Understanding the Nature of Aerobic Methane Emissions from Plants Grown under a Combination of Temperature, UVB Radiation and Watering Regime

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DEDICATION

To my two special persons, my father and my husband for their love and encouragement without their love and support I would not reach this moment and write my thesis

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ABSTRACT

Methane (CH_4) is an important greenhouse gas besides carbon dioxide, nitrous oxide, water vapour, ozone, and chlorofluorocarbons. The atmospheric concentration of CH_4 has nearly tripled since pre-industrial times. It is the most abundant organic molecule in the Earth's atmosphere and plays important roles in both the planet's radiative energy budget and global atmospheric chemistry due to its global warming potential being up to 34 times as powerful as carbon dioxide. Methane production is mainly associated with methanogenesis under anoxic conditions. A decade ago, it was reported that plants produce CH_4 under aerobic conditions by an unknown mechanism. Since then, many researchers have investigated the factors that influence CH_4 emissions from plants. Methane emissions from plants may contribute significantly to the global CH_4 budget. Most studies to date have examined the impact of single factors affecting plants, while the effects of multiple environmental factors and the interaction of abiotic stress factors on plants still need more exploration. In this thesis, pea (*Pisum sativum*) was used as a model species. Plants were grown in controlled-environment growth chambers under two temperature regimes (22/18°C and 28/24°C), two levels of ultraviolet-B radiation [0 (zero) and 5 (ambient) kJ m^{-2} d^{-1}], and two watering regimes (well-watered and water-stressed). In pea, I have confirmed that environmental stress factors, such as higher temperatures, supplemental UVB radiation, or water stress, as individual factor or in combination, can increase aerobic CH_4 emissions from plants. In addition, findings revealed interorgan and intrashoot variations in CH_4 emissions from plants. Methane emissions were highest from stem and upper part of the shoot, as these were affected the most by stress factors. I also measured CH_4 emissions from plants during vegetative and reproductive stages. In the vegetative stage, younger plants emitted more CH_4 compared to older plants. Also, in the reproductive stage, CH_4 emissions were higher from the younger pods than the older ones. In conclusion, the level of CH_4 emissions varied with plant varieties and organs, as well as with plant vegetative and reproductive stages.

Keywords: Aerobic methane, developmental stage, environmental factor, global warming, increased temperature, pea, *Pisum sativum*, plant varieties, UVB radiation, water stress.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A_{280}	Absorbance at wavelength 280 nm
ABA	Absisic Acid
A_N	Net CO_2 assimilation
ANOVA	Analysis of variance
AR1	First Assessment Report
AR2	Second Assessment Report
AR3	Third Assessment Report
AR4	Fourth Assessment Report
AR5	Fifth Assessment Report
CFCs	Chlorofluorocarbons
Chl	Chlorophyll
CO_2	Carbon dioxide
^oC	Degree Celsius
DM	Dry mass
DMSO	Dimethyl sulphoxide
DTM	days to maturity
E	Transpiration
EC	Electrical conductance
UNFCCC	United Nations Framework Convention on Climate Change
F_v/F_m	Maximum quantum yield of PSII
g	Gram
GC	Gas chromatography
GMO	Genetically modified organism
g_s	Stomatal conductance
GWP	Global warming potential
HCFC	Hydrochlorofluorocarbons
h	Hour
H_2O_2	Hydrogen peroxide
HFC	Hydrofluorocarbons
IAA	Indole-3-acetic acid
IPCC	Intergovermental Panel on Climate Change
kJ	Kilojoule
km	Kilometer
LAR	Leaf area ratio
LMA	Leaf mass per area
LMR	Leaf mass ratio
mm	Millimeter
µl	Microliter
N_2O	Nitrous oxide

NBI	Nitrogen balance index
O_2^1	Single oxygen
O_3	Ozone
OH	Hydroxyl radicals
ppb	Parts per billion
PPFD	Photosynthetic photon flux density
ppm	Part per million
ppmv	Part per million by volume
PSII	Photosystem II
RH	Relative humidity
RO	Alkoxy radicals
ROS	Reactive oxygen species
s	Second
SE	Standard error
SRR	Shoot-root mass ratio
Tg	Tera gram
UV	Ultraviolet spectroscopy
UVA	Ultra violate A
UVB	Ultra violate B
UVC	Ultra violate C
WUE	Water use efficiency
ϕPSII	Effective quantum yield of PSII
Ψ_{wmd}	Midday water potential

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CHAPTER 1

Introduction

1.1 Climate change

Life on Earth has been undergoing continuous changes due to the change in climate around it. Climate change is not just a scientific concern but encompasses a wide range of disciplines, such as economics, geopolitics, and health. The Intergovernmental Panel on Climate Change (IPCC) describes climate change as any change in climate over time due to either natural or human activity (Folland *et al*., 2001). IPCC definition of climate change is broader and differs from the one proposed by the United Nations Framework Convention on Climate Change (UNFCCC), where it is defined as "a change of climate attributed directly or indirectly to human activity that alters the composition of the global atmosphere and that is in addition to natural climate variability observed over comparable time periods" (Folland *et al*., 2001).

Climate change has affected the atmosphere, the oceans, the cryosphere, and the sea level. The Earth is predicted to warm by 1.5°C above preindustrial levels (Howarth, 2015), and may rise up to 6.4ºC more by the end of this century (Myhre *et al*., 2013). The warming primarily occurred in two phases: 1910 to 1940 and 1970 to present, with a brief cooling phase in the 1940s and 50s. The rate of warming appears to be on the increase; IPCC data revealed that each of the last three decades has been successively warmer than any other preceding decade since 1850 (IPCC, 2013). Between 1971 and 2010, the upper 120.7 km of the oceans warmed by 0.11ºC per decade. Greenland and Antarctic ice sheets have been losing mass, with an average ice loss from glacier estimated at between 91 and 361 Gt yr^{-1} over the period 1971 to 2009 (IPCC, 2013). The mean rate of global sea level rise in the same period is predicted to be between 1.5 and 1.9 mm yr^{-1}.

Climate change will have deleterious effects on the environment, plants, animals, and people. According to forecasts, the frequency and severity of storms will increase (Fealy and Maynooth, 2008). More geographical areas will be affected by droughts of greater magnitude and longer duration, and many regions in temperate zones will experience heat levels (Haines *et al.*, 2006; Myhre *et al.,* 2013). Low lying coastal regions and deltas are particularly at risk because the projected accelerated rise in sea levels will aggravate flood risk, wetland loss, erosion, and general environmental degradation (Fealy and Maynooth, 2008; Myhre *et al.,* 2013). The entire ecosystem - including agricultural systems - will be altered in some areas, resulting negative consequences for biodiversity.

These habitat changes will significantly impact plants and animals by either causing a loss of some local species or behavioral adaptations (Fealy and Maynooth, 2008; Wong *et al.*, 2015). Increased insect breeding, greater winter survival rates of invertebrates and changes in fish and bird migration are just some of the climatic change impacts on animals (Fealy and Maynooth, 2008). Changes in temperature and precipitation may also alter the global distribution of disease vectors, such as malaria (Haines *et al*., 2006).

1.2 Factors contributing to climate change

Concern about the late-20th-century increase in global temperatures has brought serious discussion on the factors contributing to climate change, including human-related causes, such as greenhouse gasses, and natural causes, such as solar irradiance, volcanic activity, the Earth's orbit around the Sun and ocean current (Crowley, 2000; Myhre *et al.,* 2013). For example, climate change is influenced by volcanic eruptions that deposit large volumes of ash, dust, hydrochloric acid and sulfur dioxide into the upper levels of the atmosphere (Atwell, 2001). These large deposits of gases and ashes affect the climate by blocking sun rays, causing a cooling effect. Blockage occurs when sulphur dioxide combines with water, forming light droplets of sulphuric acid that remain suspended in the atmosphere for years (Robock, 2000). The sulphuric acid droplets are shiny and therefore efficient reflectors of sunlight. Another factor affecting climate change is ocean currents. Oceans hold an immense amount of heat and play a vital role in regulating the planet's climate system (Rahmstorf, 2002). Evidence implicates ocean circulation in abrupt and dramatic climate shifts, such as massive surges of icebergs into the North Atlantic Ocean and temperature changes in Greenland (Rahmstorf, 2002).

Climate change in the post-Industrial Revolution period cannot, however, be fully explained by natural causes. Most evidence strongly points to anthropogenic sources, and in particular, greenhouse gases (Myhre *et al*., 2013).

1.3 Greenhouse gases

Greenhouse gases are gases that have the ability to absorb and emit long-wave radiation within the thermal infrared range (Ramanathan *et al.*, 2009), and trapping heat within the atmosphere. The most common greenhouse gases are CO_2, methane (CH_4), nitrous oxide

(N_2O), water vapour (H_2O), ozone (O_3), and a number of chlorofluorocarbons, such as fluorinated gases and hydocarbons (Jain *et al.*, 2000). Different greenhouse gases have different effects on the planet's warming system. Two fundamental ways in which these gases differ from each other are their ability to absorb energy, and how long they remain trapped in the atmosphere.

The Global Warming Potential (GWP) concept was developed to compare the global warming impacts of different gases. IPCC typically uses 100 years as the time frame for the calculation of the GWP (Myhre *et al.,* 2013). This allows us to compare the global warming impact of greenhouse gases relative to CO_2 (Table 1.1; Alvarez *et al*., 2012); GWP is the amount of energy that can be absorbed by 1 tonne of gas in a given period of time, relative to 1 tonne CO_2 (Myhre *et al.,* 2013). The global warming potential over a 100-year time horizon of some of these gases is provided in Table 1.1.

1.3.1 Carbon dioxide

Carbon dioxide is the second most abundant greenhouse gas in the Earth's atmosphere (after water vapour) (Sung *et al.,* 2009). It enters the planet's atmosphere through various natural and anthropogenic sources. Dying and dormant plants (decomposition), metamorphism, and volcanism (although the latter is a very small source) are considered natural sources of CO_2 (Burton, 2013). Anthropogenic activities, including combustion of fossil fuel (coal, oil, and natural gas), industrial chemical reactions, and solid waste, account for the largest amount of CO_2 emissions, (Casper, 2010). The four primary industrial processes responsible for the CO_2 emissions are manufacturing of metals, such as aluminum, manufacturing of chemicals, such as ammonia, use of petroleum products in feedstocks, and manufacturing of mineral products, such as cement, soda ash, and lime (Casper, 2010).

Fossil fuel combustion and deforestation alone have caused the concentration of CO_2 to increase by 43% since the beginning of the Industrial Revolution; tropospheric mixing ratio of CO_2 increased globally from 276-280 μmol mol^{-1} to 390.3-390.7 μmol mol^{-1} between 1750 and 2011 (Myhre *et al*., 2013). While all greenhouse gases cause temperature levels to increase, the emissions of CO_2 is of particular concern because of its high radiative efficiency and longer lifetime (Alvarez *et al.*, 2012).

Carbon dioxide is eliminated from the atmosphere through carbon sequestration. The processes by which CO_2 is absorbed are carbon sinks, such as forests, agricultural sinks, geologic formations, and oceanic sinks (Casper, 2010). The IPCC reports that carbon sequestration by agriculture and forestry alone considerably helps to offset CO_2 emissions that contribute to climate change (IPCC, 2013).

1.3.2 Methane

Methane occurs naturally underground and below the sea. It is the second most important gas after CO_2 (Spokas *et al.* 2006). When the gas reaches the atmosphere, it is referred to as atmospheric CH_4. A more comprehensive discussion of CH_4 emissions is found in section 1.5 below.

1.3.3 Nitrous oxide

Commonly known as the 'laughing gas', nitrous oxide is a clear, colourless gas with a slightly pleasant taste. It is a microbial product that is formed from the bio-geochemical processes of the nitrogen cycle (Reay, 2010). Nevertheless, anthropogenic activities, such as agriculture, wastewater management, fossil fuel combustion, and industrial reactions have accelerated the release of N_2O molecules in the atmosphere. Agricultural activities are the primary causative agents of N_2O emissions – through the use of nitrogen fertilizers and the breakdown of nitrogen in urine and dung excretions (Reay, 2010).

Nitrous oxide is considered important for two reasons when it comes to the climate change: (1) N_2O has the ability to absorb infrared radiation at a rate that is roughly 300 times more than CO_2, and thus contributes greatly to greenhouse effect despite its low mixing ratio; and (2) it contributes to the decrease of stratospheric ozone (Reay *et al*., 2010). Modern day concentrations of N_2O are 19% above their pre-Industrial Revolution levels; they have risen from 270 ± 7 ppb (nmol mol^{-1}) to 324.2 ± 0.1 ppb (nmol mol^{-1}) between 1750 and 2011 (Myhre *et al*., 2013). Nitrous oxide molecules stay in the atmosphere for an average of 114 years (Reay *et al*., 2010).

1.3.4 Water vapour

Water vapour, the gaseous state of water, is the dominant contributor to the greenhouse gas effect (Schmidt *et al*., 2010). Its contribution to the natural greenhouse effect relative to that of CO_2 depends on the particular accounting method, but is usually considered to be roughly two or three times greater (Myhre *et al*., 2013). Under typical conditions, water vapour is naturally produced by the process of evaporation and is eliminated from the atmosphere through condensation (Myhre *et al.*, 2013). The amount of water vapour in the atmosphere depends on air temperature. Additional water vapour is added to the atmosphere through anthropogenic activities, mostly through irrigation of crops, power plant cooling, and combustion of fossil fuels.

Water vapour is not given the same focus as CO_2 as a forcing to climate change because it behaves differently, condensing and precipitating, and because anthropogenic contributions are small relative to 'natural' evaporation. When highly humid air cools, some of the vapour condenses into ice particles or water droplets and precipitates. The average residence of water vapour in the atmosphere is only ten days (Myhre *et al.*, 2013).

Anthropogenic emissions significantly impact stratospheric water vapour that is located 10 km above the atmosphere. Increased CH_4 concentration due to anthropogenic activities creates additional sources of water through oxidation, which provides part explanation for the atmospheric changes in that layer. Stratospheric water concentrations have fluctuated across decades and the actual range of variations is not fully understood and is possibly more a feedback process than a forcing (Myhre *et al.*, 2013). The contribution of stratospheric water vapour to warming is, however, smaller compared to CO_2 and CH_4. Also, water vapour's residence in the atmosphere is primarily dependent on CO_2 and the removal of CO_2 would cause the temperature to drop sufficiently to induce decrease of water vapour, thus leading to a reduction in greenhouse effect (Myhre *et al.*, 2013).

1.3.5 Ozone

Ozone, alternatively known as tri-oxygen and denoted by O_3, is a pale blue gas with a pungent smell (Smical *et al.,* 2010). Ozone is relatively less stable than O_2 and is found in low concentrations in the stratosphere. Stratospheric ozone is helpful because it provides a barrier that prevents ultraviolet rays from damaging plants and animals (Kumbhani, 2015). Tropospheric ozone is produced by human activities, and is considered harmful for fauna and flora, because it is located at ground level. Current peak concentrations of ozone are much higher than what they were in the past (Karlsson *et al*., 2017). In Europe, before the Industrial Revolution, ozone was approximately 10 ppb (Karlsson *et al*., 2017). The time series data taken from Kap Arkona, Germany and Mace Head, Ireland indicates that the European concentrations increased by + 0.3 ppb annually between 1950s and 1970s and by + 0.5 annually between 1987 and 2003 (Karlsson *et al*., 2017). While emissions of ozone precursors somewhat subsided in Europe between the 1980s and 1990s, they have radically increased in Asia.

As a greenhouse gas, ozone absorbs infrared radiation emitted by the earth. Tropospheric ozone's annual global warming potential is estimated to be between 918-1022 tonnes (Kumbhani, 2015). This suggests that for every molecule, tropospheric ozone has a radiative forcing impact of about 1,000 times greater than CO_2. However, tropospheric ozone has a short lifetime and decays at a faster rate than CO_2. The short lifetime means that the total greenhouse effect is significantly less than that of CO_2. Still, ozone has considerably strong radiative forcing effects in some regions (Karlsson *et al*., 2017).

TABLE 1.1 Global warming potential (GWP) of selected greenhouse gases.

		GWP values for 100-year time horizon				
Industrial designation or common name	Chemical formula	First Assessment Report (AR1-1990)	Second Assessment Report (AR2-1995)	Third Assessment Report (AR3-2001)	Fourth Assessment Report (AR4-2007)	Fifth Assessment Report (AR5-2013)
Carbon dioxide	CO_2	1	1	1	1	1
Methane	CH_4	21	21	23	25	28-34
Nitrous oxide	N_2O	290	310	296	298	265

Source: (Myhre *et al*., 2013).

1.4 Methane and climate change

Methane was first identified by Italian physicist Alessandro Volta in the 18th century as a flammable gas in the bubbles that rise from waterlogged marsh (Reay *et al*., 2010). It is today globally used for sustained economic development and to provide a lower-carbon energy alternative to coal and oil in industrial and domestic settings. The United Kingdom is an example of a country that has promoted the wide use of CH_4 to meet its Kyoto Protocol commitments of reducing greenhouse gas emissions (Reay *et al*., 2010). To be precise, the UK switched to gas-fired power stations and phased out coal-fired stations. While coal-fired stations accounted for 69% of electricity generated in the country in 1990, they only accounted for 30% by 2000 (Volkmar, 2012). Methane has been demonstrated to be a good absorber of infrared rays (Reay *et al.*, 2010). Ice core records and atmospheric samples show that CH_4 emissions have considerably increased. Globally averaged CH_4 surface concentrations have increased from 722 ± 25 ppb (nmol mol^{-1}) to 1803 ± 2 ppb (nmol mol^{-1}) between 1750 and 2011 (Myhre *et al.*, 2013). Although these concentrations are lower than those of CO_2, CH_4 has more radiative efficiency. Indeed, its GWP is 34 times greater than that of CO_2 over a 100-year time horizon, as mentioned in Table 1.1 (Myhre *et al.*, 2013).

The impact of CH_4 on the climate has mainly been attributed to anthropogenic CH_4 emissions, but anthropogenic emissions of other compounds also impact CH_4 concentrations by altering its removal rate. Methane currently contributes about 20% to the total forcing by all greenhouse gases (Vigano, 2010). Between the 1990s and the first few years of the 21st century, the previously rising concentration rates of CH_4 slowed down to almost zero, but rates again picked up in 2007 and 2008 (Nisbet *et al.,* 2014). Environmental and climate change literature in the subsequent years attributed the increase to higher CH_4 emissions in the Arctic as a consequence of high temperatures in 2007 and to higher precipitation in the tropics in 2008. The attribution of higher CH_4 emissions in the Arctic to higher temperatures in 2007 represents a snapshot of a potentially large positive climate feedback. The higher temperatures predicted for high latitudes in the 21st century will increase CH_4 emissions from permafrosts, wetlands, and CH_4 hydrates (Kirschke *et al*., 2013).

There is a growing recognition that the reduction of CH_4 could provide a more cost-effective and efficient mean to mitigate climate change caused by human activities (Schwietzke *et al.*, 2016). Methane is the most unpredictable greenhouse gas and a better comprehension of the role of CH_4 in climate change will lead to better projections of greenhouse gas concentrations (Nisbet *et al*., 2014).

1.4.1 Methane sources and the global methane budget

Globally, CH_4 inputs to the atmosphere come from a large number of sources, while outputs are to a comparatively smaller number of sinks. The primary way that CH_4 is removed from the atmosphere is through destruction by hydroxyl (OH) radicals in the troposphere (Vigano, 2010; Kirschke *et al*., 2013). This process contributes to the generation of peroxy radicals, which can lead to tropospheric ozone creation thus inducing a further indirect climate-forcing effect (Nisbet *et al*., 2014). The hydroxyl reaction also decreases the atmosphere's overall oxidizing ability and producing H_2O and CO_2. It is estimated that about 429-507 Tg (1 million tonnes) of atmospheric CH_4 are eliminated in this process (Reay *et al.*, 2010).

The other two sinks are smaller. Approximately 40 Tg of CH_4 is removed from the stratosphere through reactions with OH radicals and about 30 Tg CH_4 is metabolized/absorbed by methanotrophs, a CH_4-oxidizing bacteria (Reay *et al.,* 2010). The magnitude of the soil sink is principally dependent on the local meteorology, seasonality, and human land management methods. An additional but small amount of CH_4 is metabolized/absorbed through chemical oxidation by chlorine in the air and in the surface waters of seas (Reay *et al.,* 2010).

Imbalance in sources and sinks is responsible for the variations in atmospheric concentration of CH_4. The production of CH_4 can happen following biotic or abiotic process, which are either natural or anthropogenic. Major natural sources of CH_4 emissions are wetlands, onshore and offshore geological processes, and termites. Wetland CH_4 emissions, excluding those from rice cultivation, are estimated to total between 100 and 231 Tg every year according to Reay *et al.* (2010); which represents approximately 25% of global CH_4 emissions. Methane emissions exclusively from rice cultivation are projected to range between 25 and 50 Tg annually (Reay *et al.*, 2010). With the global

population estimated to hit 9 billion by 2050, rice cultivation is likely to be escalated and as a result elevate rice cultivation as a major source of CH_4 (Reay *et al*., 2010).

Onshore and offshore geological processes are commonly cited as sources of CH_4. In recent years, clathrates or CH_4 hydrates – which are basically ice-water with trapped CH_4 molecules found in ocean sediments – have emerged as sources of climatic warming (Reay *et al.,* 2010). These sources are thought to be responsible for about 4 or 5 Tg of CH_4 emissions. On average, one termite produces a microgram of CH_4 in a day. However, when then aggregate global population of termites is considered, total CH_4 emissions from termites is estimated at 20 Tg per year to the total CH_4 budget (Ho *et al*., 2013). According to Ho *et al*. (2013), CH_4 derived from termites contributes to between 3 and 4% of the total CH_4 budget globally.

Another commonly cited and well-studied natural source of CH_4 is microbial decomposition by Archaea under anaerobic conditions (Vigano, 2010). This is the process that aides CH_4 production in rice paddies, marshes, or flooded terrains in tropical regions (Vigano, 2010). Methanogenic Archea can be found in ruminant stomachs and animal sources. Ruminant livestock – cattle, sheep, goats, and deer – were projected to produce 100 Tg in 2010 according to Reay *et al.* (2010). Microbial methanogenesis is also estimated to be responsible for between 25 and 50 Tg of CH_4 emissions per year in rice cultivation (Reay *et al*., 2010).

Major anthropogenic sources of CH_4 are landfills (solid waste disposal), biomass burning, and fossil energy use. Landfills have been established as significant sources of CH_4 generation, as they provide optimum anoxic conditions for methanogenesis to occur (Spokas *et al*., 2006). Spokas *et al*. (2006) observed that giant landfill sites with at least one million cubic meters of excretions and high biodegradable waste can emit several hundred cubic meters of gas per hectare. However, the amount of CH_4 emitted from landfills into the atmosphere can be moderated by factors, such as effectiveness of the gas collection system and type of cover on the landfill (Goldsmith *et al*., 2012).

Methane emissions from biomass burning are hugely dependent on local socioeconomic factors and the management of forest resources (Vigano, 2010). Nevertheless, biomass burning accounts between 14 - 88 Tg of CH_4 emissions each year (Reay *et al.*, 2010). Methane emissions from biomass burning are usually products of

incomplete burning and emanate from a wide array of sources that include savanna, woodland, and peatlands. Peat burning and agricultural waste particularly produce higher CH_4 emissions because of low oxygen and high water content (Reay *et al*., 2010). Differentiating CH_4 emissions from natural as opposed to anthropogenic biomass burning is, however, difficult because of coincidence and space of most events.

Finally, fossil fuel combustion emits a significant amount of CH_4 into the atmosphere. Fossil fuels are mainly decomposed organic products that have been deposited underground for many years. While global fossil fuel industry is estimated to contribute between 15 and 22% of total atmospheric CH_4 budget, Schwietzke *et al.* (2016) argue that the CH_4 emissions levels from fossil fuel industry are higher, and may be up to 60% higher than estimated.

1.4.2 Atmospheric methane emissions

Methane is the most abundant and simplest reduced organic gas in the atmosphere; it has a mixing ratio of between 1.75 and 1.8 parts per million by volume (ppmv) (Sonnemann *et al*., 2014). According Sonnemann *et al.* (2014), the current concentrations are approaching greenhouse-gas-intensive scenarios. Scientists have intensively monitored CH_4 in recent decades because of the increasing recognition that the current atmospheric concentrations are not acceptable (Reay *et al*., 2010). However, additional scientific knowledge and innovation are needed to provide accurate predictions of atmospheric emissions of CH_4, due to the complexity of the processes, and the great variability in emissions among geographic regions. For example, decadal and annual emissions from wetlands are considerably higher than for other regions (Sonnemann *et al*., 2014). Also, the average mixing ratio in the northern hemisphere is comparatively higher than in the southern hemisphere, which has been attributed to the fact that most developed countries and most of the land surface are located in the north. The seasonal cycle of CH_4 concentrations in different geographic regions is dependent on the process of oxidation (Reay *et al*., 2010).

1.5 History of aerobic methane emissions

As is obvious from the above discussion, most of the natural emissions of CH_4 is attributed to the anaerobic decomposition of organic matter carried out by methanogens in anoxic environments, such as wetlands. In the absence of oxygen, organic carbon in compounds like acetate acts as the terminal electron acceptor thus working as a source of energy (Reay *et al*., 2010). In 2006, Keppler *et al*. (2006) used stable carbon isotopes to estimate for the first time the amount of CH_4 released from terrestrial plants. They reported that a significant amount of CH_4 was released from both living plants as well as their detached leaves in *in-situ* experimentation. Based on these measures, Keppler *et al*. (2006) estimated an annual global production of CH_4 about 62-236 Tg from living plants and 1-7 Tg from plant litter. Later, Kirschbaum *et al*. (2006) re-calculated the annual global CH_4 budget and concluded that the estimation was small compared to other sources and did not have impact in the context of climate change. Dueck *et al*. (2007) investigated CH_4 emissions from six plant species at temperatures of 25 and 35^{o}C; they observed CH_4 production ranging between 10 ng g^{-1} h^{-1} and 42 ng g^{-1} h^{-1} which was significantly lower than the values reported by Keppler *et al*. (2006). Beerling *et al*. (2008) reported no emissions of CH_4 from terrestrial plants, tobacco or corn, under aerobic and controlled conditions. It was not until they exposed the plants to UVB radiation that significant CH_4 emissions were observed. From this, the researchers concluded that no observable link existed between aerobic CH_4 emissions and the photosynthetic or respiratory mechanisms; in fact, it was proposed to be associated with non-enzymatic processes dependent upon radiations of particular wavelengths like that of UV radiation.

Environmental stressors, such as high temperature (Keppler *et al.,* 2006; Qaderi and Reid, 2009a), UVB radiation (Vigano *et al.,* 2008; Qaderi and Reid, 2009b), water stress (Qaderi and Reid, 2009b; 2011) and physical injury (Wang *et al.,* 2009; Lenhart *et al.,* 2015), are reported to increase aerobic CH_4 emissions from both intact and detached leaves of plants. In contrast, several studies have suggested that the sources of CH_4 are not the plant itself (Beerling *et al.,* 2008; Nisbet *et al.,* 2009), but rather endophytic methanotrophic bacteria hosted by the plants (Raghoebarsing *et al.,* 2005). These symbiotic endophytes are typically found in CH_4-rich environments, such as soil,

freshwater and marine environments (Keppler *et al.,* 2009). Other support for this view comes from the fact that soil water contains dissolved CH_4 that is produced from biological processes, including methanogenesis in the anoxic soil microenvironment and the microbial decomposition of organic matter (Das and Baruah, 2008).

1.6 The mechanism of aerobic methane emissions from plants: the puzzle

How living plants might produce CH_4 remains a mystery. Since the observations by Keppler *et al*. (2006), many studies have tried to determine the origin of CH_4 emissions from plants. Pectin was suggested as the source of CH_4 emissions from plants because of the large amount of methoxyl groups (Bruhn *et al.,* 2009a; Keppler *et al.,* 2006; 2009). Stable isotope analysis was used to study the methoxyl groups in plant pectin and lignin for their unique carbon isotope signatures to associate them with CH_4 emissions (Keppler *et al.,* 2008). Results of the isotope analysis clearly revealed that ester methyl groups of pectin can act as CH_4 precursors. The findings by Keppler *et al*. (2008) supported those by Keppler *et al*. (2006) that heating or temperature and UV irradiation lead to CH_4 emissions from pectin. Vigano *et al.* (2009) reported that in addition to pectin, cellulose and lignin may be sources of CH_4 emissions from plants, while Bruhn *et al*. (2014) suggested that emissions may come from wax. Recently, Lenhart *et al.* (2015) suggested that the sulphur-bound methyl group (-S-CH_3) of methionine should also be considered as a source of aerobic CH_4 in plants. In addition, several stress factors, such as UV radiation, temperature, and physical injuries by insect or pathogens, have been predicted to increase in the future (Myhre *et al*., 2013); thus, direct CH_4 production by plants, animals and fungi may play an increasingly important role in the global CH_4 budget in the future. The source of aerobic CH_4 emissions from plants has yet to be definitively determined, but as plant-derived CH_4 has the potential to be significant with regard to climate change, identifying the source and mechanisms remain important goals.

Most studies examining plant CH_4 emissions to date have tested the impact of single stress factors (Bowling *et al.,* 2009). Some investigators have measured the effects of two factors (e.g., Martel and Qaderi, 2017), but very few have investigated the phenomenon using multiple factors (Qaderi and Reid, 2009; 2011). Under climate change, plants will

experience multiple stressors simulataneously, and thus it is important to study the interactive effects of the main factors likely to impact plant CH_4 emissions.

1.7 Effects of environmental factors on plants

1.7.1 Effects of high temperature on plants

Morphologically, the most obvious damage done by high temperatures is in the form of scorched leaves and stems, abscised leaves, and even premature senescence. In addition, the temperature level and duration of heat stress can cause changes in growth rate and developmental stages (Bita and Gerats, 2013) (Table 1.2). Hatfield *et al*. (2011) reported that an increase of 1^{o}C above the optimum temperature can decrease cereal yields by 4.1 to 10%. Similarly, plants facing heat stress also show compromised development in the form of shorter and thinner stems, smaller and thicker leaves, and an overall reduced biomass (Qaderi *et al*., 2006). There is usually a comparatively more extensive root system in heat stressed plants (King *et al*., 1997). Drought also causes a significant loss of cell turgidity; this loss leads to a reduction in plant growth and gas exchange (Larcher 1983; Anjum *et al.,* 2011). In addition, drought decreases chlorophyll content and fluorescence, plant height, stem diameter, total dry mass, and relative leaf expansion rate and leaf elongation (Kirnak, 2001). Apart from the superficial damage and growth inhibition, heat stress burdens the reproductive capabilities of plants. It has been reported that reproductive stages in most of plants, such as flowering and pollen production, are more sensitive to increased temperature as compared to vegetative stages (Wahid *et al*., 2007). In tomato, heat stress is reported to delay the development of both male and female reproductive organs thus stalling any exchange of genetic material in the plant (Bhatnagar-Mathur *et al*., 2008). Furthermore, heat stress may even lead to oxidative stress with the generation of reactive oxygen species (ROS), such as superoxide anion radicals (O^{-2}), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), alkoxy radicals (RO), and singlet oxygen (O_2^1) (Apel and Hirt, 2004).

