3.1 A	52.0 ± 2.1 B	61.9 ± 2.7 A	57.6 ± 2.4 A	54.1 ± 2.4 A
	Stem diam.	3.5 ± 0.1 A	2.4 ± 0.1 B	1.9 ± 0.0 C	1.4 ± 0.0 D	2.4 ± 0.1 A	2.3 ± 0.1 A	2.3 ± 0.1 A
	Leaf number	9.0 ± 0.2 A	9.4 ± 0.1 A	9.3 ± 0.1 A	8.8 ± 0.1 A	9.1 ± 0.1 A	9.1 ± 0.1 A	9.3 ± 0.1 A
	Leaf area	192.9 ± 6.1 A	110.1 ± 4.5 B	99.6 ± 6.1 B	80.6 ± 2.7 B	118.8 ± 7.1 A	130.1 ± 8.1 A	125.3 ± 7.7 A
	Growth rate	2.2 ± 0.1 A	2.5 ± 0.1 A	2.7 ± 0.1 A	2.1 ± 0.1 A	2.6 ± 0.1 A	2.4 ± 0.1 A	2.2 ± 0.1 A
Dry mass	Leaf mass	0.49 ± 0.02 A	0.17 ± 0.01 B	0.16 ± 0.01 B	0.22 ± 0.01 B	0.27 ± 0.03 A	0.25 ± 0.02 A	0.27 ± 0.03 A
	Stem mass	0.17 ± 0.01 A	0.06 ± 0.00 B	0.05 ± 0.00 B	0.06 ± 0.00 B	0.10 ± 0.01 A	0.09 ± 0.01 A	0.08 ± 0.01 A
	Root mass	0.10 ± 0.00 A	0.03 ± 0.00 B	0.04 ± 0.00 B	0.04 ± 0.00 B	0.05 ± 0.00 A	0.06 ± 0.00 A	0.05 ± 0.00 A
	Total mass	0.75 ± 0.04 A	0.27 ± 0.01 B	0.25 ± 0.01 B	0.32 ± 0.01 B	0.41 ± 0.04 A	0.40 ± 0.03 A	0.40 ± 0.00 A

Table B2. Effects of blue light and chemical application on growth indices and gas exchange of canola (*Brassica napus*) plants grown under four different levels of blue light (control, 0.4 µmol photon m^{-2} s^{-1}, 4.0 µmol photon m^{-2} s^{-1}, or 8 µmol photon m^{-2} s^{-1}) and applied with either no chemical, an ethylene promoter (10^{-4} M kinetin) or ethylene inhibitor (10^{-4} M silver nitrate). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. LMA, leaf mass per area (g m^{-2}); LMR, leaf mass ratio; LAR, leaf area ratio (cm^{-2} g^{-1}); shoot to root (S:R) mass ratio; A_N, net CO_2 assimilation (µmol CO_2 m^{-2} s^{-1}); *E*, transpiration, mmol H_2O m^{-2} s^{-1}); g_s, stomatal conductance (mol H_2O m^{-2} s^{-1}); WUE, water use efficiency (µmol CO_2 $mmol^{-1}$ H_2O).

Properties	Parameters	Blue light				Chemical application		
		Control	Lower	Medium	High	Control	Kinetin	Silver nitrate
Indices	LMA	25.0 ± 1.1 A	15.7 ± 0.6 B	17.3 ± 0.7 B	27.6 ± 1.4 A	19.7 ± 0.7 A	19.7 ± 0.7 A	20.6 ± 0.8 A
	LMR	0.6 ± 0.0 A	0.6 ± 0.0 A	0.7 ± 0.0 A	0.7 ± 0.0 A	0.6 ± 0.0 B	0.6 ± 0.0 B	0.7 ± 0.0 A
	LAR	273.6 ± 11.0 B	429.5 ± 15.4 A	397.2 ± 13.5 A	255.9 ± 9.7 B	348.6 ± 20.1 A	344.3 ± 12.1 A	348.7 ± 13.1 A
	S:R ratio	1.9 ± 0.2 A	1.8 ± 0.1 A	1.5 ± 0.1 A	1.6 ± 0.1 A	2.0 ± 0.1 A	1.6 ± 0.1 A	1.6 ± 0.1 A
Gas exchange	A_N	9.0 ± 0.3 A	5.8 ± 0.1 B	5.8 ± 0.1 B	4.9 ± 0.0 C	6.6 ± 0.4 A	6.5 ± 0.3 A	6.0 ± 0.2 B
	E	7.3 ± 0.3 A	6.7 ± 0.2 AB	6.4 ± 0.2 B	6.9 ± 0.1 AB	6.8 ± 0.2 A	7.0 ± 0.2 A	6.6 ± 0.2 A
	g_s	1.1 ± 0.1 A	0.4 ± 0.0 B	0.3 ±0.0 B	0.5 ±0.1 B	0.8 ±0.1 A	0.7 ± 0.1 B	0.3 ± 0.0 B
	WUE	1.3 ± 0.1 A	0.9 ± 0.0 B	0.9 ± 0.0 B	0.7 ± 0.0 C	1.0 ± 0.0 A	0.9 ± 0.0 A	0.9 ± 0.0 A