1.7.1.1 Plant responses and adaptations to increased temperatures

The response of plants to heat stress varies with the intensity of temperature increase, duration of exposure, and plant species. The response can be as extreme as sudden plant

death due to cell damage or an inhibition of germination, growth, development, reproduction, and yield (Apel and Hirt, 2004). In case of exposure – whether long-term or short-term – plants adapt to heat stress by opting for avoidance mechanisms or tolerance mechanisms (Kramer *et al*., 1980; Hasanuzzaman *et al*., 2013). In case of avoidance, plants may mitigate heat stress through physical alterations. The effects of overheating can be reduced by rolling or dropping leaves and temporary wilting as in Geraniaceae (*Pelargonium)* species (Nicotra *et al*., 2008). These adaptive mechanisms may result in reduced photosynthesis as they aim at blocking out excessive heat as well as incident solar radiation (Hasanuzzaman *et al*., 2013). Many species have adaptations to withstand heat, for instance, a thick waxy cuticle on the surface of leaves.

For tolerance, plants have developed different physiological, biochemical, and molecular mechanisms to respond to increased temperature. Some major ways in which plants adapt to higher temperatures are producing osmoprotectants, antioxidant defense, and factors involved in signaling cascades (Levitt, 1980; Hasanuzzaman *et al*., 2013). Primarily, plants alter their metabolism in order to produce appropriate solutes, which help them manage proteins and cellular structures during heat stress, maintain cell turgidity by osmotic adjustment, and re-establish the redox balance of cells by modifying the antioxidant system, and restore cell homeostasis (Janská *et al*., 2010). At the molecular level, plants respond to heat stress by altering the expression of genes, which are involved in protection against increased temperature (Chinnusamy *et al*., 2007). These genes are responsible for the expression of osmoprotectants (proline, glycine betaine, trehalose, etc.), detoxifying enzymes, phytohormones, transporters, and regulatory proteins (Krasensky and Jonak, 2012).

TABLE 1.2 Effects of high temperature on different crop plants.

Crops	Heat treatment	Exposure time	Growth stage	Major effects	Source
Maize	33-40ºC	15 days	During pre-anthesis and silking onwards	Severe consequences on plant and growth rates	(Zhang *et al*., 2013)
Pea	45ºC	24 h	Vegetative stages	Inhibitation of photosynthetic activity	(Georgieva *et al*., 2000)
Rice	Above 33ºC	10 days	Heading stage	Reduced rates of pollen and spikelet fertility	(Hurkman *et al*., 2009)
Rice	32ºC night	--	Reproductive stage	Decreased yield, increased spikelet sterility, decreased grain length, width, and weight	(Suwa *et al*., 2010)
Soybean	38 / 28ºC day/ night	14 days	Flowering stage	Decreased rate of photosynthesis and stomatal conductance; damaged plasma, chloroplast, and thylakoid membranes; distorted mitochondrial membranes, cristae, and matrix	(Tan *et al*., 2011)
Tobacco	43ºC	2 hours	Early growth stage	Decreased rate of photosynthesis and stomatal conductance; reduced activity of antioxidant enzymes	(Gunawardhana and De Silva, 2011)
Wheat	30 / 25ºC day/ night	60 days after sowing	Maturity stage	Reduction in leaf size, grain size, and yield	(Djanaguiraman *et al.*, 2010)
Wheat	38ºC	24 and 48 hours	Seedling stage	Decreased chlorophyll level and relative water level; diminished antioxidative capacity	(Hasanuzzaman *et al*., 2013)

Hormonal response is among the initial biochemical responses shown by plants when they face heat stress; phytohormones including abscisic acid (ABA), indole-3-acetic acid (IAA), and ethylene are the prominent ones released in times of stress (Nilsen and Orcutt, 1996). Releasing hormones helps transpiration process by controlling stomatal conductance, and thus reduced water loss (Davies, 2004). Under high temperatures, the release of ABA is increased while ethylene and IAA are decreased. Among the most prominent responses to heat stress is the development of an extensive root system that helps plant to uptake more water and compensate for water loss due to increased transpiration (Qaderi *et al*., 2006). Moreover, tolerant plants have a tendency of protecting themselves against the damaging effects of ROS, by producing enzymatic and non-enzymatic ROS scavenging products (Apel and Hirt, 2004). These antioxidant enzymes are temperature-sensitive that increase with increasing temperature. Chakraborty and Pradhan (2011) observed that enzymes, such as catalase, ascorbate peroxidase, and superoxide dismutase, exhibited an initial increase before decreasing at 50^{o}C, while peroxidase and glutathione reductase activities showed a decline at all temperatures ranging from 20 to 50^{o}C.

1.7.2 Effects of UVB radiation

Ultraviolet radiation is generally categorized into three types: UVA with wavelength (320-400 nm), UVB (280-315 nm), and UVC (200-280 nm) (Rozema *et al.,* 2002). UVA radiation reaches the earth's surface but has low DNA damaging effect because it is not absorbed by the DNA (Gill *et al*., 2015). UVB radiation also reaches the surface of the Earth, but is more damaging because it induces chemical reactions within cellular DNA (Ravanat *et al*., 2001). UVC radiation is the shortest wavelength, highest energy radiation, and is potentially very damaging, but usually has little to no impact on plants because it is normally absorbed completely by the stratospheric ozone layer (Stapleton, 1992).

1.7.2.1 Stratospheric ozone layer

The evolution of life on Earth became possible because of the formation of the stratospheric ozone layer, which covered the planet as a protective layer and prevented

harmful ultraviolet radiations from hitting the Earth (Kakani *et al.,* 2003) Unfortunately, variations in the thickness of ozone layer, both seasonal and anthropogenic, have a direct effect on the level of harmful radiation reaching the surface of the Earth (Rozema *et al.,* 2002; Qaderi *et al.,* 2007).

The protective ozone layer has decreased dramatically over some parts of the Earth (Singh, 2016). It is estimated that long-term recovery will not occur until 2050 (Weatherhead and Anderson, 2006), when the levels of ozone depleting substances, such as chlorine and bromine in the stratosphere will be stabilized to near pre-1980 values. The variation of ozone layer affects the amount of UVB radiation reaching the Earth surface. The average forcing levels of UVB reaching the surface of the earth vary between 2 and 12 kJ m^{-2}, depending on location (Kakani *et al.,* 2003). Predictive studies on the future levels of UVB radiations are still controversial; some studies predict that ozone levels will stabilize as a result of the phasing out of chlorofluorocarbons (Ballaré *et al.,* 2011). However, other studies suggest that potent ozone-depleting atmospheric pollutants, such as N_2O, will continue to cause ozone reductions (Ravishankara *et al.,* 2009). Other than the depletion of stratospheric ozone layer, specific factors, such as cloud cover, tropospheric pollution (Ballaré *et al.,* 2011), and plant canopies (Kakani *et al.,*2003) influence the amount of UVB radiation reaching the Earth (Rozema *et al.,* 2002). Seasonal variations also play a role, with as much as 14% more UVB reaching the Earth's surface during the spring, when crops are initially being planted (Kakani *et al.,* 2003). Furthermore, certain areas of the world that are already receiving higher doses of UVB radiation will be most affected by climate change (Kakani *et al.,* 2003).

1.7.2.2 Effects of UVB radiation on plant growth and development

Plants exposed to high levels of UVB radiation display an array of visible symptoms. Initially, leaves begin to turn brown due to loss of chlorophyll resulting in necrotic lesions and areas of chlorosis (Jansen *et al.,* 1998; Kakani *et al.,* 2003). As a response, leaves begin to curl inward reducing the surface area exposed to incoming radiation. However, if exposure persists, leaves can desiccate and fall, resulting in reduced productive biomass (Kakani *et al.,* 2003). Moderate UVB radiation causes an increase in leaf thickness due to the formation of extra layers of spongy mesophyll cells and wider

palisade cells (Kakani *et al.,* 2003). It additionally causes a protective waxy layer to develop that contributes to leaf thickness (Sangtarash *et al.,* 2009). Another consequence of UVB radiation on plant development is decreased height of stems and branches as well as an overall decrease in leaf area (Kakani *et al.,* 2003).

It should be kept in mind that different plants or crops show varied reactions to excessive exposure to UVB radiations depending upon their origin and sensitivities (Table 1.3). A reduction in dry matter accumulation was observed in different cultivars of canola along with a general reduction in size and height of the plants (Qaderi *et al.,* 2007). The same trend has been reported in pea plants (Mepsted *et al.,* 1996). It has been reported that temperate regions, such as the Canadian prairies, will suffer greater impacts than the alpine regions. A study that examined the effects of UVB radiation on two separate ecotypes, prairie and alpine, of *Stellaria longipes* revealed that the prairie ecotype was more sensitive to enhanced UVB stress. It showed greater reduction in biomass and a smaller increase in flavonoids (Sangtarash *et al.,* 2009).

1.7.2.3 Physiology and biochemistry of the plants under UVB radiation

Ultraviolet-B radiation affects the physiology and biochemistry of plants. One proposed mechanism behind these negative effects is the photo-oxidative degradation of the hormone auxin by UVB radiation, caused inhibitory effects on plant growth (Jansen *et al.,* 1998; Qaderi and Reid, 2005). Enhanced UVB levels can affect growth and yield of crops through direct effects on DNA, photosynthesis, membrane integrity, and chemical composition of plant tissues (Qaderi and Reid, 2005; Qaderi *et al.,* 2007; 2008). UVB radiation caused damage to DNA include DNA breaks and inaccurate base pairing. This contributes to a decrease in protein synthesis (Jansen *et al.,* 1998).

Studies have shown that UVB radiation inactivates photosystem II through the degradation of the two protein subunits, D1 and D2, that make up this system (Jansen *et al.,* 1998). Additionally, reduced activity of Rubisco is observed along with decreased chlorophyll and carotenoid concentrations (Jansen *et al.,* 1998; A-H-Mackerness, 2000), down-regulation of photosynthetic genes (Jordan *et al.,* 1994; Jansen *et al.,* 1998), and changes in chloroplast and chloroplast protein content (Jansen *et al.,* 1998; Kakani *et al.,* 2003). Reduced stomatal conductance has also been observed in some plants (Kakani *et*

al., 2003). Enhanced UVB radiation decreased stomatal number as well as leaf area with an increase in cell size of an invasive alien species (*Silene noctiflora),* suggesting that UVB may have a negative impact on cell mitosis. Decreased stomatal number reduced net photosynthesis (Qaderi *et al*., 2008), as well as decreased in net CO_2 assimilation and water use efficiency (WUE) in alien species (*Silene noctiflora)* and canola (*Brassica napus*) (Qaderi *et al.,* 2007; 2008). In contrast, some studies have shown that UVB radiation increased chlorophyll content in plants, as examined in certain cultivars of lettuce (*Lactuca sativa* L.), which resulted in improved photosynthetic capacity (Smith *et al.,* 2000). Also it was observed that UVB radiation caused reduction in number and size of anther, thus reducing in the amount of pollen available for fertilization (Kakani *et al.,* 2003).

1.7.2.4 Reproduction and survival of plants under UVB radiation

Plant reproduction is also negatively affected by exposure to UVB radiation. Through delaying of germination, alteration of flowering times and inhibition of pollen formation, UVB radiation is reported to have profound effects on plant reproduction (A-H-Mackerness, 2000; Kakani *et al.,* 2003). Reproductive stages including flowering, pollination, and seed development are all harmfully affected by UVB irradiation. It can also decrease the number of flowers and affect the time of flowering stage (Tevini and Teramura, 1989). Such effects have significant consequences for plant populations, which rely on synchrony between flowering and appropriate insect pollinators. Although pollen itself appears to be tolerant towards UVB radiations, owing to high levels of flavonoids in the anthers and pollen wall (Lois and Buchanan, 1994), growth of pollen tubes is highly sensitive to UVB level, which can lead to lowered success of pollination (Zhang *et al*., 2014) and decreased seed yield (Liu *et al*., 2013).

1.7.3 Plant responses to UVB stress

Plants have developed certain mechanisms in order to adapt to UVB stress (Caldwell and Flint, 1994). Plants exposed to enhanced UVB for longer lengths of time have been shown to respond to the stress through an increased level of defensive pigments, including phenolic compounds such as flavonoids (Jansen *et al.,* 1998). Phenolics have

the ability to absorb UVB radiation and shield the underlying productive tissue (Kakani *et al.,* 2003). Studies on soybean revealed that both nitrogen and phenolics increase under UVB stress (Hatcher and Paul, 1994). Several other compounds, including ROS, salicylic acid, jasmonic acid, and ethylene are responsible for the regulation of gene expression in response to UVB exposure (A-H-Mackerness, 2000; Qaderi *et al.,* 2007). Additionally, plants can respond to UVB by forming smaller and thicker leaves, protected by an enhanced layer of epicuticular wax (Qaderi and Reid, 2005; Qaderi *et al.,* 2007).

Plants respond to UVB radiation by increasing the activity of radical-scavenging, which helps plants to eliminate the damaging ROS caused by radiation. After a few days, these scavenger compounds decrease and other mechanisms take over to promote UVB tolerance. These mechanisms take a few days to form which can be the reason for the initial boost in the amount of scavengers (Jansen *et al.,* 1998). Higher wax production has been reported as a response in canola plants exposed to both ambient and elevated CO_2; this wax likely caused a decrease in penetration of UVB into the leaf tissue, thus lessening the damage caused by UVB, as a first line of defense (Qaderi and Reid, 2005; Qaderi *et al*., 2008).

TABLE 1.3 The varying sensitivities of cultivated crops to UVB radiation (Krupa and Kickert, 1989).

Sensitive to UVB	**Insensitive to UVB**
Cauliflower	Cabbage
Clover	Cotton
Cucumber	Lettuce
Faba Bean	Lucerne
Pea	Maize
Soybean	Rice
Spinach	Tomato
Turnip	Wheat

1.7.4 Effects of water stress on plants

Drought decreases germination potential as well as reducing plant and seedling stand establishment (Kaya *et al.,* 2006). Studies on a wide range of plants have reported varied effects of drought on plant growth and development. For instance, water stress reduced the length and fresh/dry weights of alfalfa (*Medicago sativa* L.) shoots. Also, root length was reported to be increased in this experiment (Zeid and Shedeed, 2006). Similarly, increased water stress during the vegetative stage of rice resulted in great reduction in the growth and development of this crop (Manickavelu *et al.,* 2006). Furthermore, Okcu *et al*. (2005) reported that water stress negatively affected germination and early growth of seedlings pea (*Pisum sativum* L.).

Growth of plant is dependent upon cell division and enlargement. The physiological and molecular processes in cell are highly sensitive to water stress (Shinozaki and Yamaguchi-Shinozaki, 1997). When higher plants face severe water stress, cell elongation is inhibited due to interrupted water flow from xylem to surrounding growing cells in the plant (Hsiao, 1973; Nonami, 1998). Furthermore, inadequate water delays mitosis and expansion of cells, thus resulting in plants with reduced height, leaf area, and lesser yield under drought conditions (Hussain *et al.,* 2008).

Relative water content, leaf water potential, stomatal resistance, rate of transpiration, and leaf temperature are important in defining the water relations, i.e., balance of water, in plants (Farooq *et al.,* 2009). In a study by Siddique *et al*. (2001) lower relative water content was observed in water-stressed wheat as compared to wheat grown in normal conditions. Along with this, the crop suffered from a decrease in water potential and transpiration rate with an associated increase in leaf temperature. Similarly, the water content decreased by nearly 57% of Cladodes (*Opuntia ficus-indica),* when it was grown under water stress (Nerd and Nobel, 1991). The same parameters of relative water content, turgor potential, stomatal conductance, transpiration, and water-use efficiency were reported to decrease in Hibiscus (*Hibiscus rosa-sinensis*) under water stress (Egilla *et al.,* 2005). Water stress causes a limited uptake of nutrients and subsequent smaller tissue in crop plants (Farooq *et al.,* 2009). Usually, impact on nutrient relation varies among plant species and genotypes. Water stress affects photosynthesis and causes reduction in leaf expansion, damaged photosynthetic machinery, premature leaf

senescence, and malfunctioning of food production (Pessarakli, 2016). Both stomatal and non-stomatal damage to photosynthesis occurs, however, the former is quite small. This shows that photosynthetic damage does not occur because of improper CO_2 uptake alone. Other ways in which drought stress asserts negative impacts are changes in photosynthetic pigments (Anjum *et al.,* 2003) as well as damage in the biochemical apparatus, such as enzymes (Fu and Huang, 2001) - all of which ultimately reduce the crop yield (Monakhova and Chernyad'ev, 2002). Furthermore, the accumulation of ROS under water stress acts as a mechanism behind stalling of photosynthesis (Reddy *et al.,* 2004).

One of the by-products of electron-transport chain in chloroplasts, mitochondria, and plasma membranes of plants are ROS (Apel and Hirt, 2004; Sairam *et al.,* 2005). The mitochondrial electron transport chain is considered the most active in producing ROS. Exposure of plants to environmental stress leads to formation of reactive species (Munné-Bosch and Penuelas, 2003), which can react with cellular proteins, lipids, and nucleic acids and cause oxidative damage (Foyer and Fletcher, 2001). This eventually leads to impairment of normal cellular functions, such as photosynthesis.

1.7.4.1 Plant responses to water stress

Plants respond to water stress through morphological, biochemical, and physiological mechanisms. Tolerance of plants to water stress is indicated by their ability to grow, undergo flowering, and give economically feasible yield even when they are facing water stress (Farooq *et al*., 2009).

Drought tolerance in plants involves changes in the plant morphology at multiple levels (Hsiao, 1973). An initial response by the plant against limited water supply is "escape," whereby escape from drought is achieved by shortening of life cycle of the plant, such as growing season to help plant to reproduce before water becomes too limited (Kramer *et al*., 1980; Araus *et al*., 2002). Blum (1996) revealed that flowering time is a major means of crop adaptation during terminal drought and high temperatures. Similarly, plants can also "avoid" water stress by reducing water loss through stomatal control of transpiration as well as increasing water uptake by developing an extensive and longer root system (Hsiao, 1973). Another major morphological response by plant to

drought stress occurs in the form of limited leaf area; this helps the plant to cut-down its budget of water and cost of yield loss (Schuppler *et al*., 1998). Roots helps the plant to acquire water from soil, thus increased root growth, proliferation, and density are significant responses to water stress (Kavar *et al.,* 2008). Apart from this, development of a waxy layer on the surface of leaves helps plants maintain high water potential in tissues (Richards *et al.,* 1986). Also, leaf shedding is another morphological response to save water by less transpiration (Ryan, 2011).

Plants alter their physiological response through several ways. Osmoprotection, antioxidation, and ROS scavenging systems are among the major physiological responses to tolerate and survive through water stress (Farooq *et al.,* 2009). Osmotic adjustment allows plants to increase influx of water and thus maintain turgor (Shabala and Lew, 2002). Other than osmotic adjustment, ABA and dehydrins also increase plant tolerance to drought because they maintain high tissue water potential. Another way in which plants tolerate water stress is by producing antioxidant defense. This system in the plants is constituted with the help of enzymatic and non-enzymatic resources. Enzymatic resources, such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione, while non-enzymatic components include cysteine and ascorbic acid (Farooq *et al.,* 2009). Reactive oxygen species are formed in plant cells as a result of water stress. They are removed by those antioxidant enzymes or by the in-organic scavenging molecules. According to Farooq *et al.* (2008), antioxidant enzymes help plants to overcome anti oxidative damage. In addition, Kavar *et al*. (2008) reported that various genes are activated in response to the onset of drought at transcriptional level; the products of these genes are important in establishing tolerance to drought. The genetic response may be triggered directly by water stress or as a result of secondary stress or injury; in either case, the genetic response to drought is a complex phenomenon that takes place as a concerted action of numerous genes (Agarwal *et al.,* 2006). Another major response to drought occurs in the form of special proteins, such as aquaporins. The synthesis of these proteins is a ubiquitous response that helps plants cope with stressful drought conditions by hydration of cellular structures (Wahid *et al.,* 2007) or synthesis of transcription factors required for expression of other stress proteins and genes (Legay *et*

al., 2011). Proline accumulation also increases under environmental stress; this amino acid is considered an indicator for dryness in plants (Szabados and Savoure, 2009).

1.7.5 Effects of multiple environmental factors on plants

Plant growth and development is dependent on abiotic and biotic factors (Wasternack, 2007). Abiotic factors encompass the physical and environmental conditions, whereas biotic factors refer to living organisms including animals and insects affecting plants (Abou-Hussein, 2012). Climate change, however, is a set of conditions that comprise climatic and environmental changes, such as high temperatures, drought, ultraviolet radiations - all of which have unfavorable impacts on the growth and development of plants. Abou-Hussein (2012) conducted a comprehensive review of research studies on the effects of two components of climate change – temperature and carbon dioxide – on plants to report that most plants have an optimal temperature range within which they can grow well. If the temperature around the plant increases or decreases beyond this temperature, plants are not able to cope physiologically.

When temperatures rise too high above optimum temperature of plants, heat destruction of protoplast causes cell death (Hsiao, 1973; Abou-Hussein, 2012). Heat injury symptoms in plants are the appearance of dead areas in leaves of hypocotyls and young leaves of plants. High temperature injuries in plants reduce yield and the quality of produce through two mechanisms: (1) it accelerates the reproductive rate, thereby shortening seed filling and maturation period; and (2) it prevents reproductive events in crops, such as tomatoes, when the temperatures rises a few degrees above the optimum level (Abou-Hussein, 2012). Cool-season crops in the tropics, such as cabbages and pepper fruit, are particularly be affected (Abou-Hussein, 2012). Nevertheless, Texeira *et al*. (2013) reported a few benefits of temperature increases on plants. Temperature increases may lengthen the growing seasons in temperate regions and make land in previously cold climates suitable for crop production.

Changes in the concentration of CO_2 will also affect plants. In most crops, increasing CO_2 concentration improves water use efficiency due to declines in stomatal conductance. This potentially decreases both drought susceptibility and irrigation requirements (Abou-Hussein, 2012). Absolute increases in productivity in plants as a

result of elevated CO_2 concentrations are also posited for soil nitrogen and phosphourus (Abou-Hussein, 2012). Moderate yields for vegetables and greenhouse crops cucumber, eggplant, tomato, and pepper are predicted. In essence, elevated CO_2 improves relative growth rate and increases yield and biomass; it increases number of fruits, flowers, seeds, and dry matter production by stimulating photosynthesis; and reduces transpiration (Qaderi *et al*., 2009a).

Based on the above information, crops have positive responses to elevated CO_2 and negative responses to temperature increases. Qaderi *et al*. (2009a), however, reported that when the two factors interact, elevated CO_2 can, to some extent, reduce the adverse environmental effects of salinity, high temperature, and UVB radiation on crops. This is supported by the study by Prasad *et al*. (2005), who reported that elevated CO_2 increased yields of legumes, peanut and cowpea, but the beneficial effect moderated by negative effects of above-optimum temperature. There is, however, variation in the responses of crop species to the two climate components. In fact, while the wide observation is that crop responses to CO_2 scaling are strongly related to temperature, Texeira *et al.* (2013) emphasized that higher CO_2 may not necessarily mean higher yields in all crops. Temperate and subtropical areas will suffer lower yields.

On the other hand, the direct effect of UVB radiation can be influenced by a simultaneous increase in CO_2 or drought (Turtola *et al*., 2005; Koti *et al*., 2005). UVB radiation affects the growth of plants by damaging molecular machinery and biomolecules, but an increase in CO_2 concentration or increase in temperature counters this effect by enhancing biomass production and thus growth of plant (Kellomäki and Wang, 2001). Qaderi and Reid (2005) reported that a high concentration of CO_2 reduced the negative effects that UVB radiation had on *Brassica napus* height; the researchers attributed this response to an increase in UVB-screening phenolic compounds in the plant tissue under excessive CO_2 in the surroundings. An increase in temperature on its own, unlike CO_2, may reduce the levels of phenolic compounds (Veteli *et al*., 2002). Since increase in temperature and CO_2 have opposite outcomes on the concentration of phenolic compounds in plant biomass, UVB incidence would have a more severe effect when both CO_2 concentration and temperature increase simultaneously as compared to when only CO_2 concentrations increase. According to Tegelberg *et al*. (2003), the effects

of UVB radiation, temperature and CO_2 vary with plant species, which means the interactive effects will also vary (Koti *et al*., 2005).

Plants usually adapt to drought conditions by several strategies and not just by reducing water loss from their tissues (Gitz and Liu-Gitz, 2003). These adaptive mechanisms might be affected by changes in other climatic conditions, such as UVB radiation, as well. For instance, soybean is reported to have increased root length in the presence of UVB, which helps to avoid drought stress by being able to access more water (Britz, 1990). Stem elongation in plants is also influenced by the intensity of incident light. Increasing UVB radiation inhibited stem elongation in wheat crops (Holmes and Smith, 1977), suggest that this could help plants to reduce the amount of harmful radiations that reach its canopy (Ballaré *et al*., 1992). With UVB exposure, there is a shortening in the internodes, which hinders air movement through the canopy causing the leaf boundary resistance to increase thus eventually lowering the transpiration rate from the plant (Nobel, 1999). Gitz and Liu-Gitz (2003) corroborate these findings in their review by reporting that increase in UVB radiation can cause plants to adapt in a manner that increases its water use efficiency.

1.7.6 Effects of high temperature, supplemental UVB radiation, water stress and their interactive effect on methane emissions

The majority of studies on aerobic CH_4 emissions from plants have found that high temperature enhanced CH_4 emissions from plants. Keppler *et al*. (2006) observed that CH_4 emissions rates from terrestrial plants increased with increasing temperature in the range of 30 to 70ºC. Kitaoka *et al*. (2007) found that elevated CO_2 levels increased CH_4 emissions from larch, birch and oak trees. In addition, CH_4 emissions were highest for larch, while oak produced least CH_4 when exposed to elevated CO_2. The authors attributed the differences to chemical and anatomical characters, suggesting that lower CH_4 emissions by oak may have been due to greater emissions of isoprene, which is involved in protecting photosynthesis membranes. Vigano *et al*. (2008) studied the effects of UV radiation and high temperature on the level of CH_4 emissions from plants under aerobic conditions. Emission was studied in both attached plants and detached leaves in 20 plant species. Methane emissions were observed to increase with increasing

UV radiation intensity with photochemical process involved in the emissions process. Similarly, in another study, CH_4 emissions were observed in aerobic conditions from detached leaves as well as stems of 44 native species of the Inner Mongolian Steppe (Wang *et al*., 2008). Some species were reported to release CH_4 in very small amount while other species did not release any CH_4 at all. Qaderi and Reid (2009b) evaluated the effect of UVB radiation exposure, water stress, and increased temperature on six important crops, faba bean, sunflower, pea, canola, barley, and wheat. They reported that CH_4 emission was greatest from the pea plants that were grown under higher temperatures at zero UVB and experienced water stress, but smallest from the barley plants that were grown under lower temperatures at enhanced UVB and received water to field capacity. Bruhn *et al*. (2009b) revealed that CH_4 emissions did occur from low temperatures (10°C) and increased exponentially with rising temperatures to well above any enzymatic optimum temperature (80°C) in darkness. Bruhn *et al*. (2009b) also stated that CH_4 emissions stimulated under UVB radiation more than UVA. Qaderi and Reid (2011) reported that plants grown under higher temperature and water stress emitted more CH_4 than those grown under lower temperature and received water to field capacity, but a combination of elevated CO_2, higher temperature and water stress decreased the effect of the latter two stress factors on plant. It was suggested that CH_4 emissions by terrestrial plants may also be enhanced by physiological stress factors, such as nutrient deficiency, salinity and pathogen infection (Liu *et al.*, 2015).

1.8 Pea as a model species for CH_4 emissions studies

Pea (*Pisum sativum*) has a diverse use as a pulse, fresh pea, edible pod, and even fodder (Noreen and Ashraf, 2009). Pea cultivation is currently popular in Canada, the United States, India, Russia, China, and France (McKay *et al*., 2003), due to its better growth in colder climates. Pea seed is rich in protein, carbohydrates (Noreen and Ashraf, 2009), vitamins, minerals, (Dahl *et al*., 2012), amino acids (McKay *et al*., 2003), fibers, and even anti-oxidants (Pownall *et al*., 2010). Dahl *et al*. (2012) report pea to be an inexpensive food source feeding no less than 800-900 million people's basic dietary demands globally every year.

Apart from being an important food source, pea and its different varieties are widely acknowledged among the scientific community for the suitability as a model species to study a wide range of natural phenomenon. The pea plant was used as a model in scientific investigations even before Gregor Mendel performed his legendary experiments in genetics on it (Symkal, 2014). Now that pea plant is an excellent model for modern studies on more complicated natural phenomenon too. Furthermore, other traits that make it an attractive model include its flexibility for cultivation, diverse available varieties, and its ability to self-pollinate and yield true breeding lines (Elzebroek, 2008).

1.8.1 Botanical traits and associated economic value

Pea is an annual legume, commonly called a pulse crop, belonging to the family, Fabaceae. It grows in soils with a wide range of textures, ranging from light sandy to heavy clay soil (McKay *et al*., 2003). In most soils, maximal growth of pea plants require addition of nutrients, including potassium, nitrogen, and phosphorus (Ashraf *et al*., 2011). After planting, a pea plant reaches flowering stage within 30-45 days followed by the flowers maturing to yield a fruit in the form of 3 to 10 cm long pods containing four to nine seeds with a wrinkled or smooth appearance (McKay *et al*., 2003; Pavek, 2012). Mature pea plants generally grow up to be 60 cm tall on average, but some plants may reach 3 m needing external support like a climber (Pavek, 2012).

The major botanical trait of pea is its leguminous nature, which enables it to fix nitrogen in conjunction with nitrogen-fixing bacteria from the genus *Rhizobia* (Graham and Vance, 2003). This crop can be effectively rotated, such as cereal crops, barley and wheat to improve their crop yield with lesser use of artificial fertilizers (Collins *et al*., 1992). In this way, pea plants not only increase the crop yield for the major crop grown on that land with less use of fertilizers, but may even provide 2 to 3 harvests from the same land.

1.8.2 Varieties of pea

Pea plants are classified into different varieties on the basis of different physical and botanical traits including height, means of vegetative growth; pod shape and size; season of maturity; color, shape, sweetness, and tenderness of seed; number of seeds per pod;

and average number of pods per node on the plant (Elzebroek, 2008). These pea varieties generally exhibit specificity for climate, which leads farmers and breeders around the world to carefully choose the variety that is best compatible with their regional conditions. Smykal (2014) reports further categorization of pea into varieties on the basis of market value, yield potential, and ease of harvest as well.