Table B3. Effects of blue light and chemical application on growth indices and gas exchange of canola (*Brassica napus*) plants grown under four different levels of blue light (control, 0.4 µmol photon m^{-2} s^{-1}, 4.0 µmol photon m^{-2} s^{-1}, or 8 µmol photon m^{-2} s^{-1}) and applied with either no chemical, an ethylene promoter (10^{-4} M kinetin) or ethylene inhibitor (10^{-4} M silver nitrate). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. ϕPSII, effective quantum yield of PSII; F_v/F_m, maximum quantum yield of PSII; qNP, non-photochemical quenching; qP, photochemical quenching; chl (chlorophyll) *a* (mg g^{-1}), chl *b* (mg g^{-1}), total chl (mg g^{-1}), carotenoids (mg g^{-1}).

Properties	Parameters	Blue light				Chemical application		
		Control	Lower	Medium	High	Control	Kinetin	Silver nitrate
Fluorescence	ϕPSII	0.7 ± 0.0 C	0.7 ± 0.0 BC	0.8 ± 0.0 B	0.8 ± 0.0 A	0.8 ± 0.0 A	0.8 ± 0.0 A	0.8 ± 0.0 A
	F_v/F_m	0.7 ± 0.0 A	0.7 ± 0.0 A	0.7 ± 0.0 A	0.8 ± 0.0 A	0.7 ± 0.0 A	0.7 ± 0.0 A	0.7 ± 0.0 A
	qNP	0.9 ±0.0 B	0.9 ± 0.0 B	0.8 ± 0.0 B	1.3 ± 0.0 A	1.0 ± 0.0 A	1.0 ± 0.0 A	1.0 ± 0.0 A
	qP	0.2 ±0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ±0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A
Pigments	Chl *a*	4.6 ± 0.2 B	5.3 ± 0.1 A	5.0 ± 0.1 AB	5.3 ± 0.1 A	5.0 ± 0.1 B	4.7 ± 0.1 C	5.5 ± 0.1 A
	Chl *b*	1.2 ± 0.0 C	1.5 ± 0.3 AB	1.4 ± 0.0 B	1.6 ± 0.0 A	1.4 ± 0.0 AB	1.3 ± 0.0 B	1.5 ± 0.0 A
	Total Chl	5.8 ± 0.2 B	6.8 ± 0.2 A	6.3 ± 0.1 AB	6.9 ± 0.1 A	6.4 ± 0.5 B	6.0 ±0.1 B	7.0 ± 0.0 A
	Chl *a*/*b*	3.9 ± 0.0 A	3.6 ± 0.0 B	3.6 ± 0.0 BC	3.4 ±0.0 C	3.7 ± 0.0 A	3.5 ± 0.0 B	3.7 ± 0.0 A
	Carotenoids	0.9 ± 0.0 B	1.1 ± 0.0 A	0.9 ± 0.0 AB	1.0 ± 0.0 AB	1.0 ± 0.0 AB	0.9 ± 0.0 B	1.1 ± 0.0 A

Table B4. Effects of blue light and chemical application on nutrient balance, moisture content, and protective compounds of canola (*Brassica napus*) plants grown under four different levels of blue light (control, 0.4 µmol photon m^{-2} s^{-1}, 4.0 µmol photon m^{-2} s^{-1}, or 8 µmol photon m^{-2} s^{-1}) and applied with either no chemical, an ethylene promoter (10^{-4} M kinetin) or ethylene inhibitor (10^{-4} M silver nitrate). Data are means ± SE (n = 12 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. NBI, nitrogen balance index; moisture (%); flavonoids (µg cm^{-2}); epicuticle wax (µg mg^{-1}).

Properties	Parameters	Blue light				Chemical application		
		Control	Lower	Medium	Control	Lower	Medium	Control
Nitrogen balance	NBI	78.9 ± 2.6 C	104.2 ± 2.5 B	113.7 ± 1.5 AB	123.5 ± 3.1 A	104.5 ± 2.7 A	98.3 ± 3.6 A	106.3 ± 3.7 A
Moisture	Moisture %	89.1 ± 0.2 A	88.9 ± 0.2 A	89.3 ± 0.2 A	88.8 ± 0.2 A	89.3 ± 0.3 A	89.1 ±0.2 A	88.8 ± 0.2 A
Protective	Flavonoids	0.5 ± 0.0 A	0.3 ± 0.0 B	0.3 ± 0.0 B	0.3 ±0.0 B	0.3 ± 0.0 A	0.3 ± 0.0 A	0.3 ± 0.0 A
	Wax	1.9 ± 0.1 B	1.8 ± 0.1 B	2.3 ± 0.1 B	5.1 ± 0.2 A	2.5 ± 0.2 B	3.0 ± 0.3 A	2.9 ± 0.4 AB

Table B5. Effects of blue light and chemical application on amino acid content (µmol g^{-1} DM) of canola (*Brassica napus*) plants grown under four different levels of blue light (control, 0.4 µmol photon m^{-2} s^{-1}, 4.0 µmol photon m^{-2} s^{-1}, or 8 µmol photon m^{-2} s^{-1}) and applied with either no chemical, an ethylene promoter (10^{-4} M kinetin) or ethylene inhibitor (10^{-4} M silver nitrate). Data are means ± SE (n = 9 samples). Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. Amino acid content measured in µmol g^{-1} of dry mass (DM).

Amino acid	Blue light				Chemical application		
	Control	Low	Medium	High	Control	Kinetin	Silver nitrate
Alanine	4.2 ± 0.4 B	3.5 ± 0.1 C	5.3 ± 0.6 A	4.4 ± 0.3 B	4.6 ± 0.2 AB	4.7 ± 0.4 A	3.7 ± 0.2 B
Aspartic acid	8.0 ± 1.1 B	10.9 ± 1.1 A	17.2 ± 2.9 A	14.5 ± 2.1 A	10.7 ± 0.6 A	13.0 ± 2.0 A	14.2 ± 1.8 A
Glycine	3.4 ± 0.1 C	10.3 ± 1.3 A	12.8 ± 0.6 A	7.8 ± 0.3 B	8.6 ± 0.8 A	9.5 ± 1.1 A	8.2 ± 1.0 A
Isoleucine	2.7 ± 0.0 B	2.6 ± 0.0 B	2.7 ± 0.0 B	3.2 ± 0.1 A	2.7 ± 0.1 A	2.8 ± 0.0 A	2.8 ± 0.1 A
Leucine	3.9 ± 0.1 C	5.0 ± 0.2 B	4.8 ± 0.3 B	7.2 ± 0.5 A	6.0 ± 0.5 A	4.6 ± 0.2 C	5.1 ± 0.3 B
Lysine	133.3 ± 14.4 A	53.6 ± 8.5 B	32.2 ± 5.9 C	34.0 ± 3.0 C	44.6 ± 6.5 B	67.4 ± 12.7 A	77.8 ± 13.8 A
Methionine	8.9 ± 1.8 C	22.0 ± 2.7 B	45.4 ± 4.3 A	22.1 ± 2.1 B	21.1 ± 3.0 B	23.1 ± 2.9 B	29.5 ± 4.9 A
Phenylalanine	3.6 ± 0.3 A	3.2 ± 0.0 A	3.4 ± 0.1 A	3.5 ± 0.1 A	3.3 ± 0.0 A	3.5 ± 0.2 A	3.4 ± 0.0 A
Proline	14.0 ± 1.0 B	13.3 ± 0.2 B	13.1 ± 0.4 B	30.2 ± 3.7 A	19.7 ± 3.2 A	17.7 ± 1.7 A	15.6 ± 1.0 A
Serine	24.2 ± 4.7 B	25.4 ± 4.2 B	35.7 ± 2.1 B	89.9 ± 20.6 A	27.4 ± 2.9 A	49.7 ± 7.8 A	54.3 ± 14.5 A
Threonine	5.4 ± 0.3 B	5.4 ± 0.4 B	7.5 ± 0.3 B	10.5 ± 1.4 A	6.3 ± 0.5 A	7.5 ± 0.6 A	7.8 ± 1.0 A
Tyrosine	60.0 ± 5.7 A	28.6 ± 2.4 B	22.1 ± 3.9 B	13.3 ± 0.5 C	23.4 ± 2.7 C	30.8 ± 5.5 B	38.8 ± 5.6 A
Valine	6.4 ± 0.5 B	6.1 ± 0.2 B	6.3 ± 0.3 B	9.0 ± 0.6 A	6.0 ± 0.3 B	7.1 ± 0.3 AB	7.8 ± 0.5 A