1.9 Thesis research questions

The potential of plants to release CH_4 into the atmosphere under normal or stress conditions has been investigated in this thesis using *Pisum sativum* as a model species. This is an important step towards determining the potential impact of CH_4 emissions from plants on the global CH_4 budget, and may call for a reconsideration of the contribution of natural sources of CH_4 to climate change. Further studies are required to understand the variation aerobic CH_4 emissions from plants, in terms of genotypic differences, temporal variation, and variation among organs within plants. In my work, I measured CH_4 emissions from ten pea vatieties as a first step to investigate the varieties that produce highest and lowest CH_4 emissions. I measured CH_4 emissions from plants grown in two types of environments: mixture of perlite: vermiculite: peat moss (pot) and in water (hydroponic system). Plants grown using pot were subjected to combinations of the three stress factors; high temperature, UVB radiation, and drought, and plants grown hydroponically were subjected to temperature and UVB radiation.

From this investigation, I look forward to gaining a better understanding of CH_4 emissions from different varieties of pea plants, shoot parts (upper, middle, and lower), different vegetative and reproductive stages, and plant organs (leaf, stem, root, flower, and pod).

The objectives of this study are:

1) to evaluate the level of CH_4 emission from ten pea varieties grown under a combination of temperature, UVB radiation, and watering regime.
2) to study the response of plant organs (leaf, stem, root, flower, and pods), parts (upper, middle, and lower), and developmental stages (vegetative and reproductive) under these environmental factors in respect to CH_4 emission, plant growth and physiological parameters.

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CHAPTER 2

Single and Interactive Effects of Temperature, UVB Radiation and Water Stress on Methane Emissions from Ten Pea Varieties

2.1 ABSTRACT

Environmental stress conditions, such as heat, ultraviolet-B (UVB) radiation, and drought can have devastating effects on plant growth. The effects of these stresses on plants are typically studied under controlled growth conditions in the laboratory. Few studies have measured the interactive effects of multiple stress factors on methane (CH_4) emissions from plants. We examined the effects of temperature, UVB radiation and watering regime on CH_4 emissions from ten pea varieties – 231E Cascadia, 231C Oregon giant, 237J Sundance, 237M Legacy, UT234 Lincoln, 238A Knight, 236A Paladio, 422 Ho Lan Dow, 234 Lincoln and 234B Bolero. Plants were grown in controlled-environment growth chambers under two temperature regimes (22/18°C and 28/24°C), two levels of UVB radiation [0 (zero) and 5 (ambient) kJ m^{-2} d^{-1}] and two watering regimes (well-watered and water-stressed). A gas chromatograph with a flame ionization detector was used to measure CH_4 emissions rates [ng g^{-1} DM h^{-1}] from detached fresh leaves of each variety. We found that higher temperature and water stress significantly increased CH_4 emissions. Pea varieties varied in CH_4 emissions, which was highest in 237J Sundance and lowest in 422 Ho Lan Dow. Under a combination of the three main factors, 422 Ho Lan Dow had higher stem height and diameter, leaf area and numbers, total dry mass, transpiration, the effective quantum yield of PSII, and wax content than 237J Sundance. These results show that 422 Ho Lan Dow is more resistant to stress conditions, and had lower CH_4 emissions as compared to that of 237J Sundance. We conclude that CH_4 emissions increased under climatic stress conditions and this extra source contribute to the greenhouse effect.

2.2 INTRODUCTION

At present, climate change is a very critical issue to the environment and has stimulated a lot of discussion among environmental scientists on how this change affects the growth of plants. Increasing in temperature and carbon dioxide (CO_2) within the atmosphere is resulting in increase of abiotic or environmental stressors and these all have a detrimental effect on the growth of crop plants (Abou-Hussein, 2012). Environmental stressors have a great impact on the growth as well as physiological processes of all plants. Many environmental stressors affect the growth of crop plants simultaneously (Qaderi *et al.*, 2010). The interactive process among various stressors, such as high temperature, UVB, and water stress, play a role in determining the seriousness of the stress experienced and the overall response shown by the plant (Martel and Qaderi, 2016). In comparison to other stressors, high temperature, UVB radiation, and water stress affect growth patterns, physiological and developmental aspects of plants (Gupta, 2005). Many studies have used several species of agricultural plants to examine the effects of temperature (Rosenzweig *et al.*, 2014), UVB radiation (Wargent *et al.,* 2015), and water stress (Poni, 2015).

It has been documented that the ambient world-wide temperatures are increasing in the last 100 years and it has been projected that at the end of the current century, the global air temperature may be higher by 1.2 to 6.4ºC (Sánchez *et al*., 2010). This in turn will cause changes in precipitation trends and heighten the already existing soil water level deficits (Myhre *et al*., 2013). In addition, the continued depletion of the ozone layer will result in ever increasing levels of UVB radiation that reaches the planet's surface. Plants will have to face increasing levels of this harmful radiation as one of the stressors together with water stress and high ambient temperatures (Qaderi *et al*., 2012).

High temperature as a stressor has a detrimental impact effect on several aspects of plant growth and physiology (as seen in many crop plants, such as tomato, pepper, bean and sweet-corn) (Abou-Hussein, 2012). Exposure to high temperature speeds up many metabolic-related cellular processes while making the developmental phases shorter. This causes decreased rates of growth as well as lowered yields. The accompanying decreases in plant biomass due to high temperature exposure are associated with lowered photosynthesis (Timlin *et al.*, 2006), higher transpiration (Montero *et al.*, 2001) and

lowering of a plant's water utilization efficiency (Craufurd *et al.*, 1999). Proline, as a mediator of osmotic adjustment, has been reported to accumulate under abiotic stresses, such as drought, high or low temperature, and UV radiation (Sin *et al*., 2016).

Water stress has a negative impact on the physiology, biochemistry as well as molecular and cellular processes of a plant. Water stress reduces CO_2 uptake levels due to lowered stomata conducting capabilities but it heightens WUE thus poorly affecting overall productivities of the plant (Qaderi *et al*., 2012). Plants are known to handle water stress by avoiding morphology-based alterations and by developing their tolerance abilities. Phytohormones have been associated in plant responses to combined water stress and high temperature exposure (Zandalinas *et al*., 2016).

Increased exposure to UVB radiation as an abiotic stress factor has a negative impact on plant physiology and growth rate too. In many crop plants, it results in lowered biomass accumulation (Bernal *et al*., 2015). High levels of UVB radiation have a detrimental effect on the plant's DNA, its membranes, photosynthetic ability and levels of phytohormones (Qaderi *et al*., 2010). Higher exposure to UVB radiation also affect negatively the morphogenetic processes, such as height of plant stem, leaf area, the width of the leaf (Chen *et al*., 2016), and the production of secondary metabolites, such as flavonoids that helps in protecting plants against UV radiation (Jansen *et al*., 1998).

Keppler *et al*. (2006) reported for the first time that living plants, such as trees and grasses, emit CH_4 under aerobic conditions. Methane is currently the second most important greenhouse after CO_2 (Fraser *et al*., 2015), and its global warming potential is 34 times more than CO_2 (Myhre *et al*., 2013). Recent studies have shown that CH_4 emissions increased when plants were exposed to stress factors, such as higher temperature (Keppler *et al*., 2006), UVB radiation (Vigano *et al*., 2008), and physical injury (Lenhart *et al*., 2015). However, few studies have dealt with multiple environmental factors (Vigano *et al*., 2008; Qaderi and Reid, 2009; 2011; Abdulmajeed *et al*., 2017; Martel and Qaderi, 2017).

In this study, we examined the effect of multiple factors: temperature, UVB and watering regime, on CH_4 emissions and other parameters from ten pea varieties. We hypothesized that a combination of higher temperatures, supplemental UVB radiation, and water stress influence plants to emit more CH_4, which varies with plant genotype.

The objectives of this study were to measure the rate of CH_4 emissions from different varieties of pea exposed to two temperature regimes, two UVB levels and two watering regimes, and to identify the most tolerant or sensitive variety to environmental conditions.

2.3 MATERIAL AND METHODS

2.3.1 Plant material and growth conditions

In this study, we selected pea (*Pisum sativum* L.) plants because they were one of the highest CH_4 emitters among five other crops, including faba bean, sunflower, canola, barley and wheat, used in previous studies (Qaderi and Reid, 2009; 2011).Then, ten common pea varieties in Nova Scotia, belonging to snowpea and garden pea, were selected to be examined for the highest and lowest CH_4 emissions rates. These varieties are – 231E Cascadia, 231C Oregon giant, 237J Sundance, 237M Legacy, UT234 Lincoln, 238A Knight, 236A Paladio, 422 Ho Lan Dow, 234 Lincoln and 234B Bolero (Table 2.1). Each variety was grown and examined separately three times (three trials) under similar conditions of temperature, UVB radiation and watering regime in controlled-environment growth chambers. Seeds of pea were planted in pots containing a mixture of perlite: vermiculite: peat moss (1:1:2, by volume), and modified Hoagland's solution was used as fertilizer (Zioni *et al*., 1971). Plants (one in each pot) were grown in a controlled-environment growth chamber for one week under the following conditions: temperature (24/20ºC, 16 h light/ 8 h dark), photoperiod (16 h, photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}), and relative humidity (RH, ~ 65%). Light was provided by a mix of cool white fluorescent tubes (Master TL-D-58W/840, Philips, Amsterdam, Netherlands) and incandescent lamps (Litemor, Boston, Massachusetts, USA). PPFD was measured with a quantum LI-250A radiometer/photometer (LI-COR Biosciences, Lincoln, Nebraska, USA) at the shoot apex, and RH was measured with a thermohygrometer (WD-35612-00, Oakton Instruments, Vernon Hills, Illinois, USA). Seedlings of each variety were placed under each of two temperature regimes (22/18°C and 28/24°C; 16 h light/8 h dark), and each temperature regime was supplied with two levels of biologically effective UVB (UVB_{BE}) radiation (0 and 5 kJ m^{-2} d^{-1}) for two weeks of vegetative stage of plants (Fig. 2.1). The UVB_{BE} radiation of 5 kJ m^{-2} d^{-1} is

within the range of natural solar UVB radiation measured in the summer in different areas withi Halifax, Nova Scotia (A. Abdulmajeed, pers. obs.). Under each UVB treatment there were two groups of plants; well-watered plants, which were determined by the excess water drainage, and water-stressed plants, which were determined by the sign of leaf wilting. Midday leaf water potential (Ψ_{wmd}) for the well-watered and water-stressed plants were about –1.0 and –3.0 MPa, respectively. Leaf Ψ_{wmd} was measured with a WP4C Dew Point PotentiaMeter (Decagon Devices Inc., Pullman, WA, USA). UVB radiation was supplied by four fluorescent lamps (UVB 313EL, Q-Panel, Cleveland, OH, USA), which were pre-burned for 96 h to stabilize the UVB output. Each lamp was wrapped in two layers of 0.127 mm cellulose diacetate film (Grafix Plastics, Cleveland, OH, USA) to filter radiation below 280 nm and to provide the desired UVB level (5 kJ m^{-2} d^{-1}). UVB radiation was measured with a PMA2100 photometer/radiometer, which was calibrated against a National Institute of Standards and Technology traceable standard (Solar Light Co., Philadelphia, Pennsylvania, USA). UVB_{BE} levels, measured with a biologically weighted UVB detector, were estimated using Caldwell's (1971) generalized plant damage action spectrum normalized to 300 nm. Daily UVB radiation was for 8 h around noon (Qaderi and Reid, 2005). Plants were rotated and re-organized at the same condition twice a week to reduce positional effects within treatments.

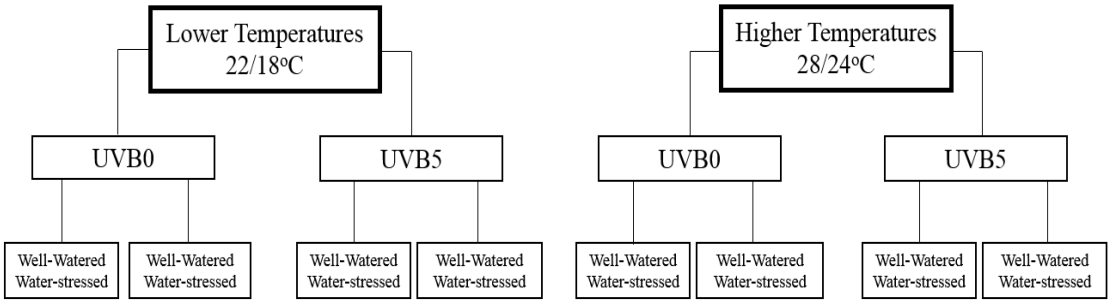
2.3.2 Aerobic methane emissions

Methane emissions rates were determined by a modification of the method that was used for measuring ethylene from plant tissues (Qaderi *et al*., 2010). From each condition, three samples of leaf of three-week-old plants (from the middle part of the plant) were detached and incubated for 2 h in 3-ml plastic syringes inside a growth chamber at 22ºC and 300-µmol photons m^{-2} s^{-1}. Previous studies have shown that 2 h incubation under the temperature of 22ºC is sufficient to detect CH_4 emissions. From each syringe, 1 mL of gas was collected and injected manually into a gas chromatograph-flame ionization detector system (GC-FID; Varian 3900 Gas Chromatograph; Varian Canada, Mississauga, ON) equipped with a capillary column (Carboxen 1006 PLOT, 30 m x 0.53 mm ID, Supelco, Bellefonte, PA, USA). The injector and detector temperatures were set

at 200 and 230ºC, respectively. Helium was used as a carrier gas at 10 mL min^{-1}. Methane was eluted with the following programmed temperature gradient: 1 min isothermal heating at 35ºC followed by a 24ºC min^{-1} oven ramp to 225ºC until the end of the 9 min run. Methane emission was measured first from surrounding air in order to know the level of ambient CH_4 in the air. The injection revealed an insignificant amount of CH_4 that was not readable (see Appendix IC). Then, CH_4 was measured from plant sample by identifying the retention time of the analyte ~2.6 min (see Appendix ID), using external standard (Air Liquide, Dartmouth, Nova Scotia, Canada), and quantified on the basis of standard curve derived from the injection of standard CH_4 gas in 5 (see Appendix IA), 10, 25 and 30 μL with three replications of each. Then, linear regression analysis was applied to generate an eq. (Y = a + bX) in which the Y was replaced by the CH_4 value (mL h^{-1}), which was then converted to ng h^{-1}. The rates of CH_4 emissions (ng g^{-1} DM (dry mass) h^{-1}) were calculated on the basis of plant tissue dry mass by drying the samples at 60°C for 96 h. Accuracy, prior to each use the GC column was conditioned for 90 min. This was done to protect the column from contaminating the ion source, to improve sensitivity and to increase maintenance intervals. Also, it was recommended to run a blank sample to test the sensitivity of the column (see Appendix IB).

FIG. 2.1 Experimental design for pea plants grown in pots. Plants were grown in growth chambers supplemented with two levels of UVB radiation [0 (zero) and 5 (ambient) kJ m^{-2} d^{-1}]. Under each UVB treatment there were two groups of plants; well-watered plants, which were determined by the excess water drainage, and water-stressed plants, which were determined by the sign of leaf wilting.

FIG. 2.1


Lower Temperatures
22/18ºC
Higher Temperatures
28/24ºC
UVB0
UVB5
UVB0
UVB5
Well-Watered
Water-stressed
Well-Watered
Water-stressed
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Water-stressed

TABLE 2.1 List of selected pea varieties under study.

Number	Variety	DTM	Attributes	Tolerance
1	231E Cascadia snowpea	58	Suitable for freezing suitable for canning open pollinated	Powdery Mildew and pea enation virus
2	231C Oregon Giant snow pea	60	As No. 1	Powdery mildew, wilt and enation mosaic
3	237J Sundance garden pea	70	As No. 1	Fusarium wilt Fusarium root rot
4	237M Legacy garden pea	59	As No. 1	Fusarium and powdery Mildew and pea enation
5	UT234 Lincoln (untreated) garden pea	65	As No. 1	Wilt
6	238A Knight garden pea	57	As No. 1	Wilt, powdery mildew and several mosaics
7	236A Paladio garden pea	62	As No. 1	Fusarium
8	422 Ho Lan Dow snow pea	65	Open pollinated	No information available
9	234 Lincoln garden pea	65	As No. 1	Tolerant to common wilt
10	234B Bolero garden pea	66	As No. 1	Best disease tolerance

DTM: Days to maturity

2.3.3 Plant growth and dry mass

From each treatment, three samples of plant were taken and their fresh weights, leaf, stem and root, were determined, using an electrobalance (Model H51, Sartorius GmbH, Goettingen, Germany). Then, the samples were dried at 60°C for 72 h in an Isotemp oven (Model 255G, Fisher Scientific, Nepean, ON, Canada) and reweighed to obtain their dry mass. Growth indices were calculated as following: leaf mass per area (also shown as SLM; specific leaf mass), leaf mass ratio [LMA (g m^{-2}) = leaf dry mass: leaf area], leaf mass ratio [LMR = leaf dry mass: plant dry mass], leaf area ratio [LAR (cm^2 g^{-1}) = leaf area:plant dry mass], and shoot:root mass ratio [SRR = shoot dry mass:root dry mass] (Qaderi *et al*., 2006).

2.3.4 Gas exchange

From each condition, three fully-expanded leaves were used to measure 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 m^{-2} s^{-1}) with a LI-COR portable photosynthesis system (model 6400XT, LI-COR Inc., Lincoln, NE, USA). The photosynthesis system was calibrated before measurements with 400 µmol mol^{-1} of CO_2 with flow rate of 400 mL s^{-1}. The water use efficiency (WUE, µmol CO_2mmol H_2O^{-1}) was calculated by dividing A_N by E (Lambers *et al*., 2008).

2.3.5 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were taken from three leaves using a Fluorpen FP 100 portable fluorometer with a standard leaf-clip (Photon Systems Instruments, Drasov, Czech Republic). Under light conditions, photosynthetic electron transport was measured to determine the effective quantum yield of PSII (ϕPSII). After this, leaves were dark-adapted within the fluorometer clamp for 30 min, which is normally sufficient period that electron carriers are in the oxidized state and the levels of proton gradient and ATP formation are minimal (Bolhar-Nordenkampf *et al*., 1989). Under dark-adapted conditions, measurements were taken of the maximum quantum yield of PSII (F_v/F_m), non-photochemical quenching (qNP), and photochemical quenching (qP) (Schreiber,

2004). In addition, the portable fluorometer has sensor responses to photons withing the 400-700 nm wavebands, the range that plants use energy during photosynthesis.

2.3.6 Proline concentration

Proline concentration was determined as explained in Bates *et al*. (1973). Leaf samples (0.3 g) were homogenized with 5 mL of 3% sulphosalicylic acid and then centrifuged at 4000×*g* for 10 min. Two mL of filtrate was reacted with 2 mL acid-ninhydrinand and 2 mL glacial acetic acid in a test tube. Reaction mixture was incubated at 98ºC for 30 min in a water bath and extracted with 5 mL of toluene. The chromophore, including toluene was aspirated from the aqueous phase, then cooled to room temperature and the absorbance was measured at 520 nm (using toluene as a blank). The proline concentration was determined from a standard curve and calculated on a fresh weight basis µmol g^{-1} FW.

2.3.7 Nitrogen balance index, chlorophyll and flavonoids

Nitrogen balance index (NBI), chlorophyll and flavonoid contents were determined using the Dualex Scientific® system (Dualex Scientific, Force-A, Orsay Cedex, France). This measurement uses the UV optical absorbance of the epidermis, which is based on the fluorescence emitted by the chlorophyll located in the mesophyll. NBI is the ratio of chlorophyll and flavonoid (flavonols). The leaf clip simulates accurately the concentration of chlorophyll and flavonoid contents in the leaf epidermis, based on different wavelengths reading (Martel and Qaderi, 2016).

2.3.8 Wax contents

Wax content was measured from three fully-expanded leaves of each experimental condition. The fresh mass and leaf area were taken immediately before doing the experiment. Each leaf was submerged into 20 mL of trichloromethane for 30 seconds, according to the method explained in Qaderi *et al*. (2002). The solution was left to evaporate until dryness. The quantity of surface wax was expressed in terms of leaf surface area (µg mm^{-2}) or leaf fresh mass (µg mg^{-1}).

2.3.9 Statistical analysis

Effects of temperature, UVB radiation, watering regime, total of ten varieties or two different varieties and their interactions on CH_4 emissions, were determined by means of analysis of variance for split-split-split-plot design (SAS institute, 2011). For such studies, this is the appropriate experimental design (Potvin, 2001; Hinkelman and Kempthorne, 2008). In addition, morphological and physiological parameters of two pea varieties, 237J Sundance and 422 Ho Lan Dow, were also determined by means of analysis of variance for split-split-split-plot design (SAS institute, 2011). In the split-split-split-plot analysis, temperature regimes were treated as the main plot, UVB radiation as the subplot, watering regime as the split-subplot, varieties as the split-split-subplot, and growth chambers as replications (SAS institute, 2011). Differences among growth conditions for each variety were determined using a one-way ANOVA, Scheffé's multiple-comparison procedure, at the 5% confidence level (SAS institute, 2011). Also, the relationship between plant parameters was determined by Pearson's correlation coefficient (Minitab, 2014). All data are reported as mean ± standard error.

2.4 RESULTS

2.4.1 Methane emissions

Methane emissions were tested from ten pea varieties. The one-way ANOVA revealed that higher temperature and water stress increased CH_4 emissions (Fig. 2.2A, C). Among the ten varieties, CH_4 emissions was highest in Sundance 237J (40.92 ± 3.52), but lowest in 422 Ho Lan Dow (25.34 ± 2.02) (Fig. 2.2D). The four-way interaction of temperature (T) × UVB radiation (U) × watering regime (W) × variety (V) significantly affected CH_4 emissions from ten varieties of pea.

Comparing between the two varieties who produced highest, 237J Sundance, and lowest, 422 Ho Lan Dow, CH_4 emissions. The three-way interaction among U × W × V significantly affected CH_4 emissions from pea plants (Table 2.4). However, on the basis of one-way ANOVA, CH_4 emissions was not affected by these factors (Table 2.2). In Fig. 2.4, no differences were found on CH_4 levels from the two varieties under experimental conditions, which are corroborated by the result of one-way ANOVA. However, in term of comparison between the two varieties within conditions, 237J Sundance had higher

CH_4 emissions than 422 Ho Lan Dow when they were grown under L0S and H0S (Fig. 2.4A, B).

2.4.2 Plant growth

Stem height was affected by the four-way interaction of T × U × W × V (Table 2.5). One-way ANOVA, showed that lower temperature and watering to field capacity increased stem height, whereas UVB radiation did not affect stem height of the two varieties. In Figs. 2.4 and 2.5, and Table 2.2, Stem was taller in 422 Ho Lan Dow than 237J Sundance. As shown in Fig. 2.6, it was observed that stem height decreased in plants under higher temperature and also when plants received water stress, regardless of UVB radiation. Only the two-way interaction of U × V affected stem diameter, however, none of the main factors had effects on stem diameter according to the one-way ANOVA. On the other hand, variety had significant effect on stem diameter with thicker stem in 422 Ho Lan Dow than in 237J Sundance (Table 2.2). In Fig. 2.5A and D, in 237J Sundance, stem height decreased when plants grown under higher temperatures, whereas plants of 422 Ho Lan Dow received well water had higher stem height that those plants received water stress.

The three-way interaction among U × W × V had significant effects on leaf area and leaf numbers. However, in one-way ANOVA, well water increased leaf area. Variety also had significant effect on leaf area and leaf number. 422 Ho Lan Dow had higher leaf area and leaf numbers than 237J Sundance (Table 2.2). Among experimental conditions, no significant differences were found in the values of stem diameter and leaf number under any of the conditions (Fig. 2.5).

2.4.3 Dry mass accumulation

Three-way interaction among U × W × V had significant effect on leaf, stem, root and total dry mass. However, one-way ANOVA revealed that only water stress decreased leaf dry mass, whereas other dry mass accumulations remained unaffected by any of the main factors (Table 2.2). Among experimental conditions, no significant differences in leaf, stem and root dry mass have been found for either 237J Sundance or 422 Ho Lan Dow (data not shown).

2.4.4 Growth index

The three-way interaction among U × W × V significantly affected LMA and LAR, whereas only variety and the two-way interaction between U × W, U × V, and W × V affected LMR. On the other hand, no significant differences were found in SRR (Table 2.7). Comparing between these two varieties, 422 Ho Lan Dow had higher LMR, LAR and SRR than 237J Sundance (Table 2.2). While on the other hand, no significant differences in growth index parameters have been found for 237J Sundance and 422 Ho Lan Dow under the eight experimental conditions (data not shown).

2.4.5 Gas exchange

On the basis of one-way ANOVA, higher temperatures decreased A_N and WUE, whereas water stress decreased A_N only. 422 Ho Lan Dow had higher *E*, but lower WUE than 237J Sundance (Table 2.2). The two-way interaction between U × W, U × V, and W × V had significant effect on *E*, while the three-way interaction among U × W × V had significant effect on A_N, g_s, and WUE (Table 2.8). Among experimental conditions, 237J Sundance showed no significant difference in A_N, *E*, and WUE under the provided growth conditions (Fig. 2.7A, B, D). However, it was noticed that *E* increased slightly when plants were exposed to higher temperatures, while WUE decreased slightly when plants were exposed to higher temperatures. In case of g_s, the highest values were obtained under H0W, while the lowest were recorded at H0S (Fig. 2.7C). 422 Ho Lan Dow also showed no significant difference among the values of g_s, *E*, and WUE (Fig. 2.7F, G, H). For A_N, the value was highest under L0W and lowest under H5S (Fig. 2.7E).

TABLE 2.2 Effects of temperature, UVB radiation, watering regime and variety on methane emissions, plant growth and physiological parameters of two pea varieties – 237J Sundance and 422 Ho Lan Dow. Means followed by different upper-case letters within each parameter and condition are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. (3), 237J Sundance; (8), 422 Ho Lan Dow

	Temperature		UVB Radiation		Watering regime		Variety	
Parameters	Lower	Higher	UVB0	UVB5	Well-watered	Parameters	(3)	(8)
Methane	32.25 ± 2.61A	34.01 ± 2.12A	33.92 ± 2.48A	32.34 ± 2.28A	30.94 ± 2.20A	35.32 ± 2.48A	40.92 ± 2.15A	25.33 ± 1.23B
Stem height	9.11 ± 0.27A	5.22 ± 0.44B	7.43 ± 0.62A	6.89 ± 0.76A	7.91 ± 0.624A	6.41 ± 0.49B	6.69 ± 0.54B	7.64 ± 0.61A
Stem diameter	1.37 ± 0.9A	1.03 ± 0.09A	1.41 ± 0.07A	1.26 ± 0.10A	1.39 ± 0.05A	1.28 ± 0.11A	1.19 ± 0.08 B	1.47 ± 0.08A
Leaf area	62.11 ± 4.97A	56.39 ± 4.46A	59.48 ± 4.69A	59.01 ± 4.83A	66.42 ± 5.17A	52.08 ± 3.76B	44.66 ± 2.95B	73.84 ± 4.26A
Leaf number	21.26 ± 1.03A	19.76 ± 1.08A	21.26 ± 1.07A	19.76 ± 1.06A	21.03 ± 1.05A	20.00 ± 1.09A	16.54 ± 0.52B	24.49 ± 0.83A
Leaf DM	171.07 ± 17.04A	159.47 ± 14.54A	162.59 ± 15.76A	167.95 ± 16A	192.43 ± 18.28A	138.11 ± 10.33B	120.06 ± 7.04B	210.48 ± 16.66A
Stem DM	163.07 ± 32.35A	147.28 ± 42.45A	138.02 ± 46.52A	173.61 ±25.78A	177.13 ± 43.98A	133.22 ± 29.63A	46.48 ± 2.83B	263.87 ± 42.64A
Root DM	123.62 ± 15.22A	163.28 ± 41.48A	157.05 ± 35.66A	129.84 ± 26.44A	150.32 ± 32.38A	136.58 ± 30.57A	201.42 ± 39.49A	85.47 ± 11.60B
Total DM	405.49 ± 56.63A	364.81 ± 63.03A	404.34 ± 67.70A	365.95 ± 51.00A	454.25 ± 69.09A	316.04 ± 45.03A	210.48 ± 16.6B	559.82 ± 65.46A
LMA	27.35 ± 0.77A	29.29 ± 1.19A	27.08 ± 0.76A	29.56 ± 1.18A	29.04 ± 1A	27.60 ± 1.03A	27.98 ± 1A	28.66 ± 1.04A
LMR	0.35 ± 0.02A	0.38 ± 0.02A	0.37 ± 0.02A	0.36 ± 0.02A	0.37 ± 0.03A	0.36 ± 0.02A	0.32 ± 0.03B	0.41 ± 0.01A
LAR	129.51 ± 8.61A	139.07 ± 10.40A	139.47 ± 10A	129.12 ± 9.04A	129.70 ± 9.76A	138.89 ± 9.34A	115.94 ± 9.16B	152.64 ± 8.42A
SRR	1.11 ± 0.08A	1.30 ± 0.12A	1.18 ± 0.08A	1.23 ± 0.11A	1.25 ± 0.11A	1.16 ± 0.08A	1.04 ± 0.11B	1.37 ± 0.07A
A_N	15.39 ± 0.40A	13.41 ± 0.39B	14.47 ± 0.46A	14.3 3± 0.41A	15.35 ± 0.42A	13.45 ± 0.36B	14.10 ± 0.35A	14.70 ± 0.50A
g_s	0.07± 0.01A	0.08 ± 0.02A	0.07 ± 0.02A	0.07 ± 0.01A	0.08 ± 0.01A	0.07 ± 0.02A	0.05 ± 0.00A	0.10 ± 0.02A
E	1.80 ± 0.17 A	1.85 ± 0.11A	1.71 ± 0.21A	1.94 ± 0.14A	1.90 ± 0.15A	1.75 ± 0.13A	1.52 ± 0.11B	2.13 ± 0.14A
WUE	10.42 ± 0.27A	7.73 ± 0.32B	9.89 ± 0.42A	8.26 ± 0.24A	9.48 ± 0.40A	8.67 ± 0.32A	10.615 ± 0.28A	7.54 ± 0.43B
ϕPSII	0.62 ± 0.02A	0.56 ± 0.03B	0.60 ± 0.02A	0.59 ± 0.03A	0.65 ± 0.01A	0.53 ± 0.03B	0.53 ± 0.03B	0.65 ± 0.02A
F_v/F_m	0.73 ± 0.02A	0.76 ± 0.01A	0.76 ± 0.01A	0.73 ± 0.02A	0.77 ± 0.01A	0.72 ± 0.02B	0.78 ± 0.01A	0.71 ± 0.01B
qNP	1.56 ± 0.04A	1.34 ± 0.07B	1.46 ± 0.06A	1.44 ± 0.06A	1.39 ± 0.08A	1.51 ± 0.04A	1.46 ± 0.07A	1.44 ± 0.05A
qP	0.19 ± 0.11B	0.32 ± 0.07A	0.25 ± 0.10A	0.26 ± 0.08A	0.24 ± 0.08A	0.27 ± 0.10A	0.27 ± 0.11A	0.24 ± 0.02A

TABLE 2.2 Continued

	Temperature		UVB Radiation		Watering regime		Varities	
Parameters	Lower	Higher	UVB0	UVB5	Well-watered	Parameters	Var (3)	Var (8)
NBI	51.38 ± 1.74A	42.52 ± 3.42B	53.01 ± 2.60A	40.90 ± 2.54B	54.76 ± 1.87A	39.15 ± 2.76B	47.28 ± 3.03A	46.62 ± 2.69A
Total chlorophyll	34.01 ± 0.59A	30.76 ± 1.57B	32.87 ± 0.95A	31.90 ± 0.99A	34.95 ± 0.50A	29.82 ± 1.05B	32.56 ± 0.91A	32.21 ± 1.04A
Flavonoids	0.67 ± 0.02B	0.77 ± 0.05A	0.64 ± 0.02B	0.80 ± 0.04A	0.65 ± 0.02B	0.79 ± 0.04A	0.70 ± 0.03A	0.74 ± 0.04A
UV-absorb comp.	0.47 ± 0.06A	0.28 ± 0.03B	0.38 ± 0.05A	0.37 ± 0.05A	0.40 ± 0.04A	0.35 ± 0.06A	0.44 ± 0.07A	0.32 ± 0.02B
Proline	3.25 ± 0.27B	3.95 ± 0.41A	3.25 ± 0.23B	3.96 ± 0.44A	2.52 ± 0.15B	4.69 ± 0.36A	3.80 ± 0.46A	3.41 ± 0.19A
Wax	3.22 ± 0.57A	2.78 ± 0.47A	3.05 ± 0.63A	3.00 ± 0.41A	2.92 ± 0.51A	3.07 ± 0.55A	2.11 ± 0.24B	3.89 ± 0.60A

TABLE 2.3 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on methane emissions from ten varieties of pea (*Pisum sativum*)

		Methane	
Source	d.f.	MS	*F*
Temperature (T)	1	227.55	28.29*
Main plot error	2	8.04	0.45
UVB radiation (U)	1	15244.01	708.52**
T × U	1	191.02	8.88
Subplot error	2	21.52	1.21
Watering regime (W)	1	15423.53	790.97****
T × W	1	229.73	11.78*
U × W	1	7721.27	395.97****
Split-subplot error	4	19.49	1.10
Variety (V)	9	22804.20	1537.06****
T × V	9	143.65	9.68*
U × V	9	14828.67	999.49****
W × V	9	14982.34	1009.85****
T × U × W	1	216.48	11.10*
T × U × V	9	105.30	7.10*
T × W × V	9	132.51	8.93*
U × W × V	9	9149.90	616.73****
T × U × W × V	9	88.64	5.97*
Split-split-subplot error	160	14.84	0.83

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 2.4 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on methane emissions from two varieties of pea (*Pisum sativum* 237J Sundance and 422 Ho Lan Dow)

	Methane		
Source	d.f.	MS	*F*
Temperature (T)	1	23.76	0.74
Main plot error	2	31.99	2.72
UVB radiation (U)	1	2569.98	83.33*
T × U	1	5.63	0.18
Subplot error	2	30.84	2.63
Watering regime (W)	1	2700.79	66.77**
T × W	1	28.02	0.69
U × W	1	2435.51	60.21**
Split-subplot error	4	40.45	3.45*
Variety (V)	1	2457.59	118.61****
T × V	1	6.94	0.33
U × V	1	2443.39	117.93****
W × V	1	2546.25	122.89****
T × U × W	1	19.83	0.49
T × U × V	1	0.00	0.00
T × W × V	1	1.32	0.06
U × W × V	1	2600.68	125.52****
T × U × W × V	1	0.00	0.00
Split-split-subplot error	8	20.72	1.77

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.000$

FIG. 2.2 Methane emissions from ten pea varieties grown under eight experimental conditions. (**A**) temperature, (**B**) UVB, (**C**) watering regime, (**D**) varities; **1**, 231E Cascadia; **2**, 231C Oregon Giant; **3**, 237J Sundance; **4**, 237M Legacy; **5**, UT234 Lincoln; **6**, 238A Knight; **7**, 236A Paladio; **8**, 422 Ho Lan Dow; **9**, 234 Lincoln; **10**, 234B Bolero.

FIG. 2.2

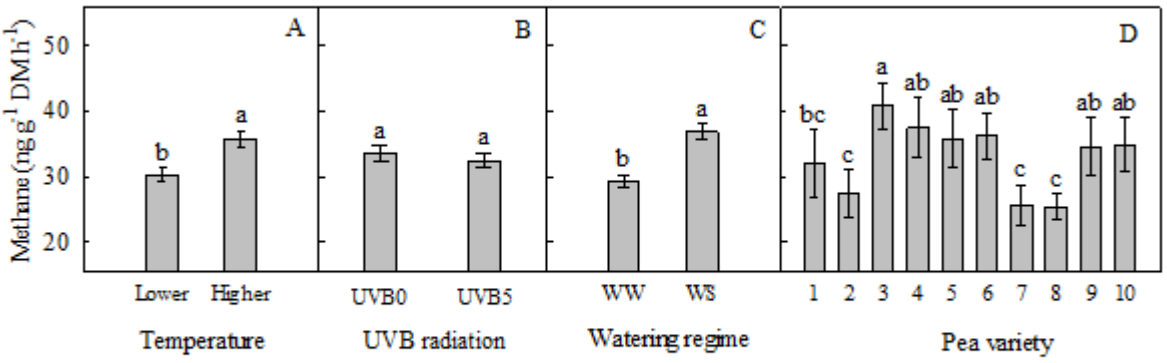

Methane (ng g-1 DM h-1)
50
40
30
20
A
B
C
D
b
a
a
a
b
a
bc
c
a
ab
ab
ab
c
c
ab
ab
Lower
Higher
UVB0
UVB5
WW
WS
1
2
3
4
5
6
7
8
9
10
Temperature
UVB radiation
Watering regime
Pea variety

FIG. 2.3 Methane emissions from (**A**) 237J Sundance and (**B**) 422 Ho Lan Dow grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC. L0W, low temperature-UVB0-well watered; L0S, low temperature-UVB0-water stressed; L5W, low temperature-UVB5-well watered; L5S, low temperature-UVB5-water stressed; H0W, high temperature-UVB0-well watered; H0S, high temperature-UVB0-water stressed; H5W, high temperature-UVB5-well watered; H5S, high temperature-UVB5-water stressed. Bars (mean ± SE) followed by different upper-case letters between varieties and lower-case letters within each variety are significantly different ($P < 0.05$) according to Scheffé's multiple comparison procedure.

FIG. 2.3

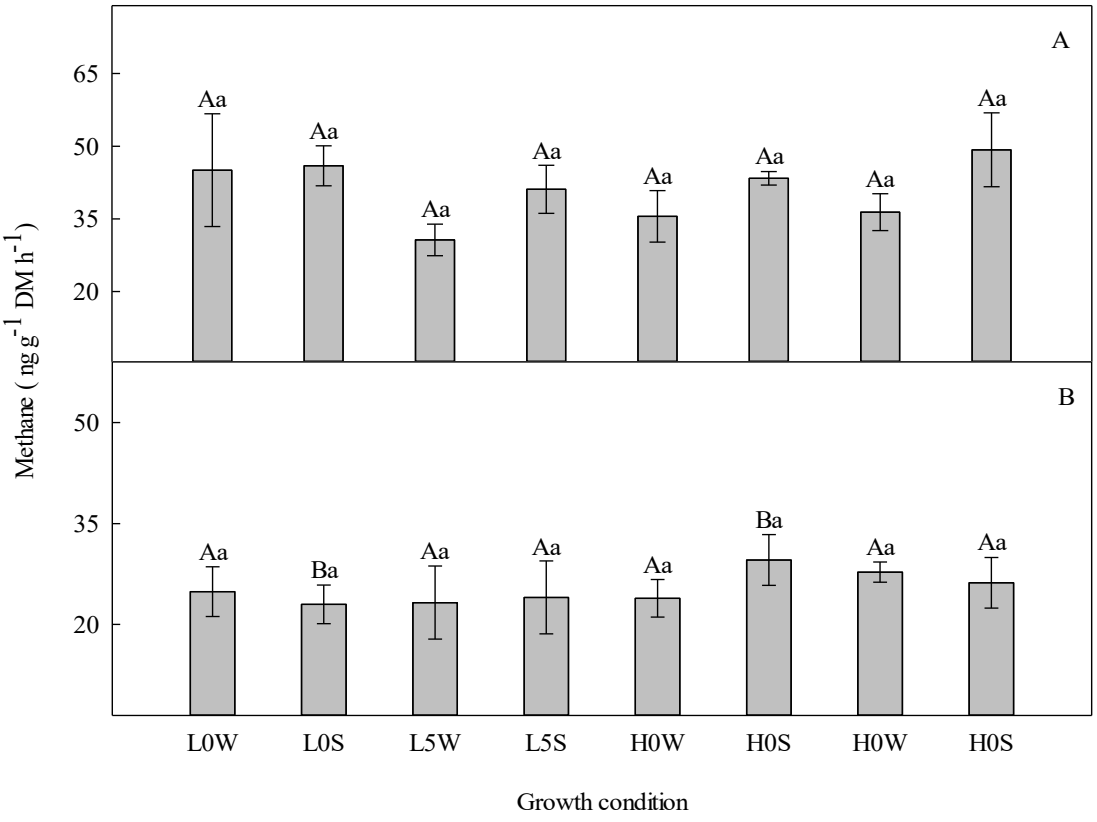

A
B
65
50
35
20
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Ba
Aa
Aa
Aa
Ba
Aa
Aa
L0W
L0S
L5W
L5S
H0W
H0S
H0W
H0S
Methane (ng g-1 DM h-1)
Growth condition

TABLE 2.5 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on stem height, stem diameter, leaf area and leaf number from two varieties of pea (*Pisum sativum* 237J Sundance and 422 Ho Lan Dow). Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

		Stem height		Stem diameter		Leaf area		Leaf number	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	2.01	1.44	0.26	1.82	9.89	0.25	0.06	0.01
Main plot error	2	1.40	4.80	0.14	0.84	39.45	0.84	7.91	2.06
UVB radiation (U)	1	93.81	221.44**	1.17	5.02	8302.9	190.95**	1083.98	126.08**
T × U	1	4.41	10.42**	0.01	0.02	35.97	0.83	0.46	0.05
Subplot error	2	0.42	1.45	0.23	1.37	43.48	0.92	8.60	2.24
Watering regime (W)	1	52.78	64.12**	2.63	10.48*	8699.02	181.26***	1143.37	168.17***
T × W	1	2.40	2.91	0.06	0.23	5.00	0.10	0.19	0.03
U × W	1	75.24	91.41***	0.00	0.00	8175.87	170.36***	979.49	144.07***
Split-subplot error	4	0.82	2.82	0.25	1.48	47.99	1.02	6.80	1.77
Variety (V)	1	71.81	41.63***	0.07	0.20	8178.33	243.31****	999.31	83.12****
T × V	1	6.38	3.70	0.25	0.70	29.51	0.88	1.98	0.16
U × V	1	44.31	25.69**	2.95	8.15*	7959.67	236.81****	1020.19	84.85****
W × V	1	97.55	56.55****	1.39	3.86	8298.79	246.90****	1062.50	88.37****
T × U × W	1	2.41	2.92	0.14	0.55	7.85	0.16	0.00	0.00
T × U × V	1	9.43	5.47*	0.29	0.81	87.26	2.60	7.54	0.63
T × W × V	1	6.43	3.73	0.15	0.43	35.48	1.06	1.83	0.15
U × W × V	1	61.90	35.89***	0.00	0.00	8121.48	241.62****	1142.26	95.01****
T × U × W × V	1	10.86	6.30*	0.32	0.89	114.14	3.40	8.43	0.70
Split-split-subplot error	8	1.72	5.90	0.36	2.13	33.61	0.71	12.02	3.13

FIG. 2.4 Pea variety (*Pisum sativum* 237J Sundance), which had highest CH_4 emissions among ten varieties. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC.

FIG. 2.5 Pea variety (*Pisum sativum* 422 Ho Lan Dow), which had lowest CH_4 emissions among ten varieties. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC.

FIG. 2.4


LT-UVB0-WW
LT-UVB0-WS
LT-UVB5-WW
LT-UVB5-WS
HT-UVB0-WW
HT-UVB0-WS
HT-UVB5-WW
HT-UVB5-WS

FIG. 2.5

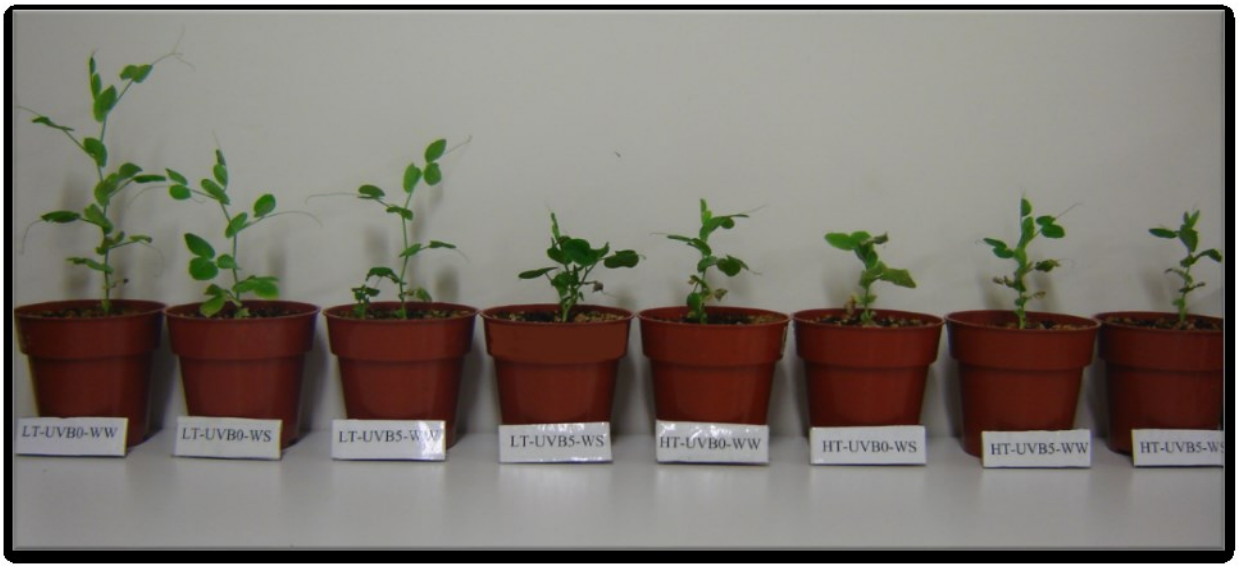
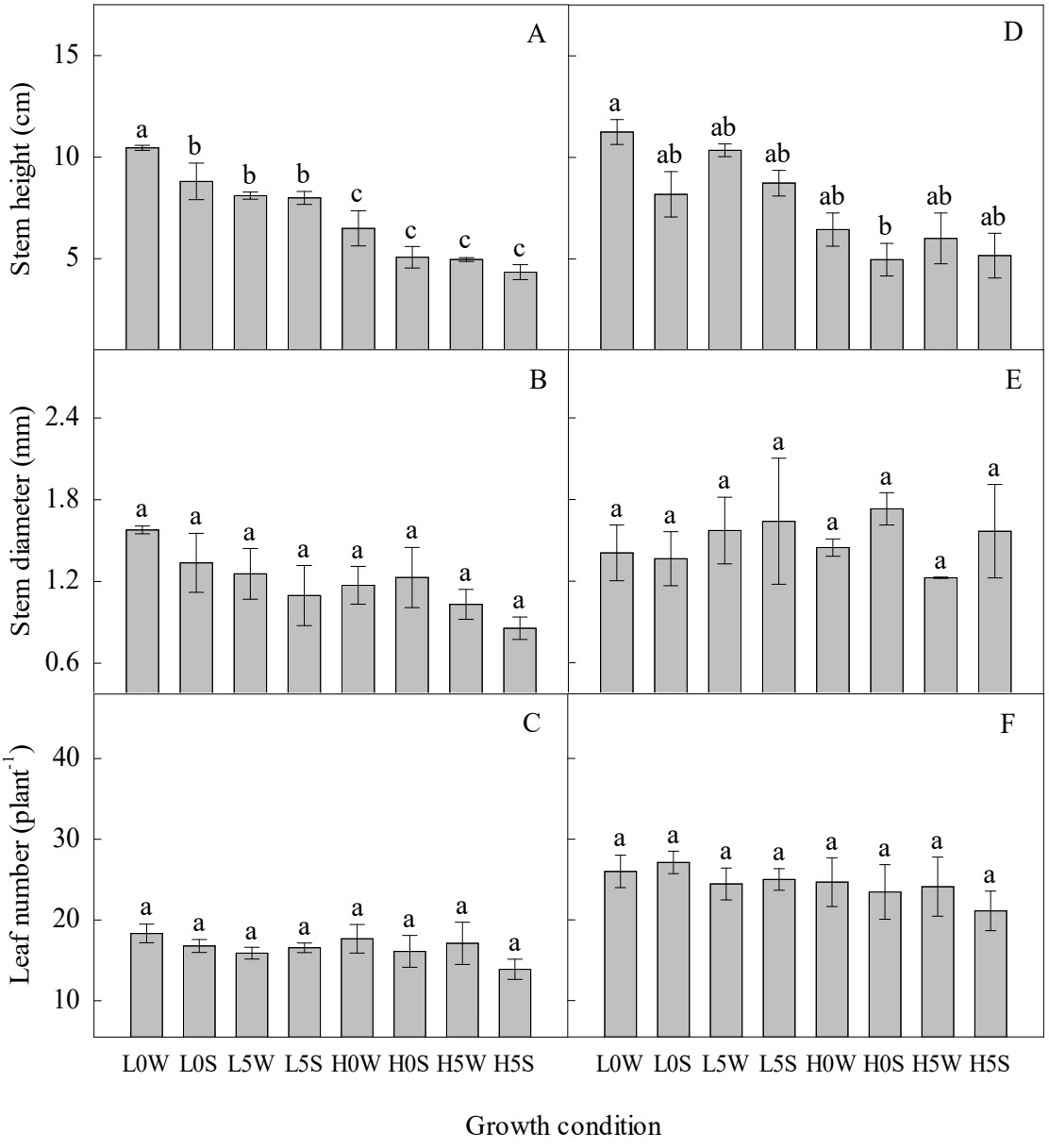

LT-UVB0-WW
LT-UVB0-WS
LT-UVB5-WW
LT-UVB5-WS
HT-UVB0-WW
HT-UVB0-WS
HT-UVB5-WW

FIG. 2.6 Stem height, stem diameter and leaf number from (**A-C**) 237J Sundance and (**D-F**) 422 Ho Lan Dow grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC.Other details are the same as in Fig. 2.4.

FIG. 2.6


A
D
B
E
C
F
Stem height (cm)
Stem diameter (mm)
Leaf number (plant-1)
15
10
5
2.4
1.8
1.2
0.6
40
30
20
10
a b b b c c c c
a ab ab ab ab b ab ab
a a a a a a a a
a a a a a a a a
a a a a a a a a
a a a a a a a a
L0W L0S L5W L5S H0W H0S H5W H5S
L0W L0S L5W L5S H0W H0S H5W H5S
Growth condition

2.4.6 Chlorophyll fluorescence

The results of one-way ANOVA revealed that water stress decreased ϕPSII and F_v/F_m, while higher temperatures decreased ϕPSII and qNP, but increased qP (Table 2.2). In the two varieties, 422 Ho Lan Dow had higher ϕPSII, whereas 237J Sundance had higher F_v/F_m (Table 2.2). The two-way interaction between U × W was significant for ϕPSII and F_v/F_m, whereas U × W, U × V, and W × V were significant for qP (Table 2.9). Among experimental conditions, the values of ϕPSII, F_v/F_m, and qNP for 237J Sundance were not different (Fig. 2.8A-C). However, water stress decreased ϕPSII, regardless of temperature and UVB radiation. For qP there were very large differences with the highest value observed for H0W and lowest for L5W (Fig. 2.8D). The values of ϕPSII, F_v/F_m, qNP and qP for 422 Ho Lan Dow did not have any significant differences among conditions (Fig. 2.8E-H).

2.4.7 Nitrogen balance index, chlorophyll, and flavonoids

The three-way interaction of U × W × V was significant for NBI (Table 2.10). One-way ANOVA showed that higher temperatures, UVB5 and water stress decreased NBI (Table 2.2). However, in Fig. 2.9A, water stress slightly decreased NBI in 237J Sundance regardless of temperature and UVB radiation. In 422 Ho Lan Dow, water stress decreased NBI in plants grown under higher temperature, regardless of UVB levels. In both varieties, NBI was lowest in H5S as compared to all other conditions that had the same level of NBI (Fig. 2.9E).

The three-way interaction among U × W × V was significant for chlorophyll (Table 2.10). However, one-way ANOVA showed that higher temperatures and water stress decreased total chlorophyll (Table 2.2). A combination of temperature, UVB and watering regimes indicated that total chlorophyll was lowest in H5S but highest in L5W (Fig. 2.9B). Similarly, in 422 Ho Lan Dow, plants showed lowest total chlorophyll under H0S, but highest in L0W and H5W (Fig. 2.9F), whereas other conditions had almost the same level of total chlorophyll.

The two-way interaction of U × W was significant for flavonoids (Table 2.10). However, one-way ANOVA showed that higher temperatures, UVB5 and water stress increased the flavonoid content (Table 2.2). However, a combination of the three factors

that showed that highest content was observed for condition H5S and lowest in H0W in 237J Sundance (Fig. 2.9C), whereas also H5S had highest flavonoids compared to other conditions in 422 Ho Lan Dow (Fig. 2.9G).

2.4.8 UV-absorbing compounds

The two-way interactions between U × W, U × V, and W × V had significant effect on UV-absorbing compounds (Table 2.10). However, one-way ANOVA indicated that higher temperatures decreased UV-absorbing compounds (Table 2.2). In Fig. 2.9D, Our result for 237J Sundance indicated that higher temperature decreased the concentration of UV-absorbing compounds, regardless of UVB radiation and watering regimes, whereas no differences in UV-absorbing compounds have been found among experimental conditions in 422 Ho Lan Dow (Fig. 2.9H).

2.4.9 Proline concentration

The three-way interaction of U × W × V was significant for proline contents (Table 2.11). However, one-way ANOVA revealed that higher temperatures, UVB5 and water stress decreased proline contents (Table 2.2). In Fig. 2.10A, water stress increased proline contents, regardless of temperature and UVB radiation and it also showed the highest value of proline was obtained under H5S as compared to the lowest value under L5W. Similarly, In 422 Ho Lan Dow, water stress increased proline contents except when plants grown under H5S (Fig. 2.10). The highest amount of proline was recorded for H0S while the lowest was recorded under L5W. Overall, no significant differences between the two varieties in proline contents were observed (Table 2.2).

2.4.10 Wax content

One-way ANOVA revealed that none of the main factors affected wax content (Table 2.2). However, the three-way interaction of U × W × V had significant effects on wax content (Table 2.11). In Fig.2.10B, D, no significant differences in wax content were observed among experimental conditions, which is not in matching with the three-way interaction. Overall, 422 Ho Lan Dow had higher wax content than 237J Sundance (Table 2.2).

2.4.11 Relationship between plant parameters

Pearson's correlation coefficients were significant for several relationships in term of physiological parameters of two varieties grown under two temperature regimes, two UVB levels and two watering regimes. For instance, CH_4 emissions was negatively correlated with leaf area ($r = -0.388$, $P = 0.006$), ϕPSII ($r = -0.302$, $P = 0.037$) and wax content ($r = -0.416$, $P = 0.003$), but positively correlated with proline ($r = 0.427$, $P = 0.002$).

TABLE 2.6 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on dry mass accumulation from two varieties of pea (*Pisum sativum,* 237J Sundance and 422 Ho Lan Dow)

		Dry mass accumulation							
		Leaf DM		Stem DM		Root DM		Total DM	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	86.79	0.30	2911.05	1.47	1781.78	0.45	7164.20	1.15
Main plot error	2	290.80	0.86	1978.66	0.71	3920.13	1.37	6213.25	1.06
UVB radiation (U)	1	62524.57	177.12**	54377.82	28.47*	55015.24	17.32	331985.90	57.65*
T × U	1	277.94	0.79	3701.12	1.94	1084.08	0.34	10335.04	1.79
Subplot error	2	353.00	1.05	1910.12	0.69	3176.85	1.11	5759.02	0.99
Watering regime (W)	1	65348.65	220.87***	56839.66	27.79**	57505.43	14.03*	346707.40	54.43**
T × W	1	41.93	0.14	2681.97	1.31	2014.02	0.49	6194.33	0.97
U × W	1	63445.20	214.44***	55110.33	26.94**	55762.27	13.60*	340141.40	53.40**
Split-subplot error	4	295.87	0.88	2045.56	0.74	4098.87	1.43	6369.71	1.09
Variety (V)	1	63037.20	172.52****	54771.07	30.51***	55417.67	19.93**	337226.90	61.72****
T × V	1	174.65	0.48	3323.79	1.85	1428.97	0.51	8441.10	1.54
U × V	1	60336.03	165.12****	52460.13	29.22***	53076.31	19.09**	321049.50	58.75***
W × V	1	62930.92	172.22****	54715.43	30.48***	55358.18	19.91**	334898.80	61.29****
T × U × W	1	65.40	0.22	2930.56	1.43	1715.73	0.42	7130.01	1.12
T × U × V	1	576.9	1.58	4599.38	2.56	483.50	0.17	13861.04	2.54
T × W × V	1	222.69	0.61	3569.28	1.99	1184.57	0.43	9437.08	1.73
U × W × V	1	59497.17	162.83****	51804.41	28.86**	52406.47	18.85**	313072.30	57.30****
T × U × W × V	1	786.10	2.15	5353.37	2.98	0.00	0.00	17039.81	3.12
Split-split-subplot error	8	365.40	1.09	1795.13	0.65	2780.18	0.97	5464.22	0.94

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 2.7 Summary of split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on growth indices from two varieties of pea (*Pisum sativum,* 237J Sundance and 422 Ho Lan Dow)

		Growth indices							
		LMA		LMR		LAR		S:R mass ratio	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	11.88	4.76	0.63	2.00	433.78	2.73	0.93	1.26
Main plot error	2	2.49	0.61	0.31	1.13	158.90	0.59	0.74	2.50
UVB radiation (U)	1	2121.06	533.91**	3.21	13.82	56978.84	277.83**	0.10	0.56
T × U	1	0.58	0.14	0.25	1.09	115.95	0.57	0.37	2.16
Subplot error	2	3.97	0.97	0.23	0.83	205.09	0.76	0.17	0.57
Watering regime (W)	1	2230.56	1080.49****	2.73	2.03	59556.38	446.41****	0.02	0.01
T × W	1	14.48	7.01	0.36	0.27	546.91	4.10	0.63	0.41
U × W	1	1992.46	965.15****	15.83	11.82*	57770.84	433.03****	6.14	4.04
Split-subplot error	4	2.06	0.50	1.34	4.79	133.41	0.49	1.52	5.16
Variety (V)	1	2014.59	281.42****	12.08	14.70**	57409.77	211.85****	3.91	4.07
T × V	1	1.22	0.17	0.50	0.60	267.71	0.99	0.29	0.31
U × V	1	2012.78	281.17****	4.96	6.03*	54974.56	202.87****	0.59	0.62
W × V	1	2097.28	292.97****	5.3	6.45*	57338.27	211.59****	0.67	0.69
T × U × W	1	8.99	4.36	0.33	0.24	387.16	2.90	0.55	0.36
T × U × V	1	0.00	0.00	0.33	0.40	0.00	0.00	0.22	0.23
T × W × V	1	0.00	0.00	0.52	0.63	148.78	0.55	0.30	0.31
U × W × V	1	2162.83	302.13****	0.00	0.00	54260.97	200.23****	2.30	2.39
T × U × W × V	1	0.00	0.00	0.33	0.40	0.00	0.00	0.17	0.17
Split-split-subplot error	8	7.16	1.74	0.82	2.94	270.99	1.00	0.96	3.26

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 2.8 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on gas exchange from two varieties of pea (*Pisum sativum,* 237J Sundance and 422 Ho Lan Dow)

		Gas exchange							
		A_N		E		g_s		WUE	
Source	d.f.	MS	F	MS	F	MS	F	MS	F
Temperature (T)	1	0.02	0.01	0.57	2.53	0.84	4.55	0.40	0.24
Main plot error	2	1.23	1.99	0.23	0.81	0.18	0.55	1.66	1.24
UVB radiation (U)	1	482.75	398.48**	5.30	17.97	0.24	0.54	183.10	533.38**
T × U	1	0.39	0.32	0.24	0.81	0.28	0.63	1.77	5.14
Subplot error	2	1.21	1.96	0.29	1.05	0.44	1.32	0.34	0.26
Watering regime (W)	1	511.88	299.92***	4.72	3.58	0.46	0.34	196.06	103.15***
T × W	1	0.05	0.03	0.31	0.24	0.57	0.42	0.45	0.23
U × W	1	406.84	238.37***	20.23	15.31*	2.79	2.04	134.48	70.75**
Split-subplot error	4	1.71	2.76	1.32	4.71	1.37	4.09	1.90	1.42
Variety (V)	1	421.96	170.37***	15.94	19.26**	1.37	1.68	144.26	71.1****
T × V	1	2.17	0.88	0.55	0.66	0.34	0.42	4.87	2.40
U × V	1	447.80	180.8***	7.45	9.00*	0.01	0.01	165.24	81.44****
W × V	1	465.97	188.13***	7.93	9.58*	0.01	0.01	171.67	84.61****
T × U × W	1	0.00	0.00	0.29	0.22	0.48	0.35	0.65	0.34
T × U × V	1	5.50	2.22	0.34	0.41	0.28	0.34	7.40	3.65
T × W × V	1	2.13	0.86	0.58	0.7	0.33	0.41	5.03	2.48
U × W × V	1	538.04	217.23***	0.00	0.00	5.44	6.68*	225.70	111.24****
T × U × W × V	1	5.98	2.41	0.36	0.44	0.19	0.23	8.14	4.01
Split-split-subplot error	8	2.48	4.01	0.83	2.95	0.81	2.43	2.03	1.51

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 2.7 Gas exchange parameter from (**A-D**) 237J Sundance and (**E-H**) 422 Ho Lan Dow grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC. Other details are the same as in Fig. 2.4.

FIG. 2.7

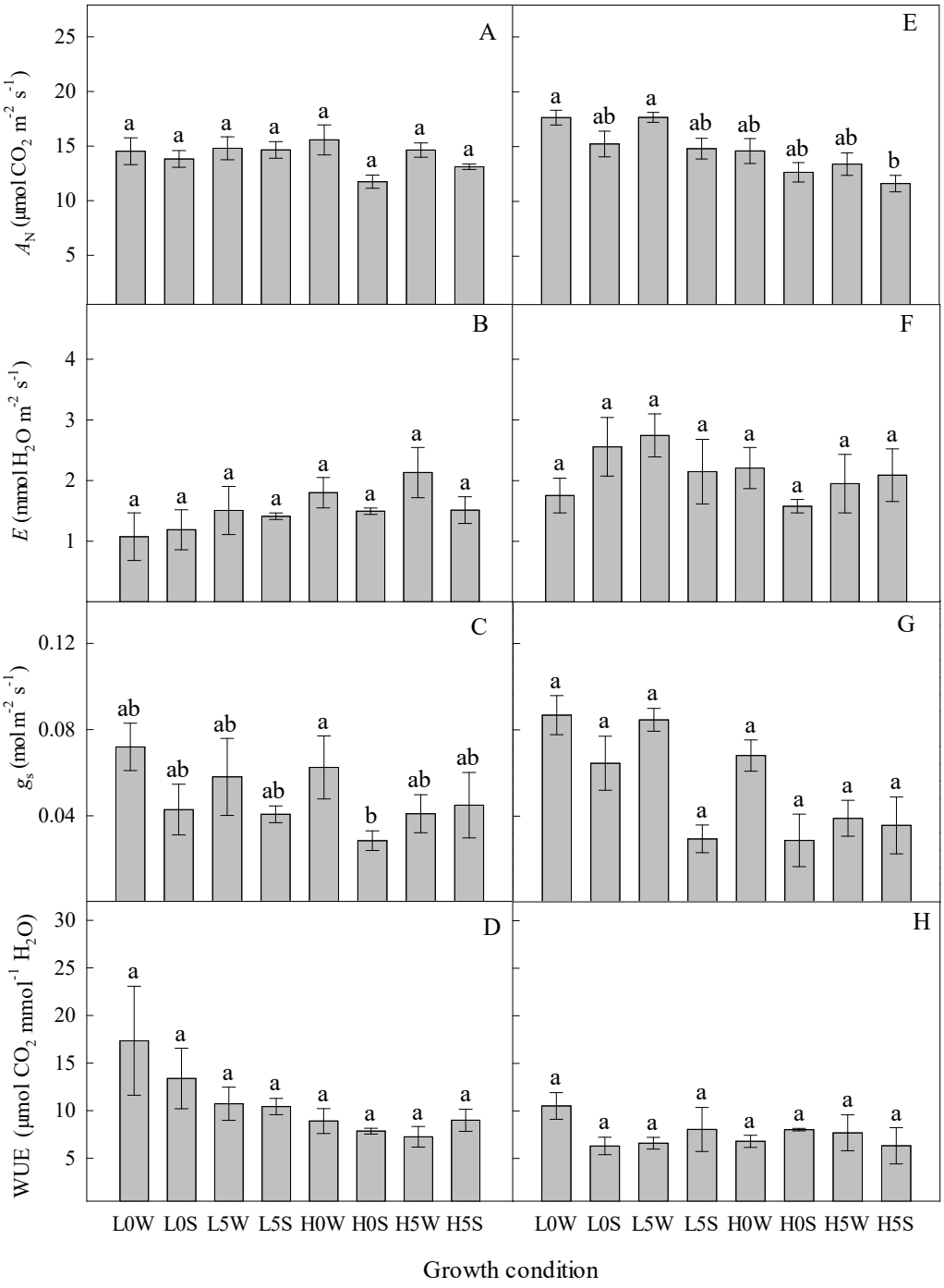

A
E
B
F
C
G
D
H
A_N (μmol CO_2 m^{-2} s^{-1})
E (mmol H_2O m^{-2} s^{-1})
g_s (mol m^{-2} s^{-1})
WUE (μmol CO_2 mmol^{-1} H_2O)
L0W L0S L5W L5S H0W H0S H5W H5S
L0W L0S L5W L5S H0W H0S H5W H5S
Growth condition

Table 2.9 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on chlorophyll fluorescence from two varieties of pea (*Pisum sativum* 237J Sundance and 422 Ho Lan Dow)

		Chlorophyll fluorescence							
		ϕPSII		*Fv/Fm*		qNP		qP	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	0.44	1.46	0.65	2.50	0.46	2.04	0.74	3.58
Main plot error	2	0.30	1.08	0.26	0.94	0.23	0.78	0.21	0.74
UVB radiation (U)	1	2.19	8.94	1.39	5.47	0.01	0.03	4.05	12.72
T × U	1	0.14	0.56	0.24	0.95	0.09	0.34	0.33	1.04
Subplot error	2	0.24	0.87	0.25	0.91	0.27	0.94	0.32	1.14
Watering regime (W)	1	1.78	1.33	1.05	0.80	0.08	0.06	3.53	2.67
T × W	1	0.23	0.17	0.39	0.30	0.26	0.20	0.45	0.34
U × W	1	13.41	10.01*	11.28	8.56*	4.27	3.27	17.68	13.37*
Split-subplot error	4	1.34	4.76	1.32	4.71	1.30	4.48	1.32	4.73
Variety (V)	1	9.99	12.18**	8.17	9.80*	2.46	2.89	13.70	16.56**
T × V	1	0.68	0.83	0.48	0.57	0.66	0.78	0.41	0.50
U × V	1	3.69	4.50	2.64	3.17	0.14	0.17	5.98	7.23*
W × V	1	3.96	4.83	2.85	3.42	0.17	0.20	6.38	7.72*
T × U × W	1	0.2	0.15	0.34	0.26	0.21	0.16	0.41	0.31
T × U × V	1	0.48	0.58	0.33	0.40	0.55	0.65	0.26	0.31
T × W × V	1	0.69	0.84	0.49	0.59	0.66	0.77	0.44	0.54
U × W × V	1	0.00	0.00	0.24	0.29	3.75	4.41	0.00	0.00
T × U × W × V	1	0.46	0.56	0.31	0.38	0.48	0.57	0.28	0.34
Split-split-subplot error	8	0.82	2.91	0.83	2.98	0.85	2.92	0.83	2.96

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 2.8 Chlorophyll fluorescence from (**A-D**) 237J Sundance and (**E-H**) 422 Ho Lan Dow grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC. Other details are the same as in Fig. 2.4.

FIG. 2.8

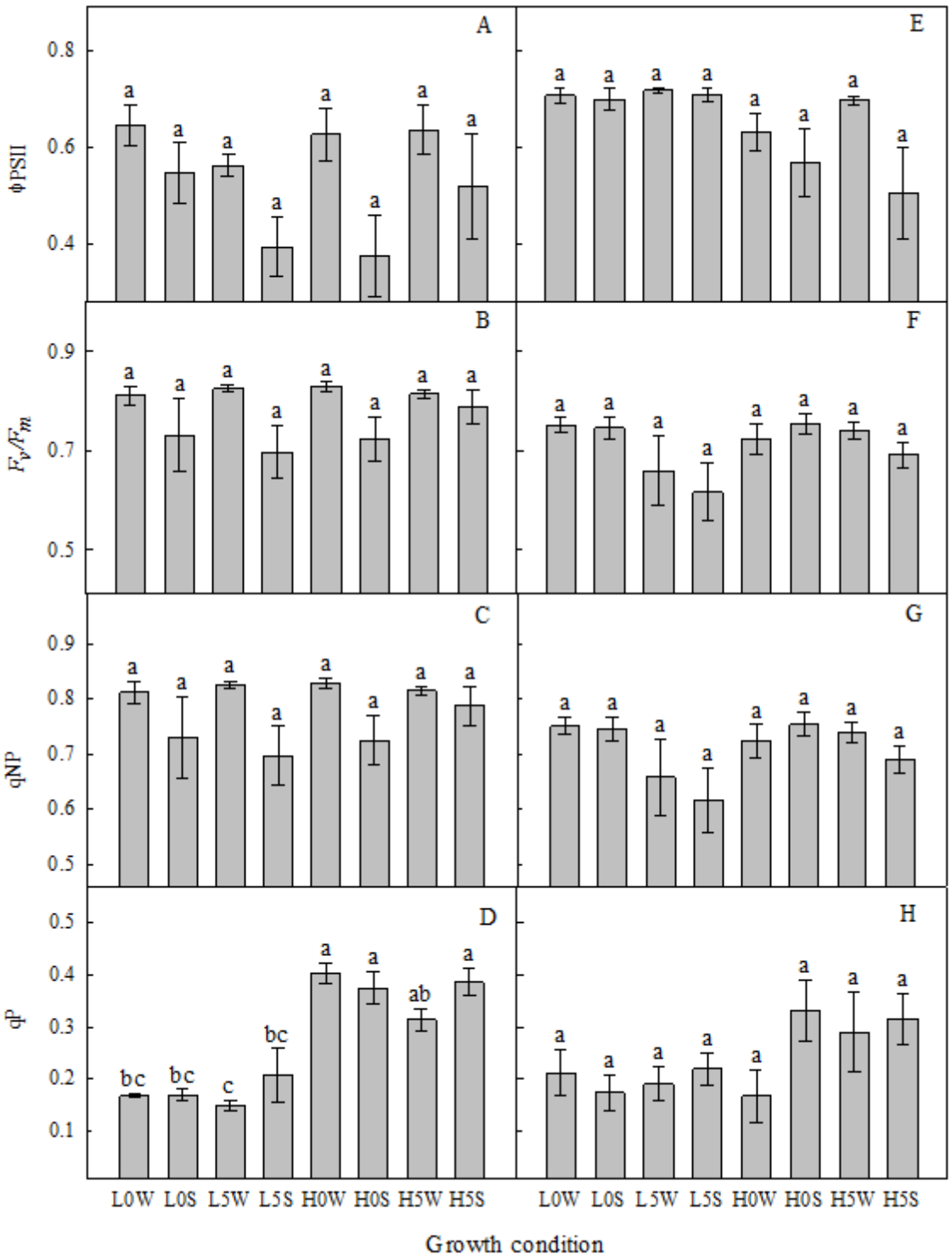

A
E
B
F
C
G
D
H
ΦPSII
F_v/F_m
qNP
qP
L0W L0S L5W L5S H0W H0S H5W H5S
L0W L0S L5W L5S H0W H0S H5W H5S
Growth condition

TABLE 2.10 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on NBI, chlorophyll, flavonoids and proline from two varieties of pea (*Pisum sativum* 237J Sundance and 422 Ho Lan Dow)

		NBI		Total chlorophyll		Flavonoids		UV-absorbing compounds	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	66.97	0.48	14.81	0.47	0.77	5.46	0.34	1.17
Main plot error	2	138.45	10.15****	31.29	13.24****	0.14	0.50	0.29	1.03
UVB radiation (U)	1	5554.45	36.52*	2555.06	78.91*	1.35	3.44	3.31	13.71
T × U	1	114.01	0.75	31.71	0.98	0.31	0.80	0.10	0.40
Subplot error	2	152.09	11.15****	32.38	13.70	0.39	1.39	0.24	0.86
Watering regime (W)	1	5824.39	46.15**	2685.16	101.74***	1.02	0.77	2.83	2.13
T × W	1	57.04	0.45	12.02	0.46	0.49	0.37	0.15	0.11
U × W	1	5411.65	42.88**	2420.75	91.72***	11.16	8.45*	16.07	12.08*
Split-subplot error	4	126.20	9.25****	26.39	11.16****	1.32	4.68	1.33	4.71
Variety (V)	1	5426.32	31.22***	2442.84	58.53****	8.06	9.50*	12.29	14.93**
T × V	1	107.41	0.62	33.39	0.80	0.39	0.46	0.82	1.00
U × V	1	5312.53	30.56***	2429.07	58.20****	2.58	3.04	5.09	6.18*
W × V	1	5538.12	31.86***	2531.32	60.65****	2.79	3.29	5.44	6.61*
T × U × W	1	69.09	0.55	15.69	0.59	0.43	0.33	0.13	0.10
T × U × V	1	185.82	1.07	63.35	1.52	0.27	0.32	0.57	0.69
T × W × V	1	119.89	0.69	37.41	0.90	0.41	0.48	0.83	1.01
U × W × V	1	5485.20	31.56***	2586.15	61.96****	0.27	0.32	0.00	0.00
T × U × W × V	1	228.02	1.31	77.99	1.87	0.25	0.30	0.55	0.67
Split-split-subplot error	8	173.82	12.74****	41.74	17.65****	0.85	3.01	0.82	2.91

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 2.9 Nitrogen balance index, chlorophyll, flavonoids and UV-absorbing compounds. (**A-D**) 237J Sundance and (**E-H**) 422 Ho Lan Dow under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC. Other details are the same as in Fig. 2.4.

FIG. 2.9

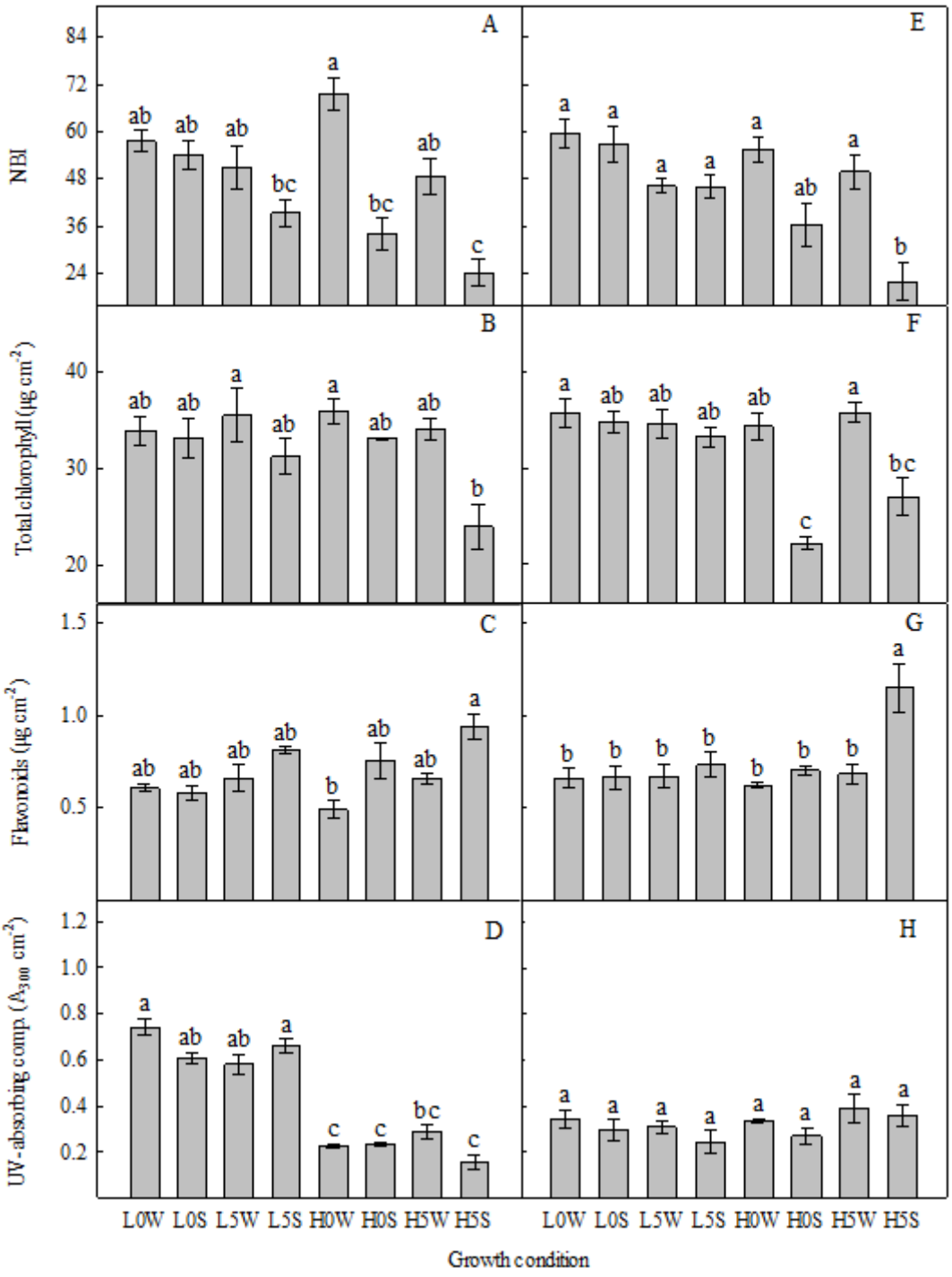

A
E
B
F
C
G
D
H
NBI
Total chlorophyll (μg cm-2)
Flavonoids (μg cm-2)
UV-absorbing comp. (A300 cm-2)
L0W L0S L5W L5S H0W H0S H5W H5S
L0W L0S L5W L5S H0W H0S H5W H5S
Growth condition

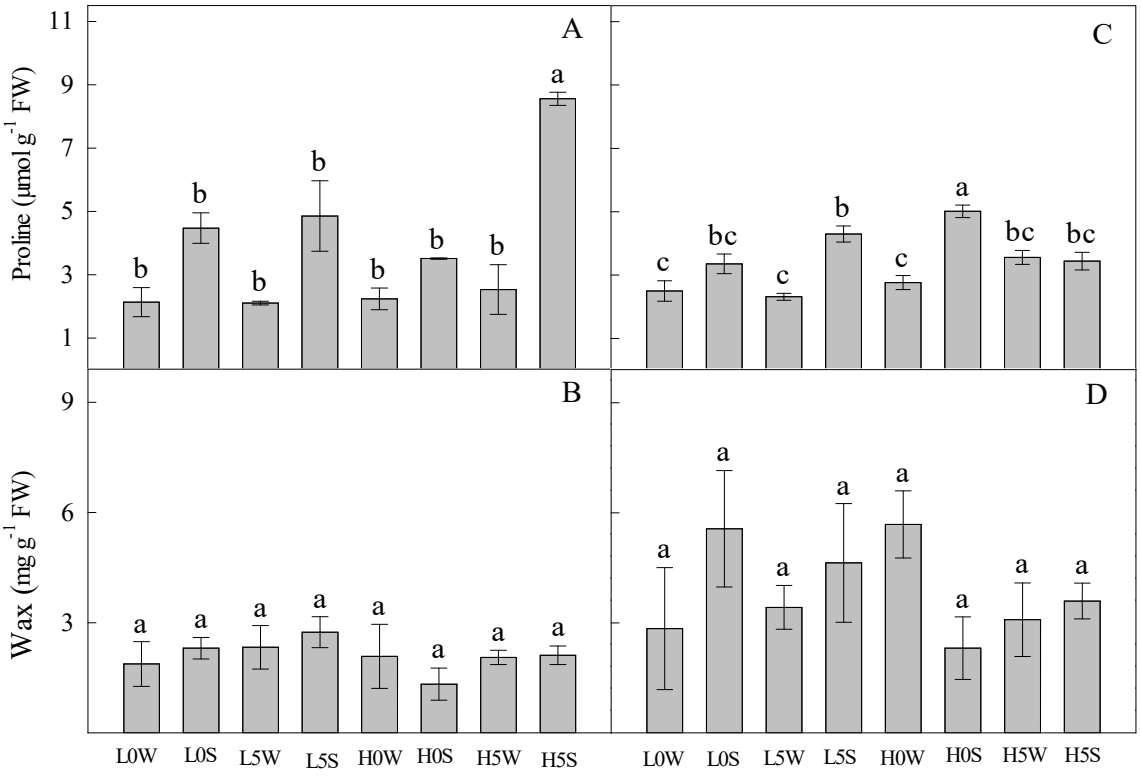
TABLE 2.11 Summary of split-split-split plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on proline and wax from two varieties of pea (*Pisum sativum* 237J Sundance and 422 Ho Lan Dow)

		Proline		Wax	
Source	d.f.	MS	*F*	MS	*F*
Temperature (T)	1	2.36	2.52	0.06	0.10
Main plot error	2	1.49	2.22	0.65	1.01
UVB radiation (U)	1	17.98	5.31	6.90	2.79
T × U	1	0.97	0.29	0.44	0.18
Subplot error	2	4.12	6.12	2.47	3.85
Watering regime (W)	1	20.34	6.00	8.19	3.13
T × W	1	2.02	0.60	0.16	0.06
U × W	1	4.70	1.39	0.32	0.12
Split-subplot error	4	4.12	6.12***	2.62	4.07
Variety (V)	1	6.87	2.85	0.98	0.58
T × V	1	0.00	0.00	3.06	1.80
U × V	1	13.77	5.71*	4.51	2.65
W × V	1	14.16	5.87*	4.59	2.70
T × U × W	1	1.69	0.5	0.23	0.09
T × U × V	1	0.00	0.00	3.13	1.84
T × W × V	1	0.00	0.00	3.04	1.78
U × W × V	1	36.15	14.99**	19.71	11.58**
T × U × W × V	1	0.00	0.00	3.10	1.82
Split-split-subplot error	8	3.00	4.46****	1.70	2.65

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 2.10 Proline and wax content from (**A-B**) 237J Sundance and (**C-D**) Ho Lan Dow under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/ 8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 14 days, after one week of initial growth under 22/18ºC. Other details are the same as in Fig. 2.4.

FIG. 2.10


A
B
C
D
Proline (µmol g-1 FW)
Wax (mg g-1 FW)
11
9
7
5
3
1
9
6
3
b b b b b b b a
a a a a a a a a
c bc c b c a bc bc
a a a a a a a a
L0W L0S L5W L5S H0W H0S H5W H5S
L0W L0S L5W L5S H0W H0S H5W H5S

2.5 DISCUSSION

It has been confirmed through many studies that CH_4 could be produced from plants under aerobic conditions (Keppler *et al.,* 2006; Martel and Qaderi, 2017), but the environmental factors that direct these emissions are still to be explored, and leads us to study this phenomenon further. On the basis of one-way ANOVA, our results from screening ten pea varieties for CH_4 emissions revealed that higher temperatures and water stress increased aerobic CH_4 emissions from plants (Fig. 2.2A, C). This is supported by other studies reporting that plants under stressed conditions would increase CH_4 emissions (Abdulmajeed *et al*., 2017), as compared to plants grown under normal conditions. However, CH_4 emissions rates varied significantly among the ten pea varieties that were grown under a combination of the three factors. Methane emissions were highest from 237J Sundance and lowest from 422 Ho Lan Dow (Fig. 2.2D). We found that a CH4 emission is 1.6 times higher in 237J Sundance than 422 Ho Lan Dow (Table 2.2). Thus, these two varieties were selected for further experiments. This study indicated that higher temperature and water stress decreased stem height, whereas water stress alone decreased leaf area as well. This finding corroborates earlier observation that stem height decreased in plants grown under a combination of higher temperature and water stress (Fig. 2.6). It has been observed previously that higher temperatures (Qaderi *et al*., 2010) and water stress (Shao *et al*., 2008) reduce plant biomass, as these two factors inhibit stem elongation (Cope *et al*., 2014). In the two chosen varieties, 422 Ho Lan Dow was taller (Fig. 2.3), had a thicker stem and it produced larger leaves, which led to greater biomass compared to 237J Sundance (Fig. 2.2). However, the latter variety had greater root dry mass, suggesting that this variety is more sensitive to stress conditions, that influence the plants to increase its root length thus increasing root-water-uptake (Li *et al*., 2006).

Our experiments also showed that there were no differences in the growth indices parameters under a combination of the three factors. However, the three-way interaction, U × W × V, affected LMA and LAR, but overall 422 Ho Lan Dow had higher LMR, LAR, and SRR, which are all associated with dry mass accumulation reported in this study (Table 2.2). Reduction in biomass under stress conditions, such as higher temperatures and water stress, may be attributable to decrease A_N (Qaderi *et al*., 2010).

Plants under these two stress factors might have partial stomatal closure (Kargar *et al*., 2017), which would affect the photosynthetic activity. In addition, higher temperatures inhibit photosynthesis by deactivating the activity of Rubisco (Way and Oren, 2010). Here, higher temperatures were recorded to decrease WUE (Table 2.2), an observation that is in agreement with previous studies (Martel and Qaderi, 2016). On the other hand, neither UVB radiation nor water stress affected WUE, which is not similar to previous findings (Chen and Zhang, 2007; Qaderi and Reid, 2009). This suggests that UVB radiation and water stress influenced stomatal closure of plants and caused reduction in evapotranspiration, therefore improving WUE. As documented in earlier studies, WUE and soil water content had a significant relationship (Chen and Zhang, 2007), which is supported by our findings too; it assumed that more water content in soil led to higher WUE as compared to plants growing in water-stressed areas. A combined application of the three factors, however, showed no difference in photosynthetic parameters, A_N, E, and WUE, for both varieties under each experimental condition (Fig. 2.7). Overall, 237J Sundance had higher WUE than 422 Ho Lan Dow (Table 2.2). These results suggest that, in combination, the three factors had less effect than each factor alone, with a small variation in plant response to each factor.

We also found that higher temperatures and water stress decreased ϕPSII (Table 2.2), suggesting that drought inhibits the PSII electron transport (Nogués and Baker, 2000), and therefore causes damage to D1 protein in the PSII reaction center (Berry and Björkman, 1980), which lead to decreased photosynthetic activity (Jansen *et al*., 1998). Therefore, water stress also decreases F_v/F_m (Table 2.2). It has been previously suggested that decrease in F_v/F_m is caused mainly by increased dissipation of excitation energy at PSII, which is controlled by the carotenoid zeaxanthin (Demming *et al*., 1988). Non-photochemical quenching and qP are defense mechanism to avoid photodamage to the photosynthetic apparatus (Li *et al*., 2008). Our experiment shows that higher temperatures decreased qNP and increased qP. Any decrease in qNP reduces the chance of plants to dissipate the extra light as heat (Ballottari *et al*., 2016). On the other hand, increased qP indicates that under higher temperatures plants promote the transfer of energy between two molecules to a substance that later will return to its ground state by losing the excess energy via dissipation (Buschmann, 1999). In the two studied varieties,

422 Ho Lan Dow had higher ϕPSII, which explains why this variety was more tolerant under stress conditions; due to high efficiency of PSII (Maxwell and Johnson, 2000). By contrast, 237J Sundance had a higher F_v/F_m, which is an indicator of the maximum quantum yield of PSII chemistry (Ballottari *et al.,* 2016). Therefore, a combination of the three main factors had no effect on ϕPSII, F_v/F_m, and qNP (Fig. 2.8). In contrast, differences were found in qP with significantly higher qP in 237J Sundance plants under higher temperature and slight increase in 422 Ho Lan Dow under the same conditions.

Photosynthesis depends on the ability of plants to capture light and convert it into biomass (Confalone *et al*., 2010). Chlorophyll allows plants to obtain energy from light in order to do photosynthesis (Jiang *et al*., 2007). Our results revealed that higher temperatures and water stress decreased chlorophyll content (Table 2.2), which was one of the reasons for the decrease in A_N; this result is in agreement with the finding reported by Anjum *et al.* (2011). Under a combination of the three factors, the effect of water stress was prominent and influential (Fig. 2.9B, F) on both varieties under higher temperature.

Reactive oxygen species (ROS) are enhanced by heat (Munné-Bosch and Penuelas, 2003), UVB (Hideg *et al.,* 2013) and water stress (Yokawa *et al.,* 2015). These compounds cause DNA and cellular damage, and oxidative stress in the cell (Schieber and Chandel, 2014). This in turn negatively affects the metabolism of plants and as part of the adaptation process, plants started to produce secondary ROS scavenging compounds, such as flavonoids, in order to mediate the effect of ROS (Fini *et al*., 2011). Flavonoids also play a role as UV-B screening pigments (Fini *et al*., 2011). Our results show that higher temperatures, UVB5 and water stress increased the concentration of flavonoids (Table 2.2), which is in agreement with the study of Tevini *et al.* (1983) in cucumber (*Cucunis sativus*) and radish (*Raphanus sativus*). However, the effect of only water stress under higher temperatures was noticeable in 237J Sundance, while no difference was observed in the level of flavonoids in 422 Ho Lan Dow except under H5S (Fig. 2.9C, G). UV-absorbing compounds are also known to play a role in protective mechanisms. On the basis of one-way ANOVA, our results revealed that higher temperatures decreased UV-absorbing compounds, which is in agreement with the results obtained when 237J Sundance plants were exposed to multiple factors, but not in the case

of 422 Ho Lan Dow (Table 2.2). This result differs from previous reports, which showed that UVB increased the concentration of UV-absorbing compounds (Häder *et al.,* 2015). We also measured nitrogen balance index (NBI) in plants, we found that higher temperature, UVB5, and water stress decreased NBI. This is an indicator that nitrogen nutrition has a big role in plant growth and development (Bojović and Marković, 2009). In addition, nitrogen is involved in synthesizing chlorophyll (Tremblay *et al.,* 2012). A combination of the three factors showed the same pattern for both varieties: NBI decreased in plants exposed to higher temperature under UVB5 and water stress.

Proline, another indicator for plant under stress, has been also measured in this study. This amino acid is involved in osmotic adjustment to maintain the water potential in plant cells during stress period (Cvikrová *et al*., 2013). Our findings indicate that higher temperatures, UVB5 and water stress increase proline contents (Table 2.2), which is reported in many previous studies. Harsh *et al*. (2016) stated that exposed moth bean (*Vigna aconitifolia*) under high temperatures of up to 42ºC for short term, as well as under drought conditions, resulted in increase in proline contents (Cvikrová *et al*., 2013). Also it has been reported that UV radiation induced proline accumulation in plants that protects them against UV radiation by inducing peroxidative processes (Saradhi *et al*., 1995). Under multiple stress factors, it was observed that water stress increased proline content regardless of temperatures and UVB radiation.

Positive correlation between proline and CH_4 emissions may suggest that plants under stress conditions severe cell damage, which in turn caused releasing of CH_4 emissions and as a part of protective system plant able to accumulate proline in order to alleviate the stress level on plants. Furthermore, content was higher in 422 Ho Lan Dow plants than in 237J Sundance plants (Table 2.2); this may be the result of increased plant dry mass in 422 Ho Lan Dow, which increased tolerance to stress conditions, in turn, 422 Ho Lan Dow emitted less CH_4. Wax prevents harmful UVB from entering the leaf tissue as well as water loss through transpiration (Pilon *et al.,* 1999). Earlier studies have documented such variations in wax content due to stress conditions, including high temperature and water stress, in pea and other plant species (Shepherd and Griffiths, 2006). That is why 422 Ho Lan Dow had lower CH_4 than 237J Sundance.

2.6 CONCLUSION

In our study, besides the main factors, the two-way, three-way, and four-way interactions of temperature, UVB, watering regime, and variety of plants revealed that plant responses may vary with the combination of environmental factors. We found that higher temperature, UVB5, and water stress significantly increased CH_4 emissions, which varied with pea variety, and supported our original hypothesis. In addition, the growth and physiological performance of pea varieties were also studied and found to be affected by these factors. It is important to determine plant response to multiple co-occurring environmental factors because such conditions are more realistic simulation for plant life (Qaderi *et al.*, 2012). In addition, plants were observed to be influenced by the interactive effect of those the three factors. Plants that experienced water shortage responded by having shorter stems, lower leaf dry mass, and smaller leaves than those that received water to field capacity (Table 2.2). In addition, the water-stressed plants had lower A_N, ϕPSII, F_v/F_m, and NBI than the well-watered plants. In contrast, water shortage increased CH_4 emissions as well as flavonoid and proline content. From the two varieties, 422 Ho Lan Dow produced lowest CH_4 emissions, meaning they were less stressed and more resistant. Also, 422 Ho Lan Dow had higher stem height, diameter, leaf area, leaf number, total dry mass, growth index, E, and ϕPSII. Increasing emissions of CH_4 and other greenhouse gases leads to global warming that plays a major role in any human being activity (Houghton *et al*., 2001). However, more studies on plant species are required with application of a number of environmental factors in order to extrapolate such finding. When applying a combination of factors, patterns of the effect of each factor need to be investigated more, this is considered the most challenging aspect of this type of experiments.

2.7 LITERATURE CITED

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CHAPTER 3

Interactive Effects of Temperature and UVB radiation on Methane Emissions from Different Organs of Pea Plants Grown in a Hydroponic System

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3.1 ABSTRACT

There is no information on variation of methane (CH_4) emissions from plant organs exposed to multiple environmental factors. We investigated the interactive effects of temperature and ultraviolet-B (UVB) radiation on CH_4 emissions from different organs of pea (*Pisum sativum* L. UT234 Lincoln). Plants were grown hydroponically under two temperatures (22/18°C and 28/24°C; 16 h day/8 h dark) and two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] in controlled-environment growth chambers for ten days, after two weeks of initial growth under ambient temperatures. Methane emissions, dry mass, growth index, electrical conductivity (EC), pectin, total chlorophyll content, gas exchange and flavonoids were measured in the appropriate plant organs – leaf, stem and root. Higher temperatures increased CH_4 emissions, leaf mass ratio, and shoot: root mass ratio. Neither temperature nor UVB had significant effects on leaf, stem, root and total dry mass, EC, pectin, total chlorophyll, as well as leaf mass per area. Among plant organs, there were differences in CH_4, EC, pectin and total chlorophyll. Methane and EC were highest for the stem and lowest for the leaf; leaf had highest, but stem had lowest, pectin content; total chlorophyll was highest in the leaf but lowest in the root. Higher temperatures decreased leaf flavonoids, net carbon dioxide assimilation, and water use efficiency. Overall, environmental stressors increased aerobic CH_4 emissions rates, which varied with plant organs.

3.2 INTRODUCTION

Atmospheric carbon dioxide (CO_2) is an important factor in global climate change. On the basis of climate models, the current concentration of atmospheric CO_2 (404.02 µmol mol^{-1}), measured at Mauna Loa Observatory (Tans and Keeling, 2014), is predicted to reach 700 µmol mol^{-1} by the end of this century (Long *et al*., 2004). Atmospheric CO_2 can increase the air temperature by 1.8-6.4°C by 2100 (Sánchez *et al*., 2014), and that may lead to drought conditions (Naumburg *et al*., 2004). Also, ozone-depleting substances, such as chlorofluorocarbons (CFCs) and nitrous oxide (N_2O) (Ravishankara *et al*., 2009), can increase the amount of solar ultraviolet-B radiation (280–315 nm) reaching the Earth's surface (Llabrés *et al.,* 2013*)*. Thus, elevated atmospheric CO_2, increased air temperature, alteration in precipitation patterns, and enhanced UVB radiation are the main components of global climate change (Long *et al*., 2004). The last three factors may lead to increased aerobic methane (CH_4) emissions (Keppler *et al*., 2006; Nisbet *et al*., 2009; Qaderi and Reid, 2009; 2011; Wang *et al*., 2009). Methane is a long-lived greenhouse gas and it is second to CO_2 in importance regarding the greenhouse effect (Kasimir-Klemedtsson *et al*., 1997), although its trapping heat potential is 28 times more than CO_2 (Myhre *et al*., 2013).

Many studies have reported an increase of global CH_4 concentrations at roughly three-fold more than the pre-industrial times (Butenhoff and Khalil, 2007). This increase in CH_4 in the Earth's atmosphere may be attributed to the fact that CH_4 emissions arise from multiple sources. For instance, Blaha *et al*. (1999) reported that CH_4 has both natural and anthropogenic sources. In 2006, for the first time, Keppler *et al*. (2006) reported that living plants, including tree and grass leaves from C_3 and C_4 plants, can also emit CH_4 under aerobic conditions. Later, Brüggemann *et al*. (2009) found that, in Grey poplar (*Populus* × *canescens*, syn. *Populus tremula* × *Populus alba*) plants, CH_4 emissions may occur from plants with nonmicrobial origin. Keppler *et al*. (2006), using isotope labelling, suggested that methoxyl groups of plant pectins could be the precursor of aerobic CH_4. It has been suggested that, under UV radiation, reactive oxygen species (ROS) may affect pectic polysaccharides to release CH_4 (McLeod *et al*., 2008). In addition to pectin, other chemicals, such as cellulose, lignin *(*Vigano *et al*., 2009) and wax (Bruhn *et al*., 2014) have been suggested as sources of aerobic CH_4 emissions in plants. Recently, Lenhart *et*

al. (2015), using lavender (*Lavandula angustifolia*), suggested that CH_4 emissions may arise from methionine, especially under stress conditions.

Environmental stressors, such as higher temperature (Qaderi and Reid, 2009) and UVB radiation (Vigano *et al*., 2009), or physical injury (Wang *et al*., 2009), have been shown to increase aerobic CH_4 emissions from plants. Nisbet *et al*. (2009), using several plant species including *Arabidopsis thaliana*, argued that under normal conditions plants emit dissolved CH_4 from the soil to the atmosphere through transpiration stream, whereas under certain stress conditions (e.g., enhanced UV or high temperature) CH_4 may be generated by the breakdown of plant material. On the basis of their findings, these authors concluded that there is no known biochemical pathway through which plants can effectively synthesize CH_4. However, studies have been mounting in support of the finding that plants produce and release CH_4 under aerobic conditions (Bruhn *et al*., 2014, Lenhart *et al*., 2015). Despite the argument that CH_4 emissions from living plants has minimal or no impact on global CH_4 (Dueck *et al*., 2007; Beerling *et al*., 2008; Kirschbaum and Walcroft, 2008; Bowling *et al*., 2009; Nisbet *et al*., 2009; Smeets *et al*., 2009) many studies have suggested that the new source has an important implications in global CH_4 budget (Crutzen *et al*., 2006; Sanhueza and Donoso, 2006; Bruhn *et al*., 2009; Wang *et al*., 2009).

Environmental stress factors can affect aerobic CH_4 emissions by altering phenological, morphological and physiological characteristics of plants. High temperatures disrupt structural and functional properties of chloroplasts, reduce chlorophyll a and chlorophyll b, and alter chlorophyll/ carotenoids ratio (Cui *et al*., 2006), which lead to reduced photosynthesis (Björn *et al*., 1999) and, in turn, to decreased biomass (Lafta and Lorenzen, 1995). On the other hand, enhanced UVB radiation harms living organisms by damaging their proteins, DNA, lipids and membranes (Hollósy, 2002). Enhanced UVB also affects the production of the protective compounds, such as flavonoids and epicuticular wax (Treutter, 2005), and decreases photosynthesis and developmental rate (Kootstra, 1994). As shown, enhanced UVB reduced crop growth and biomass (Kootstra, 1994) by decreasing plant height and altering leaf anatomy, leaf thickness and branch length (Kakani *et al*., 2003). High temperature (Sharkey, 2005), enhanced UVB radiation (A-H-Mackerness *et al*., 2001), or

water stress (Jiang and Zhang, 2002) increases the production of reactive oxygen species (ROS) in plants. Sharma *et al*. (2012) have shown that increased ROS caused oxidative damage to the membrane and led to increased membrane fluidity and permeability, which can be indicated by increased electrical conductivity (Pavlin *et al*., 2005).

Earlier studies have considered the effects of one (e.g., UV (Moschini *et al*., 2005) and physical injury (Wang *et al*., 2009)) or two (e.g., UV and temperature (Vigano *et al*., 2009)) factors on CH_4 emissions from plants; however, few studies have used multiple factors (Qaderi and Reid, 2009; 2011). Different stress factors, such as increased air temperature and enhanced UVB radiation reaching the Earth's surface, might affect global CH_4 emissions (Qaderi and Reid, 2009). It has been shown that higher temperatures can significantly increase CH_4 emissions from plants compared to lower temperatures (Qaderi and Reid, 2009). We were interested in investigating the effects of temperature and UVB radiation on CH_4 emissions from different organs of pea plants, using hydroponic system to eliminate the possibility of anaerobic CH_4 production. We hypothesized that higher temperature and UVB radiation would increase CH_4 emissions rates from plants and that the emissions will vary with plant organs. The objectives of this study were (1) to investigate the interactive effects of temperature and UVB radiation on CH_4 emissions from vegetative organs of plants, and (2) to elucidate nonmicrobial origin of CH_4 emissions from plants grown hydroponically.

3.3 MATERIAL AND METHODS

3.3.1 Plant material and growth conditions

For this study, we selected pea plants because in a previous study (Qaderi and Reid, 2009), which was conducted to examine the effects of temperature, UVB radiation and watering regime on aerobic CH_4, pea had the highest emissions among the six crop species used. Also, the use of pea may help us understand the contribution of agricultural crops in global CH_4 budget. Pea has been widely cultivated in Canada and around the world, where it is used not only as a crop to feed humans, but is considered an excellent source of protein and energy (due to high starch content) for raising poultry (Moschini *et al*., 2005). In Canada, the area seeded with pea will increase to 1.7 Mha in 2016–2017, up 16% from 2015 to 2016, due to higher returns relative to other crops (Morgan, 2016).

Therefore, pea can be an excellent model plant to be used for such studies. Seeds of pea (*Pisum sativum* cv. UT234 Lincoln, Stokes Seeds Ltd., Thorold, ON, Canada) were germinated in each of three Petri dishes in controlled-environment growth chambers (model ATC26, Conviron, Controlled Environments Ltd, Winnipeg, MB, Canada) and kept there until the development of true leaves. Then, seven-day-old seedlings of similar size In order to make the containers appropriate for hydroponic system, 12 holes of equal distance apart, were made on the lid of each container. Air pumps were connected to these containers to make sure that the roots of seedlings receive sufficient oxygen (see Appendix II). Then, containers were placed in controlled-environment growth chambers for one week with the following conditions: temperature (24/20°C, 16 h day/8 h dark), photoperiod (16 h, photosynthetic photon flux density of 300 μmol $m^{-2}s^{-1}$), and relative humidity of 40–46% (see Appendix III). At least 12 two-week-old seedlings were placed under each of two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark). Each chamber was supplied with two levels of biologically effective UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days. The UVB_{BE} radiation of 5 kJ m^{-2} d^{-1} is within the range of natural solar UVB radiation measured in the summer in Halifax, Nova Scotia (A. Abdulmajeed, pers. obs.). UVB radiation was supplied by four fluorescent lamps (UVB 313EL, Q-Panel, Cleveland, OH, USA), which were placed on top of a wooden frame that was transversely partitioned into two compartments with barriers of white cardboard. The lamps were pre-burned for 96 h to stabilize the UVB output, and each lamp was wrapped in two layers of 0.127 mm cellulose diacetate film (Grafix Plastics, Cleveland, OH, USA) to filter radiation below 280 nm. Daily UVB radiation was for 8 h around noon (Qaderi *et al*., 2006). Biologically effective UVB radiation (UVB_{BE}) was measured with a PMA2100 photometer/radiometer, which was calibrated against a National Institute of Standards and Technology traceable standard (Solar Light Co., Philadelphia, PA, USA). UVB_{BE} levels were estimated using Caldwell's generalized plant damage action spectrum normalized to 300 nm (Caldwell, 1971). The nutrient solution was replenished every week. Plants were kept under the experimental conditions for ten days (Fig. 3.1). At this time, the plants were 24 days old with sufficient exposure to experimental conditions at the vegetative stage, and were suitable to be used for measurements. One group of plants was used to examine CH_4 emissions, pectin content, electrical conductivity (EC) and total

chlorophyll (from leaf, stem and root). Another group of plants was used to determine flavonoids and gas exchange in the leaves. The experiments were conducted six times for methane emissions and three times for other experiments under the same environmental conditions.

3.3.2 Determination of aerobic methane emissions

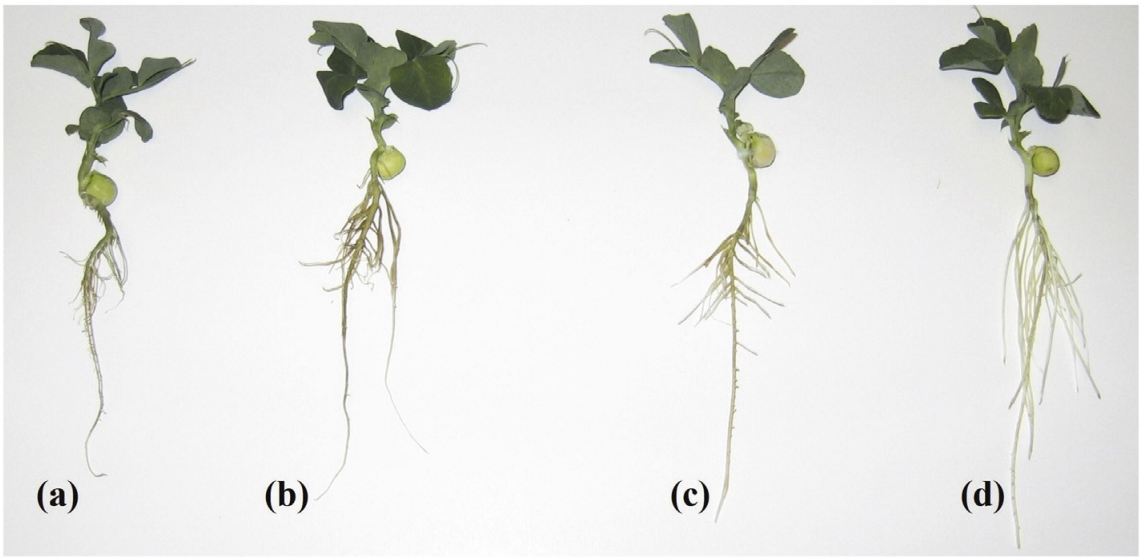
Methane emissions rates were determined by using a modification of the method that was used for measuring ethylene evolution from plants (Qaderi and Reid, 2011; Emery *et al*., 1994). From each condition, six samples detached and surface dried by paper towel to ensure no water is introduced to syringes. Then, the samples were incubated in a growth chamber under 22°C at 300 μmol photons m^{-2} s^{-1} for 2 h in 3-mL plastic syringes, flushed with CH_4-free air. A previous study showed that 2 h incubation period at 22°C is suitable for the measurement of CH_4 emissions (Qaderi and Reid, 2009). From each syringe, 1 mL of gas was collected and injected manually into a gas chromatograph-flame ionization detector system (GC-FID; Varian 3900 Gas Chromatograph; Varian Canada, Mississauga, ON) equipped with a capillary column (Carboxen 1006 PLOT, 30 m × 0.53 mm ID, Supelco, Bellefonte, PA, USA). The injector and detector temperatures were set at 200 and 230°C, respectively. Helium was used as a carrier gas at 10 mL min^{-1}. Methane was eluted with the following programmed temperature gradient: 1 min isothermal heating at 35°C followed by a 24°C min^{-1} oven ramp to 225°C until the end of the 9 min run. Methane was identified by the retention time of the analyte (~2.6min), using external standard (Air Liquide, Dartmouth, Nova Scotia, Canada), and quantified on the basis of standard curve (Qaderi and Reid, 2011) derived from the injection of three replications of 5, 10, 25 and 30 μL of standard CH_4 gas. Linear regression analysis was applied on data to generate an eq. (Y = a + bX) in which the Y was replaced by the CH_4 value (mL h^{-1}), which was then converted to ng h^{-1}. The rates of CH_4 emissions (ng g^{-1} DM (dry mass) h^{-1}) were calculated on the basis of plant tissue dry mass by drying the samples at 60°C for 96 h.

3.3.3 Determination of growth and dry mass

From each treatment, three samples of leaf, stem, and root were taken and their fresh weights were determined, using an electrobalance (Model H51, Sartorius GmbH, Goettingen, Germany). Then, the samples were dried at 60°C for 72 h in an Isotemp oven (Model 255G, Fisher Scientific, Nepean, ON, Canada) and reweighed to obtain their dry mass. Growth indices were calculated as following (Qaderi *et al*., 2006): leaf mass per area [LMA (gm^{-2}) =leaf dry mass:leaf area], leaf mass ratio [LMR=leaf drymass:plant dry mass], leaf area ratio [LAR ($cm^2\ g^{-1}$) = leaf area:plant dry mass], and shoot:root mass ratio [SRR = shoot dry mass:root dry mass].

FIG. 3.1 Picture of 24-day-old pea (*Pisum sativum*) plants that were grown under two temperature regimes (22/18°C, lower, and 28/24°C, higher; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature. (**a**) lower temperature at UVB0, (**b**) lower temperature at UVB5, (**c**) higher temperature at UVB0, and (**d**) higher temperature at UVB5.

FIG. 3.1



3.3.3 Determination of membrane permeability

Electrolyte leakage was used to measure membrane permeability of fresh leaf, stem and root (Anjum *et al*., 2012). Three plant samples (0.2 g) were rinsed and immersed in 20 mL deionized water, and agitated on a shaker (ThermoFisher Scientific Inc., Marietta,OH, USA) for 24 h. First, the electrical conductivity of the samples was measured with an HI 98311 DiST® 5 EC/TDS/Temperature Tester (Hanna Instruments Inc., Woonsocket, RI, USA). Then, plant samples were autoclaved at 120°C for 20 min, and the maximum conductivity was measured for dead tissues. Electrolyte leakage was calculated on the basis of initial conductivity (of fresh tissue) and maximum conductivity (of incubated tissue) and reported as percentage.

3.3.4 Measurement of pectin

Pectin was extracted from three samples of leaf, stem, and root tissues. For pectin extraction the method of Kim *et al*. was followed with minor modifications, as explained by Iglesias and Lozano (2004). Plant samples were ground with mortar and pestle. Pectin was extracted by shaking ground samples in distilled water at 75°C for 15 min – a process proven to remove most pigmentation. Mixture was then filtered and the remaining solid was treated with 0.75% sodium hexametaphosphate at 75°C for 1 h. The ratio of solid to liquid was 20:1 and with 6 N HCl the solution was brought to pH 3.5. The pectin solution was then filtered using a Büchner funnel and Whatman No. 4 filter paper, and subsequently cooled at 20°C. Filtrate was precipitated with 500 mL HCl (1 N) and stirred for 15 min. To ensure quality, the solution was cooled and stored at 3 °C for 22 h. The solution was then filtered, re-suspended in 70% ethanol, re-filtered, washed in 70% ethanol, rinsed with 95% ethanol, dried at 50°C for 19 h, and passed through a 100-mesh sieve. From each condition, the powder of each organ was weighed to determine pectin content (mg g^{-1} DM).

3.3.5 Measurement of total chlorophyll

From each condition, three replications of fresh leaf discs (25 mg), and stem and root segments (200 mg) were placed in 12 mL vials, containing 5 mL of dimethyl sulphoxide, as described in Qaderi *et al*. (2006). Plant samples were incubated in the absence of light

for 24 h, at room temperature, allowing for a complete extraction of chlorophyll and carotenoids. Then, a 1 mL of extract was used to measure absorbance of samples at three wavelengths (648, 664, and 470 nm) against a control solution of dimethyl sulphoxide with a UV/Visible spectrophotometer (model Ultraspec 3100 pro, Biochrom Ltd., Cambridge, UK). On the basis of absorbance, total chlorophyll and carotenoids (μg mg^{-1} FM) were calculated (Chappelle *et al*., 1992).

3.3.6 Measurement of gas exchange

From each condition, three fully-expanded leaves were used to measure net CO_2 assimilation (A_N, μmol CO_2 m^{-2} s^{-1}), transpiration (E, mmol H_2Om^{-2} s^{-1}) and stomatal conductance (g_s, mol m^{-2} s^{-1}) with a LI-COR portable photosynthesis system (model 6400XT, LI-COR Inc., Lincoln, NE, USA). Before measurements, the photosynthesis system was calibrated with 400 μmol mol^{-1} of CO_2 with flow rate of 400 mL s^{-1}. The water use efficiency (WUE, μmol CO_2 $mmol^{-1}$ H2O) was calculated by dividing A_N by E (Lambers *et al*., 1998).

3.3.7 Measurement of flavonoids

From each condition, the concentration of flavonoids was determined in three leaf samples, using a Dualex Scientific® system (Dualex Scientific, Force-A, Orsay Cedex, France), which measures optical absorbance. The leaf clip accurately simulates the concentration of flavonoids (μg cm^{-2}) in the leaf epidermis (Martel and Qaderi, 2016).

3.3.8 Statistical dnalysis

Effects of temperature, UVB radiation and their interactions on CH_4 emissions, EC, pectin, and total chlorophyll in different plant organs (leaf, stem, and root) were determined by means of analysis of variance for split-split-plot design (SAS institute, 2011). In the split-split-plot analysis, temperature regimes were treated as the main plot, UVB radiation as the subplot, plant organs as the split subplot, and growth chambers as replications (Hinkelmann and Kempthorne, 1994). Differences among growth conditions within plant organs or differences among plant organs within growth conditions were determined by Scheffé's multiple-comparison procedure at the 5% confidence level.

Effects of temperature, UVB radiation and their interactions on dry mass, growth index, gas exchange and flavonoids of leaves were determined by means of analysis of variance for split-plot design (SAS institute, 2011). In the split-plot analysis, temperature regimes were treated as the main plot, UVB radiation as the subplot, and growth chambers as replications (Potvin, 2001). Differences among treatments were determined by Scheffé's multiple comparison procedure at the 5% confidence level (SAS institute, 2011). Also, the relationship between plant parameters was determined by Pearson's correlation coefficient (Minitab, 2014). All data are reported as mean ± standard error.

3.4 RESULTS

3.4.1 Methane emissions

We found that temperature, plant organ and the two-way interactions between temperature (T) × UVB radiation (U) and T × P (plant organ) had significant effects on aerobic CH_4 emissions. However, UVB radiation had no significant effects on CH_4 emissions from pea plants (Table 3.1). Higher temperatures significantly increased (44.21 ± 4.73 ng g^{-1} DM h^{-1}) CH_4 emissions compared to lower temperatures (35.18 ± 4.74 ng g^{-1} DM h^{-1}). Methane emissions was highest for the stem (65.08 ± 4.12 ng g^{-1} DM h^{-1}) and lowest for the leaf (18.08 ± 0.96 ng g^{-1} DM h^{-1}). Within plant organs, CH_4 emissions was relatively higher from plants grown under higher temperatures at UVB5 than plants from other conditions (Fig. 3.2). The two-way interaction between T × U indicates that plants under higher temperatures at UVB5 had highest CH_4 emissions, whereas plants under lower temperatures at UVB5 had the lowest emissions. Also, the two-way interaction between T × P indicates that stem under higher temperatures had highest CH_4 emissions, whereas leaf under lower temperatures had lowest emissions.

3.4.2 Plant growth and index

Overall, temperature and UVB radiation did not significantly affect dry mass accumulation, LMA and LAR (Table 3.2). Higher temperatures increased LMR and SRR (15.4 and 47.9 times, respectively) than lower temperatures (Fig. 3.3).

FIG. 3.2 Methane emissions rates from different organs of 24-day-old pea (*Pisum sativum*) plants. Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature. (**A**) leaf, (**B**) stem, and (**C**) root. Bars (mean ± SE) surmounted by different upper-case letters among plant organs or by different lower-case letters within plant organ are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure.

FIG. 3.2

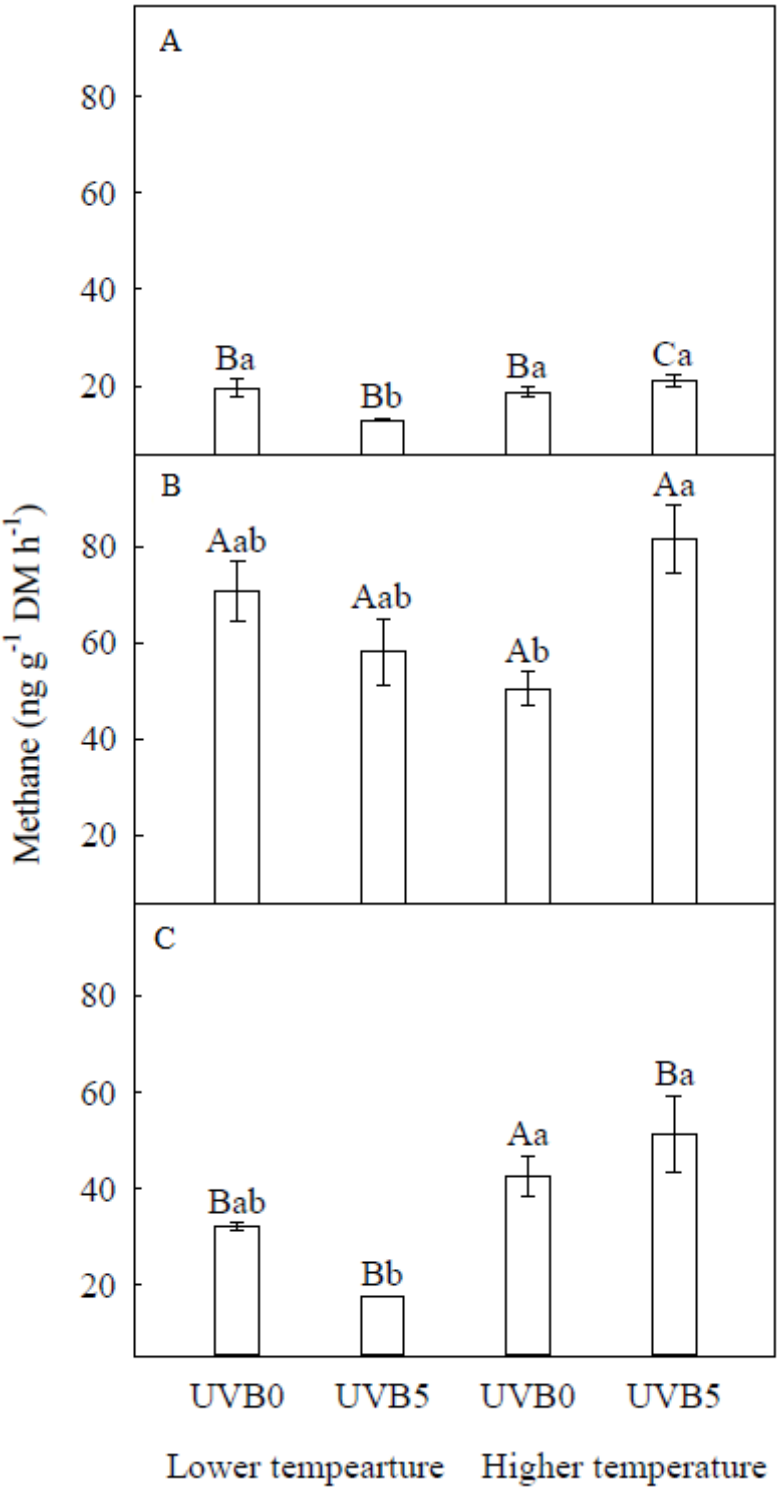

A
B
C
Methane (ng g-1 DM h-1)
80
60
40
20
Ba
Bb
Ba
Ca
Aab
Aab
Ab
Aa
Bab
Bb
Aa
Ba
UVB0
UVB5
UVB0
UVB5
Lower tempearture
Higher temperature

TABLE 3.1 Analysis of variance (*F* value) for effects of temperature, UVB radiation and their interactions on methane from different organs of pea (*Pisum sativum*) plants. Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature

Source	d.f.	Methane
Temperature (T)	1	48.38^{**}
Main plot error	3	...
UVB radiation (U)	1	0.40
T × U	1	34.21^{**}
Subplot error	6	...
Plant organ (P)	2	94.67^{****}
T × P	2	5.39^{*}
U × P	2	1.93
T × U × P	2	3.23
Split-subplot error	24	...

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

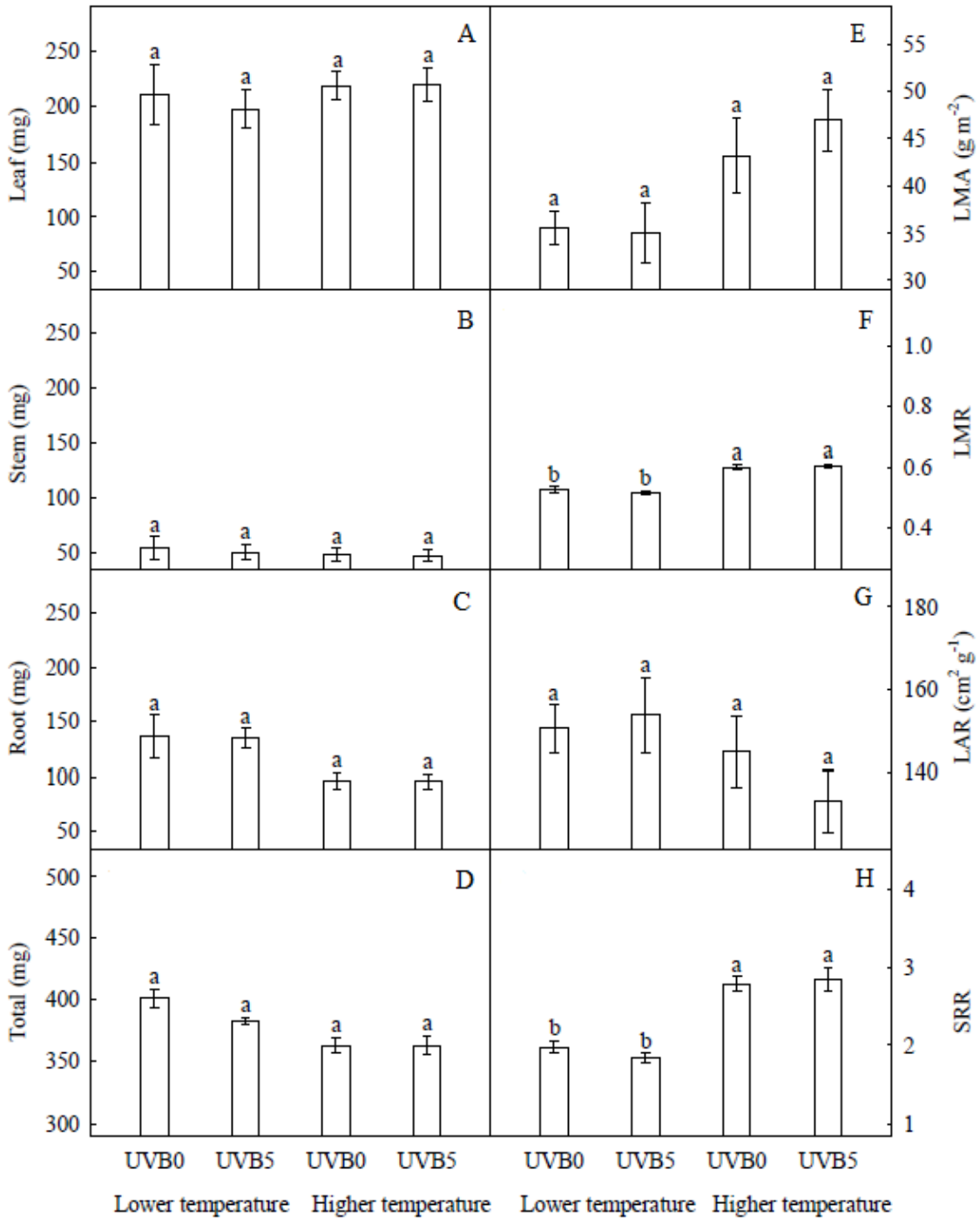
TABLE 3.2 Analysis of variance (*F* value) for effects of temperature, UVB radiation and their interactions on dry mass and growth index of pea (*Pisum sativum*) plants

Source	d.f.	Dry mass				Growth index			
		Leaf DM	Stem DM	Root DM	Total DM	LMA	LMR	LAR	SRR
Temperature (T)	1	0.50	0.24	7.06	0.42	2.17	94.31***	0.56	536.91****
Main plot error	4	…	…	…	…	...	...	...	...
UVB radiation (U)	1	0.13	0.26	0.01	0.10	3.76	0.23	2.78	0.14
T × U	1	0.18	0.13	0.00	0.10	6.96	1.15	8.95*	0.92
Subplot error	4	…	...	...	...	...	...	...	...

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 3.3 Dry mass accumulation and growth index for 24-day-old pea (*Pisum sativum*) plants. (**A**) leaf DM, (**B**) stem DM, (**C**) root DM, (**D**) total DM, (**E**) leaf mass per area (LMA), (**F**) leaf mass ratio (LMR), (**G**) leaf area ratio (LAR), and (**H**) shoot: root mass ratio (SRR). Other details are the same as in Fig. 3.2 Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJm^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature.

FIG. 3.3


A
B
C
D
E
F
G
H
Leaf (mg)
Stem (mg)
Root (mg)
Total (mg)
LMA (g m-2)
LMR
LAR (cm2 g-1)
SRR
UVB0
UVB5
UVB0
UVB5
UVB0
UVB5
UVB0
UVB5
Lower temperature
Higher temperature
Lower temperature
Higher temperature

3.4.3 Electrical conductivity

Plant organ and the two-way interaction between T × P had significant effects on EC (Table 3.3). Overall, EC was highest for the stem (64.33 ± 6.89%) and lowest for the leaf (8.82 ± 1.35%). Within plant organs, in leaf and stem, EC was relatively higher for plants grown under higher temperatures at UVB5 than for plants grown under other conditions; however, in the root, it was not different among treatments. Among plant organs, under higher temperatures, stem had highest EC but leaf had lowest, whereas under lower temperatures, both stem and root had higher EC than leaf, regardless of UVB level (Fig. 3.4). The two-way interaction between T × P showed that stem of plants grown under higher temperatures had highest EC, whereas leaf of plants grown under lower temperatures had lowest EC.

3.4.4 Pectin content

Plant organ, but not temperature or UVB, had significant effects on pectin content (Table 3.3). Among plant organs, leaf had the highest pectin content (195.48 ± 16.45 mg g^{-1} DM), while stem had the lowest content (37.51 ± 5.94 mg g^{-1} DM). However, there were no significant differences in pectin content within each plant organ (Fig. 3. 4).

3.4.5 Total chlorophyll

Plant organ, but not temperature or UVB, had significant effects on the total chlorophyll concentration (Table 3.3). There were no significant differences in total chlorophyll within organs. Among plant organs, total chlorophyll was significantly higher in leaves than in stems and roots, except in stems of plants grown higher temperatures at UVB5 (Fig. 3.4). Overall, total chlorophyll concentration was highest for the leaf (1.58 ± 0.15 μg mg^{-1} FM), but lowest for the root (0.02 ± 0.00 μgmg^{-1} FM).

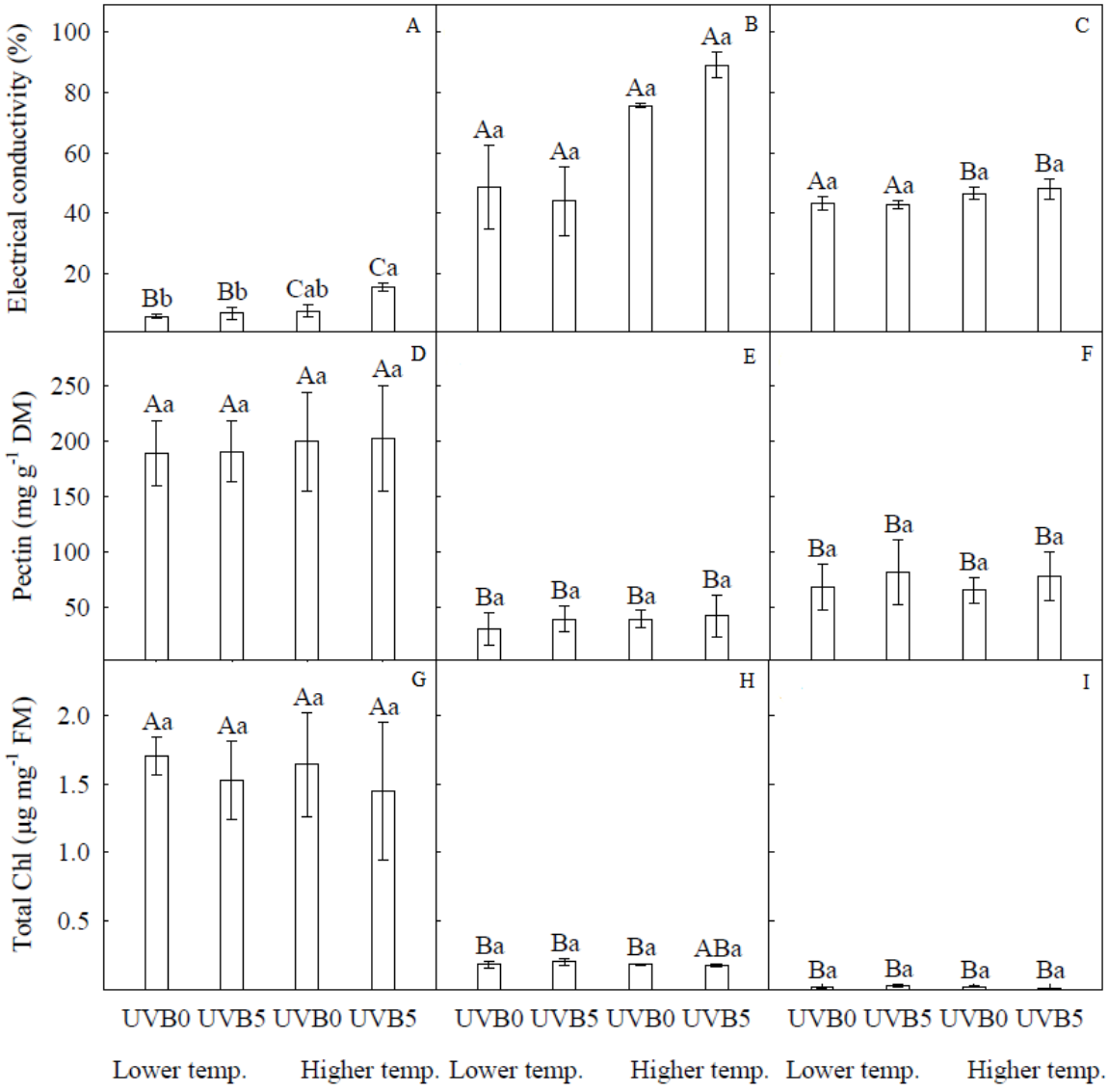
TABLE 3.3 Analysis of variance (*F* value) for effects of temperature, UVB radiation and their interactions on electrical conductivity, pectin and total chlorophyll of pea (*Pisum sativum*) plants. Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature

Source	d.f.	Electrical conductivity	Pectin	Total chlorophyll
Temperature (T)	1	11.66	0.06	0.09
Main plot error	2	...	...	...
UVB radiation (U)	1	3.89	1.36	1.07
T × U	1	7.66	0.03	0.03
Subplot error	4	...	...	...
Plant organ (P)	2	90.70^{****}	59.82^{****}	70.09^{****}
T × P	2	9.31^{**}	0.12	0.03
U × P	2	0.15	0.07	0.28
T × U × P	2	0.49	0.01	0.00
Split-subplot error	16	...	...	...

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 3.4 Electrical conductivity, pectin and total chlorophyll concentration from different organs of 24-day-old pea (*Pisum sativum*) plants. Electrical conductivity: (**A**) leaf, (**B**) stem, and (**C**) root; Pectin: (**D**) leaf, (**E**) stem, and (**F**) root; Total chlorophyll concentration: (**G**) leaf, (**H**) stem, and (**I**) root. Other details as in Fig. 3.2.

FIG. 3.4


A
B
C
D
E
F
G
H
I
Electrical conductivity (%)
Pectin (mg g-1 DM)
Total Chl (µg mg-1 FM)
100
80
60
40
20
250
200
150
100
50
2.0
1.5
1.0
0.5
Bb Bb Cab Ca
Aa Aa Aa Aa
Aa Aa Ba Ba
Aa Aa Aa Aa
Ba Ba Ba Ba
Ba Ba Ba Ba
Aa Aa Aa Aa
Ba Ba Ba ABa
Ba Ba Ba Ba
UVB0 UVB5 UVB0 UVB5
UVB0 UVB5 UVB0 UVB5
UVB0 UVB5 UVB0 UVB5
Lower temp.
Higher temp.
Lower temp.
Higher temp.
Lower temp.
Higher temp.

3.4.6 Gas exchange

Temperature, but not UVB, had significant effects on A_N, g_s and WUE (Table 3.4); however, the effect of temperature on g_s was not significant on the basis of one-way ANOVA. Higher temperatures significantly decreased both A_N and WUE (9.18 ± 0.34 μmol CO_2 m^{-2} s^{-1} and 1.4 ± 0.12 μmol CO_2 $mmol^{-1}$ H_2O, respectively) compared to lower temperatures (5.90 ± 0.22 μmol CO_2 m^{-2} s^{-1} and 0.81 ± 0.07 μmol CO_2 $mmol^{-1}$ H_2O, respectively). The two-way interaction between T × U was significant for *E* and WUE (Table 3.4). Plants grown under higher temperatures had lower A_N than those grown under lower temperatures, irrespective of UVB level. Plants grown under lower temperatures at UVB5 had lower E than plants grown under lower temperatures at UVB0 or those grown under higher temperatures at UVB5. Plants grown under lower temperatures at UVB5 had lower g_s than plants grown under higher temperatures at the same UVB level (Fig. 3.5). Overall, plants grown under lower temperatures at UVB5 had higher WUE than plants grown under other experimental conditions. The two-way interaction between T × U shows that, at UVB5, plants grown under lower temperatures had highest WUE, whereas plants grown under higher temperatures had lowest WUE (Fig. 3.5).

3.4.7 Flavonoids concentration

Temperature and the two-way interaction between T × U had significant effects on flavonoids (Table 3.4). Higher temperatures significantly decreased flavonoids (0. 50 ± 0.02 μg cm^{-2}) than lower temperatures (0. 67 ± 0.02 μg cm^{-2}). Plants grown under lower temperatures at UVB0 had higher flavonoids than plants grown under higher temperatures at both UVB levels (Fig. 3.6).

3.4.8 Relationship between Plant Parameters

In this study, Pearson's correlation analysis revealed 58 significant relationships between different measured parameters. Among them, methane emissions from leaf, stem and root were correlated with four, five and six parameters, respectively. Here, we report only the relevant relationship between CH_4 and other plant parameters. Methane emissions from leaf had positive correlation with CH_4 from stem (r = 0.715, P = 0.009) and WUE (r=

0.627, P = 0.029). Methane emissions from stem was also positively correlated with WUE (r = 0.644, P = 0.024). Pectin from stem had positive correlation with pectin from leaf (r= 0.589, P = 0.044) and root (r= 0.779, P = 0.003).

TABLE 3.4 Analysis of variance (*F* value) for effects of temperature, UVB radiation and their interactions on gas exchange and flavonoids from leaves of pea (*Pisum sativum*) plants. Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h day/8 h dark) at two levels of UVB radiation [0 and 5 kJ m^{-2} d^{-1}] for 10 days, after two weeks of initial growth under lower temperature

		Gas exchange					
Source	d.f.	A_N	E	g_s	WUE	d.f.	Flavonoids
Temperature (T)	1	83.78****	0.35	9.94*	29.36***	1	24.02****
Main plot error	8	...	...	...	...	24	...
UVB radiation (U)	1	2.41	1.02	0.57	2.24	1	1.66
T × U	1	1.23	10.81*	1.90	11.27*	1	8.35**
Subplot error	8	...	...	...	...	24	...

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

FIG. 3.5 Gas exchange in leaves of 24-day-old pea (*Pisum sativum*) plants. (**A**) net CO_2 assimilation (A_N), (**B**) transpiration (E), (**C**) stomatal conductance (g_s), and (**D**) water use efficiency (WUE). Other details are the same as in Fig. 3.2.

FIG. 3.5

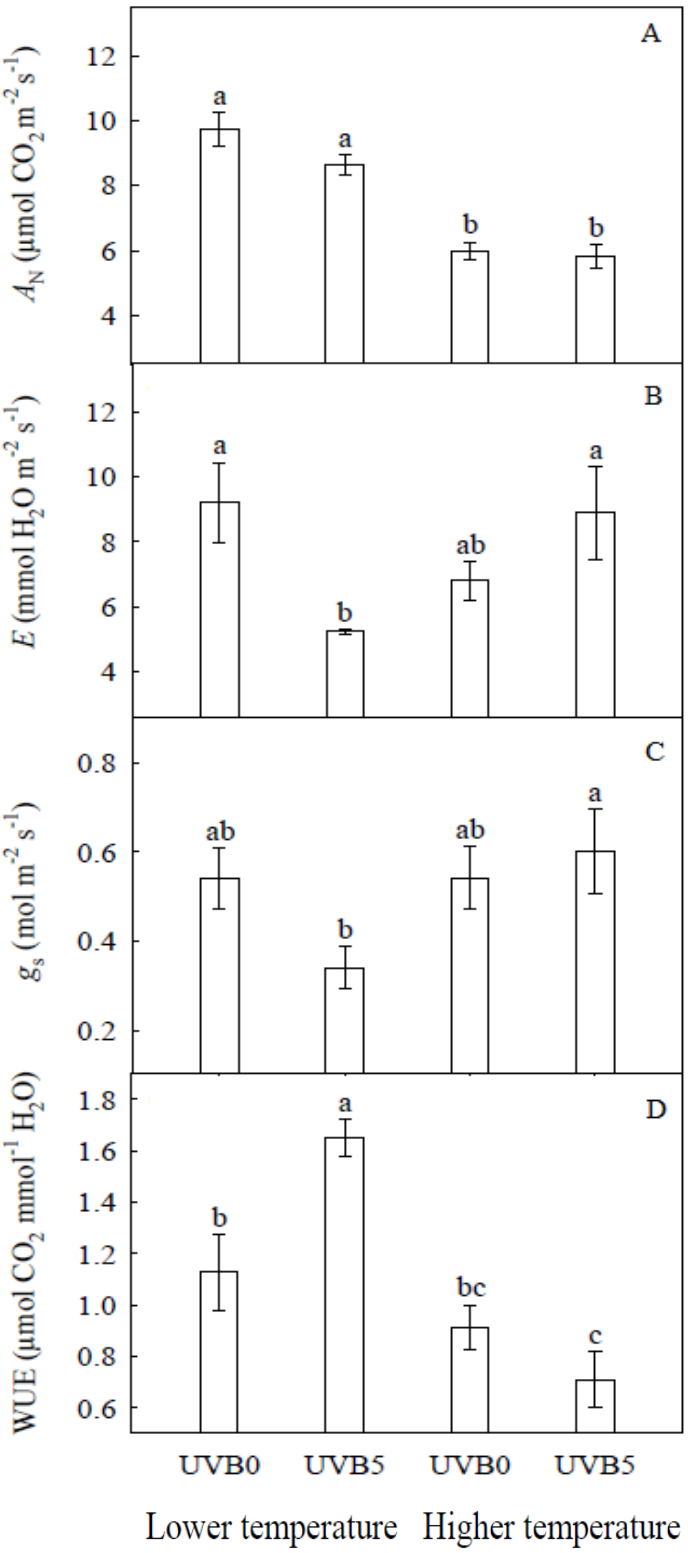
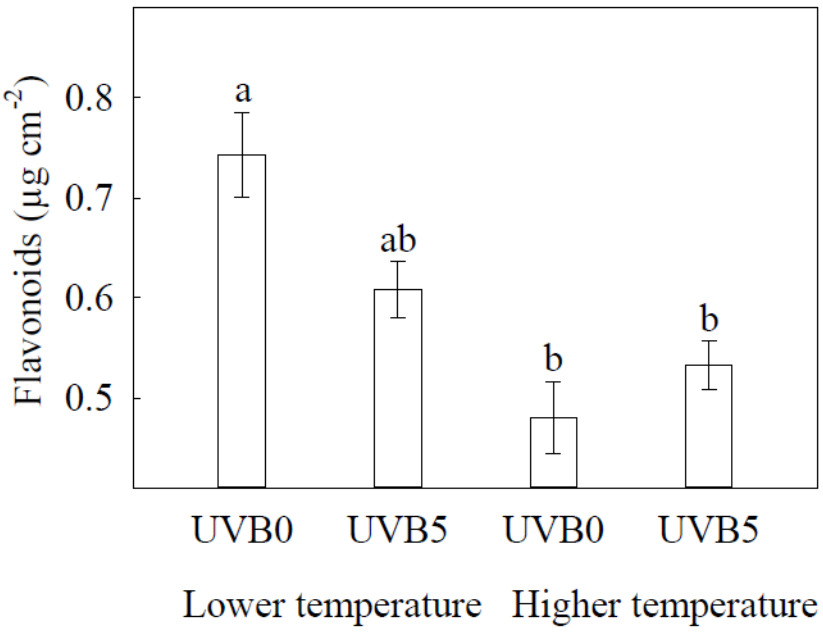

A
B
C
D
A_N (µmol CO_2 m^{-2} s^{-1})
E (mmol H_2O m^{-2} s^{-1})
g_s (mol m^{-2} s^{-1})
WUE (µmol CO_2 $mmol^{-1}$ H_2O)
a
a
b
b
a
b
ab
a
ab
b
ab
a
b
a
bc
c
UVB0
UVB5
UVB0
UVB5
Lower temperature
Higher temperature

FIG. 3.6 Flavonoids in leaves of 24-day-old pea (*Pisum sativum*) plants. Other details are the same as in Fig. 3.2.

FIG. 3.6


Flavonoids (µg cm-2)
0.8
0.7
0.6
0.5
a
ab
b
b
UVB0
UVB5
UVB0
UVB5
Lower temperature
Higher temperature

3.5 DISCUSSION

For the first time, Keppler *et al*. (2006) demonstrated that plants can emit CH_4 under aerobic conditions. Later, Nisbet *et al*. (2009) suggested that stress conditions, such as UV radiation, can stimulate plants to emit more CH_4 due to breakdown of plant material. Rice *et al*. (2010) reported that some species of broadleaf riparian trees, grown under flooded conditions, can transport CH_4 from aerobic surfaces into the atmosphere. Also, Gauci *et al*. (2010) have reported CH_4 emissions from stems of mature wetland alder (*Alnus glutinosa*) trees. We found that plants grown hydroponically under either stressed or stress-free conditions can emit CH_4 of nonmicrobial origin. Since our plants were kept in a well-oxygenated environment, the detected CH_4 should have plant origin.

Higher temperatures significantly increased CH_4 emissions. This environmental factor might have increased emissions by increasing stress on plants through causing damage to DNA (Stapleton and Walbot, 1994) or through the production of reactive oxygen species, which could have stimulated CH_4 emissions from plants by affecting pectic substances (McLeod *et al*., 2008).

Methane emissions rates varied significantly among plant organs (Fig. 3.2), with stems having the highest emissions rates and leaves the lowest. The result suggests that pea stems might have been more sensitive to the imposed stress factors than its leaves (Leymarie *et al*., 1999; Zhuang *et al*., 2011). In our study, all components of plant dry mass, LMA and LAR were not significantly affected by temperature, UVB or their interactions (Table 3.2; Fig. 3.3). This suggests that plants in hydroponic system received enough water, which might have alleviated the negative effects of higher temperatures and UVB5 on their growth and development.

Electric conductivity was not significantly affected by temperature or UVB. This finding is different from that of Zhang *et al*. (2005), in which high temperature increased membrane permeability, indicating increased conductivity. Different results could be related to plant species and temperature. Zhang *et al*. exposed grape plants (*Vitis vinifera* L. cv. Jingxiu) to 45°C, whereas we exposed pea plants to 28°C. High temperature has been shown to increase the concentration of lipid peroxidation – a process that plays a role in the damage of cell membrane (Butow *et al*., 1998; Yajima *et al*., 2009) and affects

conductivity. In our study, there are similar patterns for EC and CH_4 emissions, as both parameters are highest in the stem and lowest in the leaf tissue (Figs. 2 and 4).

Pectin content was significantly higher in the leaf and lower in the stem (Fig. 3.4); this result is the opposite of those found for the CH_4 and EC (Figs. 2 and 5). It is likely that increased CH_4 emissions from the stem has led to reduced pectin deposition in this plant organ, indicating this polysaccharide as possible precursor. Nevertheless, the results suggest that pectin (Keppler *et al*., 2006) is one of the potential sources of CH_4 in plants in addition to lignin, cellulose (Vigano *et al*., 2009), wax (Bruhn *et al*., 2014) and methionine (Lenhart *et al*., 2015). Our finding is similar to that of Ghasemi *et al*. (2013) who showed that, in rice varieties, leaves had higher concentration of cell wall carbohydrates than stem. It is because pectin consists of a complex set of polysaccharides, such as cellulose and hemicellulose (Verhertbruggen *et al*., 2009).

Neither temperature nor UVB radiation significantly affected total chlorophyll concentration, but plant organ did. Total chlorophyll was highest in the leaf and lowest in the root (Fig. 3.4). The result suggests that root may capture some light that enter through the small holes located at the lid of the containers, and may stimulate chloroplast formation in the root of pea plants (Richter, 1969). Rich *et al*. (2012) reported that roots of some aquatic plants, such as *Cotula coronopifolia* and *Meionectes brownie*, growing in lower light levels would contain less chlorophyll than leaves, stems or roots of plants growing in higher light.

We also found that higher temperatures decreased both A_N and WUE (Fig. 3.5); this could have been because of higher transpiration under this condition (Lambers *et al*., 1998). This stress factor caused plants to have reduced A_N and WUE and, in turn, increased CH_4. This is in agreement with previous findings on pea and other crop species (Qaderi and Reid, 2009; 2011).

In the current study, higher temperatures decreased flavonoids concentration compared to lower temperatures (Fig. 3.6). This suggests that the stress caused by higher temperatures might have damaged the D1 and D2 proteins of photosystem II (Yamashita *et al*., 2008) and decreased plant protective capability through reduced chlorophyll (Yang *et al*., 2005), phenolics and flavonoids (Bettaieb *et al*., 2011). It has been shown that flavonoids have the ability to protect an organism against free radicals and oxygenated

reactive species that cause damage to the cell (Sharma *et al*., 2012) and lead to stress tolerance (Zhishen *et al*., 1999). Thus, plants under higher temperatures with reduced flavonoids might have been stressed and emitted more CH_4 than plants under lower temperatures with increased flavonoids (Fig. 3.6).

3.6 CONCLUSION

This study revealed that higher temperatures stimulate plant CH_4 emissions, which vary with plant organs, whereas UVB radiation does not affect the emissions. Also, the use of hydroponic system once again confirmed the nonmicrobial origin of CH_4 emissions from plants. In this study, the use of vegetative plant organs provided novel information regarding aerobic methane emissions; however, a detailed study of the reproductive plant organs should also be considered. Also, testing plants under two environmental factors is useful; however, in the future multiple factors should be used to provide plants with more realistic growth conditions. This practice will lead us to a more comprehensive understanding of the effects of climate change components on CH_4 emissions from plants.

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CHAPTER 4

Intrashoot Variation in Aerobic Methane Emissions from Pea Plants Exposed to Multiple Abiotic Stresses

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4.1 ABSTRACT

Methane (CH_4) emissions from plants have been shown to increase with stress factors. However, the effects of multiple environmental stressors on CH_4 emissions from various shoot parts have not been studied. Peas (*Pisum sativum* L. cv. 237J Sundance) were used to determine CH_4 emissions from the upper, middle and lower parts of shoot. Plants were grown in controlled-environment chambers under temperature regime of 22/18ºC or 28/24ºC (16 h light/8 h dark), ultraviolet-B (UVB) level of 0 kJ m^{-2} d^{-1} or 5 kJ m^{-2} d^{-1}, and watering to field capacity (well watered) or at wilting point (water stressed). Methane emissions, photosynthetic parameters (A_N, net CO_2 assimilation; E, transpiration; g_s, stomatal conductance; WUE, water use efficiency), chlorophyll fluorescence (ϕPSII, effective quantum yield of PSII; F_v/F_m, maximum quantum yield of PSII; qNP, non-photochemical quenching; qP, photochemical quenching), total chlorophyll and flavonoids were measured in shoots of one-month-old plants. Higher temperatures and UVB increased CH_4 emissions, which were higher from stem than leaf, and from upper shoot than lower shoot. Lower leaves emitted more CH_4 than upper leaves. Methane emissions were increased by higher temperatures with water stress from both shoot and stem, by UVB5 with water stress from stem, and by higher temperatures with UVB0 from leaf. Water stress decreased all photosynthetic parameters. Higher temperatures and UVB5 decreased WUE, whereas UVB5 increased E and g_s. UVB5 and water stress decreased ϕPSII, but water stress increased qNP. A_N, E, g_s, ϕPSII and chlorophyll were highest in the upper leaves. All the main factors decreased chlorophyll. UVB5 decreased flavonoids, which were lowest in the lower leaves. Methane emissions from the stem had a positive correlation with E and g_s, but a negative correlation with WUE. Overall, stress factors increased CH_4 emissions, which varied with shoot parts.

4.2 INTRODUCTION

Greenhouse gases in the atmosphere can have natural and/or anthropogenic origins (Myhre *et al*., 2013). Carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are among the important greenhouse gases that are influenced by human activities (Houghton, 2015). It is predicted that the current concentration of atmospheric CO_2 (404.21 μmol mol^{-1}; Tans, 2017) may surpass 700 μmol mol^{-1} by 2100 (Myhre *et al*., 2013). Elevated CO_2 will probably increase air temperature by 1.8-4.0ºC (possibly up to 6.4ºC) by 2100, if industrial emissions continue at current rates (Sánchez *et al*., 2014). Global warming may cause drought in many areas (Vicente-Serrano *et al*., 2014). Also, chlorofluorocarbons (CFCs) and nitrous oxide (N_2O) have depleted stratospheric ozone (Ravishankara *et al*., 2009), increasing the level of solar ultraviolet-B (UVB) radiation on the Earth's surface (Costa *et al*., 2012). All these factors may affect atmospheric CH_4 budget (Bruhn *et al*., 2012).

Methane, with both natural and anthropogenic sources (Blaha *et al*., 1999), is one of the primary greenhouse gases causing global warming (Popa *et al*., 2014). Its global warming potential is about 28-34 times more than CO_2 (Myhre *et al*., 2013). The concentration of CH_4 gas in the Earth's atmosphere has doubled since the pre-industrial times (Butenhoff and Khalil, 2007). It has been known, for decades, that the synthesis of CH_4 is carried out by microorganisms in anaerobic environments (Shah *et al*., 2014). In 2006, Keppler and his colleagues showed that CH_4 can also be produced and released by plants under aerobic conditions. Many studies have focused on this new source of CH_4 (Lowe, 2006). In some studies, CH_4 emissions was measured from attached leaves (Zhang *et al*., 2014), whereas in some others from detached leaves (Qaderi and Reid, 2011; Bruhn *et al*., 2012; Fraser *et al*., 2015). However, the source of CH_4 remains unclear. Initially, the methoxyl groups of plant pectin were suggested as the source of aerobic CH_4 (Keppler *et al*., 2008), and this source has also been considered a potential precursor by other researchers (McLeod *et al*., 2008; Bruhn *et al*., 2009; Keppler *et al*., 2009; Messenger *et al*., 2009). Lignin, cellulose (Vigano *et al*., 2008), leaf surface wax (Bruhn *et al*., 2014), and methionine (Lenhart *et al*., 2015) have also been suggested as precursors of aerobic CH_4 in plants.

Abiotic stressors, such as higher temperature (Ghaffarian, 2008), UVB radiation (McLeod *et al*., 2008), and drought (Qaderi and Reid, 2009), may stimulate the production of CH_4 in plants. Environmental factors can affect plants by altering their phenological, morphological and physiological characteristics. Higher temperature reduces chlorophyll *a*, chlorophyll *b* and chlorophyll/carotenoid ratio (Cui *et al*., 2006) and, in turn, plant photosynthetic activity, which can be accompanied by an increase in transpiration (*E*) and stomatal conductance (g_s; Jones, 2014), leading to decreased plant biomass.

Enhanced UVB radiation harms living organisms by damaging their DNA, protein, lipids and membranes (Hollósy, 2002). It decreases plant height and changes leaf anatomy, leaf thickness and branch length (Kakani *et al*., 2003). Enhanced UVB decreases plant growth, biomass, and slows developmental rates (Ballaré *et al*., 2011). It also affects the production of protective compounds, such as flavonoids and epicuticular wax (Treutter, 2005). Plants cope with stressful conditions through multiple detoxification and repair mechanisms. They adapt to stress by increasing the chlorophyll concentration in leaves, and producing lignin (Shulaev *et al*., 2008) and waxy layer on the surface, which provide protection from UVB radiation (Bruhn *et al*., 2014). Besides the waxy layer, plants produce phenolic compounds, such as flavonoids and anthocyanins, which are secondary metabolites that protect plants from UVB damage (Liang *et al*., 2006).

Water stress decreases plant height, stem thickness, total dry mass, relative expansion rate and elongation of leaves, chlorophyll fluorescence, and total chlorophyll (Kirnak *et al*., 2001). Water stress affects photosynthetic parameters, protein production, accumulation of metabolites (Ohashi *et al*., 2006), and enhances ROS (reactive oxygen species) production that influences plant growth (Reddy *et al*., 2004*b*).

Many studies have measured CH_4 emissions from plants grown under single (Bowling *et al*., 2009; Fraser *et al*., 2015) or double stress factors (Vigano *et al*., 2008). Few studies have considered multiple stress factors (Qaderi and Reid, 2009; 2011). Also, none of the earlier studies has focused on measuring CH_4 emissions from different parts of plant shoot. In this study, we aimed to determine CH_4 emissions from various parts of plant shoot exposed to multiple environmental factors, i.e., temperature, UVB radiation, and

watering regime. Our hypothesis was that UVB radiation, higher temperature and water stress would influence the upper parts of the shoot to emit more CH_4 than the other parts, as new developed plant parts can be more susceptible to stress conditions.

4.3 MATERIALS AND METHODS

4.3.1 Plant material and growth conditions

In this study, we selected pea plants because they were one of the highest CH_4 emitters among six crop plants used in previous studies (Qaderi and Reid, 2009; 2011). Also, pea has been widely cultivated in Canada and around the world for both feeding humans and raising poultry (Moschini *et al*., 2005). Initially, we examined 10 pea cultivars and among them a cultivar with highest CH_4 emissions rate was selected and used as a model plant for the current study. Seeds of pea (*Pisum sativum* L. cv. 237J Sundance; Stokes Seeds Ltd, Thorold, ON, Canada) were planted (22/18°C, 16 h light/8 h dark; photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}), and relative humidity (RH ~ 65%). The light source was a mix of Litemor incandescent lamps (Boston, MA, USA) and cool white Philips Master TL-D-58W/840 fluorescent tubes (Amsterdam, the Netherlands). PPFD was measured with a quantum LI-250A radiometer/photometer (LI-COR Biosciences, Lincoln, Nebraska, USA) at the shoot apex, and RH was measured with an Oakton WD-35612-00 thermohygrometer (Vernon Hills, IL, USA). Then, 72 plants were randomly assigned to eight treatments with the following combinations: two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two levels of biologically effective UVB (UVB_{BE}) radiation (0 and 5 kJ m^{-2} d^{-1}), and two watering regimes (watering to field capacity, well-watered; and watering at wilting point, water-stressed). Plants were kept under each treatment for three weeks. Midday leaf water potential (Ψ_{wmd}) for the well-watered and water-stressed plants were about –1.0 and –3.0 MPa, respectively. Leaf Ψ_{wmd} was measured with a WP4C Dew Point PotentiaMeter (Decagon Devices Inc., Pullman, WA, USA). In the water-stressed plants, a low moisture content was maintained in pots throughout the experimental duration. UVB radiation was supplied by four fluorescent lamps (UVB 313EL, Q-Panel, Cleveland, OH, USA), which were pre-burned for 96 h to stabilize the UVB output. The desired level of UVB radiation (5 kJ m^{-2} d^{-1}) was achieved by filtering radiation below 280 nm with wrapping the lamps

with two layers of 0.127 mm cellulose diacetate film (Grafix Plastics, Cleveland, OH, USA). UVB radiation was measured with a PMA2100 photometer/radiometer, which was calibrated against a National Institute of Standards and Technology traceable standard (Solar Light Co., Philadelphia, Pennsylvania, USA). UVB_{BE} levels were estimated by following the Caldwell's (1971) procedure. Daily UVB radiation was for 8 hours around noon (for details see Qaderi and Reid, 2005). Within each treatment, plants were rotated twice a week to minimize positional effects. Experiments were repeated twice under the same experimental conditions.

4.3.2 Measurement of methane emissions

From each condition, at least three samples of fresh leaf and stem (see Appendix IV) of one-month-old plants were detached and incubated inside 3-ml plastic syringes for 2 h under the temperature of 22ºC and PPFD of 300 µmol m^{-2} s^{-1}. As previously shown, the incubation of plant sample for 2 h at 22ºC is sufficient to detect CH_4 emissions (Qaderi and Reid, 2009). After collecting 1 ml gas from each syringe, it was injected manually into a Varian 3900 gas chromatograph equipped with a flame ionization detector (Varian Canada, Mississauga, ON, Canada) and a Carboxen 1006 PLOT capillary column (30 m length x 0.53 mm ID; Supelco, Bellefonte, PA, USA). The injector temperature was 200ºC and that of detector 230ºC. The carrier gas was helium at 10 ml min^{-1}. Methane elution was achieved by the following programmed temperature gradient: first 35ºC isothermal heating for 1 min, then increasing temperature to 225ºC using an oven ramp of 24ºC min^{-1}, and finally keeping at this temperature until the end of run (9 min). The retention time (~2.6 min) of external standard (Air Liquide, Dartmouth, NS, Canada) was used to identify CH_4, and the standard curve of CH_4 gas was used to quantify its emissions rate on dry mass basis (ng g^{-1} DM h^{-1}) by drying the leaf tissue at 60ºC for 96 h (Qaderi and Reid, 2011).

4.3.3 Measurement of photosynthetic parameters

For each treatment, three fully-expanded leaves from each of the upper, middle and lower parts of shoot were used to measure 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}) with a LI-COR 6400XT photosynthesis system (LI-COR Inc., Lincoln, NE, USA). Prior to

measurements, the photosynthesis system was calibrated with 400 µmol mol^{-1} of CO_2, which had a flow rate of 400 ml s^{-1}. Leaf chamber was the same as the growth chamber temperature, either lower or higher, in the light. Measurements were performed between 10:00 h and 14:00 h. The photosynthesis system calculated A_N, E, and g_s on the basis of leaf total surface area within the leaf chamber (Qaderi *et al*., 2006). The instantaneous water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O) was calculated by dividing A_N by E (Martin *et al*., 1988).

4.3.4 Measurement of chlorophyll fluorescence

For each treatment, at least three fully-expanded leaves from each of the upper, middle and lower parts of shoot were used to measure chlorophyll fluorescence with a FluorPen FP 100 portable fluorometer (Photon Systems Instruments, Dräsov, Czechia). First, the effective quantum yield of PSII (ϕPSII) was measured in the light. Then, the maximum quantum yield of PSII (F_v/F_m), non-photochemical quenching (qNP), and photochemical quenching (qP) were measured for the dark-adapted leaves (Schreiber, 2004), which had been kept within the fluorometer clamp for 30 min (Bolhar-Nordenkampf *et al*., 1989).

4.3.5 Measurement of chlorophyll and flavonoids

Total chlorophyll and flavonoids were determined for six leaves from each treatment with a Dualex Scientific $+^{TM}$ (Force-A, Orsay Cedex, France). The leaf clip performs non-destructive measurements of chlorophyll (µg cm^{-2}) and flavonoid (µg cm^{-2}) contents in the leaf epidermis, based on reading of different wavelengths (Martel and Qaderi, 2016).

4.3.6 Statistical analysis

Effects of temperature, UVB and watering regime on methane emissions and physiological parameters of different shoot parts of pea plants were determined by analysis of variance (ANOVA) for split-split-plot design (SAS Institute, 2011). In the split-split-plot analysis, temperature, UVB, watering regime and growth chamber were considered, respectively, as the main plot, subplot, split-subplot and replication (Hinkelmann and Kempthorne, 1994). Differences among treatments were determined by a one-way ANOVA, using Scheffé's multiple-comparison procedure at the 5% level

(SAS Institute, 2011). Relationship between plant parameters was determined by Pearson's correlation coefficient (Minitab, 2014). Data are reported as mean ± standard error.

4.4 RESULTS

4.4.1 Methane emissions

Higher temperature regime and UVB5 increased shoot (leaf and stem) and stem CH_4 emissions, which were significantly higher from the upper part of shoot or stem than the middle and lower parts. However, CH_4 emissions were highest from the lower leaves but lowest from the middle leaves (Table 4.1). Methane emissions were significantly affected by temperature regime, UVB level (for shoot), and the two-way interactions of T × W (shoot, leaf and stem) and U × W (shoot); Table 4.2). In the shoot and stem, the T × W interaction revealed that the water-stressed plants had highest CH_4 emissions under higher temperature regime, but lowest emissions under lower temperature regime. In the shoot, the U × W interaction indicated that CH_4 emissions were highest from the well-watered plants grown at UVB5, but lowest from plants experienced the same watering regime and grown at UVB0. In the leaf, the T × W interaction showed that higher temperature regime at UVB0 led to highest CH_4 emissions, whereas lower temperature regime at UVB0 led to lowest emissions.

In the leaf, differences in CH_4 emissions were significant for the UVB levels (all leaf positions), and the two-way (T × U, upper leaves; T × W, upper and middle leaves; U × W, upper leaves) and three-way (T × U × W, middle leaves) interactions (Table 4.5). UVB5 increased CH_4 emissions from the upper and middle leaves, but decreased it from the lower leaves ($P < 0.05$). In the upper leaves, the two-way interactions indicated that the water-stressed plants grown under higher temperature regime or those grown under lower temperature regime at UVB5 had highest CH_4 emissions, whereas the water-stressed plants grown under lower temperature regime or those grown under lower temperature regime at UVB0 had lowest emissions. In the middle leaves, the two-way and three-way interactions revealed that CH_4 emissions from the water-stressed plants grown under higher temperature regime at UVB5 were highest, but from the water-

stressed plants grown under lower temperature regime at UVB5 were lowest. In the lower leaves, no interactions were significant (Fig. 4.1A).

In the stem, differences in CH_4 emissions were significant for the temperature regimes (middle and lower parts), UVB levels (upper and middle parts), and the two-way interactions of T × U (middle and lower parts), T × W (upper and middle parts), and U × W (upper and middle parts; Table 4.5). Higher temperature regime increased CH_4 emissions from the middle and lower parts of stem, whereas UVB5 increased CH_4 emissions from the upper and middle parts of stem ($P < 0.05$). In the upper part of stem, on the basis of two-way interactions, the water-stressed plants under higher temperature regime or at UVB0 had highest CH_4 emissions, but the water-stressed plants under lower temperature regime or the well-watered plants at UVB0 had lowest emissions. In the middle part of stem, the two-way interactions revealed that the water-stressed plants had highest CH_4 emissions under higher temperature regime at UVB5, but lowest emissions under lower temperature regime at UVB0. In the lower part of stem, on the basis of T × U interaction, CH_4 emissions were highest from plants that were grown under higher temperature regime at UVB0, but lowest from plants that were grown under lower temperature regime at UVB0 (Fig. 4.1B).

Lower leaves of the well-watered plants that were grown under either lower or higher temperature regime at UVB0, and lower leaves of the water-stressed plants that were grown under lower temperature regime at UVB0 emitted more CH_4 than leaves from the other shoot parts. However, upper leaves of the well-watered plants that were grown under either lower or higher temperature regime at UVB5 had highest CH_4 emissions (Fig. 4.1A). In the stem, CH_4 emissions were highest from the upper part; the only exception was the lower stem part of the well-watered plants, which had highest emissions under higher temperature regime at UVB0 (Fig. 4.1B).

Table 4.1 Effects of temperature, UVB radiation, and watering regime on methane emissions, photosynthetic parameters, chlorophyll fluorescence, total chlorophyll and flavonoids in pea (*Pisum sativum*) plants

Parameter	Temperature		UVB radiation		Watering regime		Shoot part		
	Lower	Higher	UVB0	UVB5	Well-watered	Water-stressed	Upper	Middle	Lower
CH_4-shoot	32.20 ± 2.90B	54.56 ± 5.03A	37.04 ± 4.37B	49.72 ± 5.86A	41.89 ± 3.92A	44.87 ± 4.67A	60.04 ± 7.51A	34.40 ± 3.28B	35.63 ± 2.89B
CH_4-leaf	21.00 ± 1.75A	26.86 ± 2.73A	23.07 ± 3.85A	24.54 ± 4.13A	23.67 ± 1.97A	24.20 ± 2.67A	23.32 ± 2.39AB	18.94 ± 1.95B	29.53 ± 3.62A
CH_4-stem	43.40 ± 4.88B	82.26 ± 7.17A	51.01 ± 12.44B	74.65 ± 9.20A	60.12 ± 6.29A	65.54 ± 7.53A	96.76 ± 10.37A	49.99 ± 4.35B	41.74 ± 4.23B
A_N	11.32 ± 0.90A	8.94 ± 0.90A	11.07 ± 1.00A	9.19 ± 0.96A	12.34 ± 1.08A	7.92 ± 0.67B	14.77 ± 1.47A	9.14 ±0.63B	6.48 ± 0.53B
E	3.94 ± 0.38A	4.73 ± 0.62A	3.48 ± 0.33B	5.19 ± 0.62A	5.20 ± 0.58A	3.47 ± 0.31B	6.14 ± 0.83A	3.86 ± 0.39B	3.00 ± 0.39B
g_s	0.14 ± 0.01A	0.15 ± 0.02A	0.12 ± 0.01B	0.18 ± 0.02A	0.18 ± 0.02A	0.11 ± 0.02B	0.22 ± 0.03A	0.13 ± 0.02B	0.10 ± 0.02B
WUE	3.25 ± 0.45A	2.07 ± 0.30B	3.43 ± 0.43A	1.90 ± 0.35B	2.84 ± 0.45A	2.49 ± 0.35B	2.69 ± 0.22A	2.80 ± 0.30A	2.50 ± 0.19A
ϕPSII	0.64 ± 0.03A	0.62 ± 0.02A	0.68 ± 0.01A	0.60 ± 0.03B	0.68 ± 0.02A	0.59 ± 0.02B	0.68 ± 0.02A	0.65 ± 0.02AB	0.59 ± 0.03B
F_v/F_m	0.68 ± 0.02A	0.70 ± 0.02A	0.70 ± 0.01A	0.69 ± 0.02A	0.70 ± 0.01A	0.69 ± 0.02A	0.68 ± 0.03A	0.71 ± 0.01A	0.69 ± 0.02A
qNP	1.65 ± 0.05A	1.59 ± 0.06A	1.59 ± 0.06A	1.62 ± 0.06A	1.50 ± 0.05B	1.70 ± 0.07A	1.76 ± 0.09A	1.49 ± 0.05A	1.55 ± 0.09A
qP	0.66 ± 0.13A	0.80 ± 0.13A	0.77 ± 0.14A	0.69 ± 0.12A	0.71 ± 0.12A	0.75 ± 0.14A	0.79 ± 0.14A	0.74 ± 0.13A	0.65 ± 0.13A
Total Chl.	22.55 ± 1.85A	15.51 ± 0.68B	21.00 ± 1.95A	17.07 ± 0.77B	23.13 ± 1.71A	14.94 ± 0.85B	22.10 ± 1.30A	19.66 ± 1.68AB	15.34 ± 2.24B
Flavonoids	0.94 ± 0.06A	0.95 ± 0.04A	1.10 ± 0.03A	0.78 ± 0.05B	0.96 ± 0.05A	0.93 ± 0.04A	0.93 ± 0.05A	1.02 ± 0.04A	0.88 ± 0.07A

Table 4.2 Summary of split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on CH_4 emissions from pea (*Pisum sativum*) plants

Treatment	Plant part								
	Shoot (leaf and stem)			Leaf			Stem		
	d.f.	MS	*F*	d.f.	MS	*F*	d.f.	MS	*F*
Temperature (T)	1	17999.14	125.97**	1	616.83	5.72	1	27190.69	18.22
Main plot error	2	-	-	2	-	-	2	-	-
UVB radiation (U)	1	5788.79	43.91**	1	53.42	0.05	1	10058.18	5.38
T × U	1	549.05	4.16	1	684.52	0.65	1	48.64	0.03
Subplot error	4	-	-	4	-	-	4	-	-
Watering regime (W)	1	318.30	3.14	1	5.06	0.05	1	528.15	0.38
T × W	1	6836.70	67.43****	1	783.32	8.01*	1	7911.27	5.65*
U × W	1	1844.62	18.19**	1	112.71	1.15	1	2512.28	1.79
T × U × W	1	338.00	3.33	1	418.81	4.28	1	30.63	0.02
Split-subplot error	8	-	-	8	-	-	8	-	-

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

TABLE 4.5 Summary of split-split-plot analysis of variance (*F* value) for effects of temperature, UVB radiation, watering regime, and their interactions on methane emissions, photosynthetic parameters, chlorophyll fluorescence, total chlorophyll and flavonoids in pea (*Pisum sativum*) plant

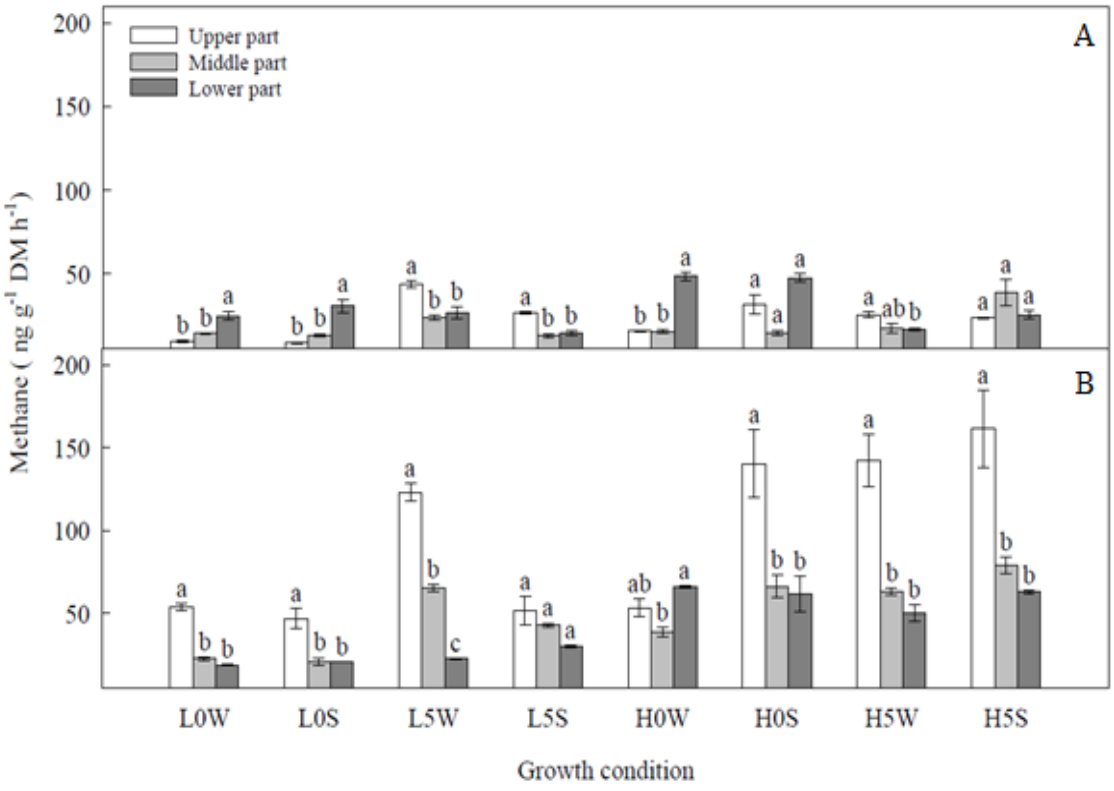
Treatment	d.f.	Methane source		Photosynthetic parameters				Chlorophyll fluorescence				Total Chl.	Flavonoids
		Leaf	Stem	A_N	E	g_s	WUE	ϕPSII	F_v/F_m	qNP	qP		
Upper part													
Temperature (T)	1	0.94	14.62	31.13*	96.68*	62.63*	422.29**	4.75	0.44	0.98	0.80	612.89**	5.05
Main plot error	2	-	-	-	-	-	-	-	-	-	-	-	-
UVB radiation (U)	1	74.28**	41.79**	304.76****	20.87*	24.56**	254.82****	0.80	0.17	0.32	3.80	29.57**	49.18**
T x U	1	66.95**	1.57	141.45***	7.96*	13.25*	0.72	0.66	0.00	0.10	2.99	137.07***	0.48
Subplot error	4	-	-	-	-	-	-	-	-	-	-	-	-
Watering regime (W)	1	0.29	0.92	165.12****	136.00****	86.83****	39.55***	5.85*	0.78	3.41	0.04	281.39****	31.11***
T × W	1	23.59**	40.50***	26.15***	57.83****	25.40**	44.85***	0.02	0.31	5.61*	1.27	54.57****	16.73**
U × W	1	26.12***	20.73**	3.46	37.90***	39.81***	35.12***	0.18	0.25	0.28	5.75*	27.92***	3.62
T × U × W	1	0.11	0.02	0.22	0.74	5.60*	83.90****	0.27	1.21	0.09	3.88	3.80	32.10***
Split-subplot error	8	-	-	-	-	-	-	-	-	-	-	-	-
Middle part													
Temperature (T)	1	8.10	622.12**	33.22*	2.12	4.71	99523.4****	0.30	4.74	1.82	0.32	77.07*	1.06
Main plot error	2	-	-	-	-	-	-	-	-	-	-	-	-
UVB radiation (U)	1	23.30**	446.55***	20.53*	17.98*	10.66*	716.17****	5.02	3.78	0.37	0.17	146.10***	14.84*
T × U	1	5.13	31.78**	39.39**	9.51*	13.27*	171.34***	0.17	1.96	0.76	5.03	436.86****	9.45*
Subplot error	4	-	-	-	-	-	-	-	-	-	-	-	-
Watering regime (W)	1	0.64	2.89	83.96****	2.21	20.99**	935.48****	12.75**	0.14	1.40	0.33	715.25****	1.28
T × W	1	9.69*	35.32***	55.24****	11.48**	9.53*	166.07****	3.67	1.43	1.81	0.00	388.05****	0.00
U × W	1	1.38	8.26*	57.36****	38.94***	37.71***	113.12****	5.52*	0.31	0.24	0.56	140.21****	0.17
T × U × W	1	9.72*	0.52	5.50*	51.54****	73.15****	1410.26****	0.43	0.91	0.16	2.32	336.51****	4.61
Split-subplot error	8	-	-	-	-	-	-	-	-	-	-	-	-

Treatment	d.f.	Methane source		Photosynthetic parameters				Chlorophyll fluorescence				Total Chl.	Flavonoids
		Leaf	Stem	A_N	E	g_s	WUE	ϕPSII	F_v/F_m	qNP	qP		
Lower part													
Temperature (T)	1	2.95	69.87*	26.09*	20.23*	169.12**	125931****	1.72	1.57	0.96	17.39	705.59**	2.26
Main plot error	2	-	-	-	-	-	-	-	-	-	-	-	-
UVB radiation (U)	1	9.90*	0.06	14.78*	0.23	1.05	628.58****	3.07	1.71	0.43	0.26	39.05**	176.61***
T × U	1	3.39	15.41*	27.59**	29.57**	22.88**	392.29****	0.28	1.19	0.89	0.05	109.37***	36.32**
Subplot error	4	-	-	-	-	-	-	-	-	-	-	-	-
Watering regime (W)	1	0.00	2.81	6.40*	0.57	1.83	17.99**	5.59*	0.01	20.04	0.09	329.92****	15.82**
T × W	1	0.30	0.00	41.53***	11.59**	14.88**	9.53*	0.02	1.20	0.92	0.73	110.01****	73.78****
U × W	1	0.10	4.51	39.89***	37.98***	30.32***	201.44****	1.94	0.96	0.03	2.59	177.51****	1.29
T × U × W	1	1.12	1.09	0.42	0.99	0.01	3.64	0.00	1.10	0.76	1.57	250.58****	29.16***
Split-subplot error	8	-	-	-	-	-	-	-	-	-	-	-	-

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

FIG. 4.1 Methane emissions from the (**A**) leaves and (**B**) stems of one-month-old pea (*Pisum sativum*) plants at three shoot parts (upper, middle, lower).

FIG. 4.1


Upper part
Middle part
Lower part
A
B
Methane (ng g-1 DM h-1)
200
150
100
50
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

4.4.2 Photosynthetic parameters

Water stress significantly decreased A_N, which was lower in the middle and lower leaves than in the upper leaves (Table 4.1). UVB5 increased, but water stress decreased, E and g_s, which were lower in the middle and lower leaves than in the upper leaves (Table 4.1). Higher temperature regime, UVB5 and water stress decreased WUE, which did not differ significantly among shoot parts (Table 4.1). All photosynthetic parameters were significantly affected by temperature, UVB, and watering regime (Table 4.3). The T × W interaction was significant for A_N, E and g_s; the U × W interaction for all photosynthetic parameters; and the T × U and three-way interactions for WUE (Table 4.3). The T × W interaction indicated that the well-watered plants under lower temperature regime had highest A_N and WUE, and the water-stressed plants under higher temperature regime had lowest A_N and WUE. On the other hand, E was highest in the well-watered plants under higher temperature regime and lowest in the water-stressed plants under lower temperature regime. The U × W interaction showed that A_N was highest in the well-watered plants at UVB0 and lowest in the water-stressed plants at UVB5; E and g_s were highest in the well-watered plants at UVB5 and lowest in the water-stressed plants at UVB0; and WUE was highest in the well-watered plants at UVB0, but lowest in plants experienced the same watering regime and grown at UVB5. On the basis of three-way interaction, the well-watered plants under lower temperature regime at UVB0 had highest WUE, whereas the water-stressed plants under higher temperature regime at UVB5 had lowest WUE (Fig. 4.2).

In the upper leaves, higher temperature regime increased E, but decreased WUE; UVB5 increased E and g_s, but decreased WUE; and water stress decreased all photosynthetic parameters ($P < 0.05$). In the middle leaves, higher temperature regime decreased A_N and WUE; UVB5 increased E and g_s, but decreased WUE; and water stress decreased WUE ($P < 0.05$). In the lower leaves, higher temperature regime, UVB5 and water stress decreased WUE, but had no significant effects on A_N, E and g_s ($P < 0.05$).

In all leaf positions, A_N was significantly affected by temperature, UVB, watering regime, T × U and T × W; in the middle and lower leaves by U × W; and in the middle leaves also by the three-way interaction (Table 4.5). On the basis of these interactions, in the upper leaves, A_N was highest in the well-watered plants under lower temperature

regime at UVB0 and lowest in the water-stressed plants under higher temperature regime at UVB5; in the middle leaves, A_N was highest in the well-watered plants under higher temperature regime at UVB0 and lowest in the water-stressed plants under higher temperature regime at UVB5; and in the lower leaves, A_N was highest in the well-watered plants under lower temperature regime at UVB5, but lowest in the water-stressed plants under higher temperature regime at the same UVB level (Fig. 4.2A).

In the upper leaves, *E* was significantly affected by temperature, UVB, watering regime, and all two-way interactions. In the middle leaves, *E* was significantly affected by UVB, and all two-way and three-way interactions. In the lower leaves, *E* was significantly affected by temperature and all two-way interactions (Table 4.5). On the basis of these interactions, in the upper leaves, *E* was highest in the well-watered plants grown at UVB5, but lowest in the water-stressed plants grown at UVB0, both under higher temperature regime; in the middle leaves, *E* was highest in the water-stressed plants grown at UVB5, but lowest in the well-watered plants grown at UVB0, both under lower temperature regime; and in the lower leaves, *E* was highest in the water-stressed plants grown under higher temperature regime, but lowest in the well-watered plants grown under lower temperature regime, both at UVB0 (Fig. 4.2B).

In the upper leaves, g_s was significantly affected by the main factors and all two-way and three-way interactions. In the middle leaves, g_s was significantly affected by UVB, watering regime, and all two-way and three-way interactions. In the lower leaves, g_s was significantly affected by temperature, and all two-way interactions (Table 4.5). On the basis of these interactions, in the upper leaves, the well-watered plants grown under higher temperature regime at UVB5 had highest g_s, whereas the water-stressed plants grown under the same temperature regime at UVB0 had lowest g_s; in the middle leaves, the well-watered plants grown under lower temperature regime at UVB5 had highest g_s, but the water-stressed plants grown under higher temperature regime at UVB0 had lowest g_s ; and in the lower leaves, the well-watered plants grown under lower temperature regime at UVB5 had highest g_s, but the water-stressed plants grown under lower temperature regime at UVB5 had lowest g_s (Fig. 4. 2C).

In all leaf positions, WUE was significantly affected by temperature, UVB, watering regime, and the two-way and three-way interactions, except for the T × U (upper leaves)

and three-way (lower leaves) interactions (Table 4.5). On the basis of these interactions, in the upper and middle leaves, WUE was highest in the well-watered plants under lower temperature regime at UVB0, and lowest in the water-stressed plants under higher temperature regime at UVB5; and in the lower leaves, WUE was highest in the well-watered plants grown under lower temperature regime at UVB0, similar to upper and middle leaves, but lowest in plants experience the same watering regime and grown under higher temperature regime at UVB5 (Fig. 4.2D).

4.4.3 Chlorophyll fluorescence

UVB5 and water stress decreased ϕPSII, which was lower in the lower leaves than in the upper leaves (Table 4.1). Water stress increased non-photochemical quenching (qNP; Table 4.1). Overall, ϕPSII by watering regime, ϕPSII and qP by U × W, and qP by the three-way interaction were significantly affected (Table 4.3). On the basis of U × W, the leaves of well-watered plants grown at UVB0 had highest ϕPSII, and the leaves of water-stressed plants grown at UVB5 had lowest ϕPSII. The two-way and three-way interactions revealed that the water-stressed plants under higher temperature regime at UVB0 had highest qP, but plants experienced the same watering regime and grown under lower temperature regime at UVB5 had lowest qP (Fig. 4.3).

In the leaf position, differences were significant in ϕPSII between watering regimes for all leaves and in U × W for middle leaves (Table 4.5). Water stress decreased ϕPSII in the middle leaves ($P < 0.05$), but had no effect on it in the upper and lower leaves. Interaction of U × W showed that ϕPSII was highest in the leaves of well-watered plants at UVB0, and lowest in the leaves of water-stressed plants at UVB5. On the basis of significant T × W of qNP in the upper leaves (Table 4.5), it was highest in the water-stressed plants, but lowest in the well-watered plants, both under higher temperature regime. Also, the significant U × W interaction of qP in the upper leaves (Table 4.5) indicated that the water-stressed plants had highest qP at UVB0, but lowest at UVB5 (Fig. 4.3).

Regardless of watering regime, lower leaves of plants under lower temperature regime at UVB0 had lowest ϕPSII (Fig. 4. 3A). Also, lower leaves of the well-watered plants under lower temperature regime at UVB5 had lowest F_v/F_m and qNP (Fig. 4.3B and C).

TABLE 4.3 Summary of split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on leaf photosynthetic parameters and chlorophyll fluorescence in pea (*Pisum sativum*) plants

Treatment	d.f.	Photosynthetic parameters							
		A_N		E		g_s		WUE	
		MS	F	MS	F	MS	F	MS	F
Temperature (T)	1	101.55	31.41*	11.13	159.13**	0.00	223.89**	25.06	4942.92***
Main plot error	2	-	-	-	-	-	-	-	-
UVB radiation (U)	1	63.29	186.87***	52.19	24.78**	0.06	21.11*	42.32	6037.90****
T × U	1	2.49	7.36	3.74	1.77	0.01	2.46	6.43	917.61****
Subplot error	4	-	-	-	-	-	-	-	-
Watering regime (W)	1	352.07	179.18****	53.91	77.77****	0.09	57.13****	2.30	196.88****
T × W	1	21.28	10.83*	9.79	14.13**	0.00	3.00	1.09	93.48****
U × W	1	11.25	5.72*	32.94	47.52***	0.06	36.00***	3.78	323.98****
T × U × W	1	0.74	0.38	0.04	0.06	0.00	0.00	2.77	237.49****
Split-subplot error	8	-	-	-	-	-	-	-	-
Treatment	d.f.	Chlorophyll fluorescence							
		ϕPSII		F_v/F_m		qNP		qP	
		MS	F	MS	F	MS	F	MS	F
Temperature (T)	1	0.0012	0.08	0.0201	1.81	0.1754	2.09	0.3431	2.58
Main plot error	2	-	-	-	-	-	-	-	-
UVB radiation (U)	1	0.1295	5.28	0.0009	0.06	0.0152	0.07	0.1209	1.35
T × U	1	0.0154	0.63	0.0000	0.00	0.1734	0.81	0.2767	3.09
Subplot error	4	-	-	-	-	-	-	-	-
Watering regime (W)	1	0.1434	29.14***	0.0050	0.43	0.7510	4.08	0.0369	0.30
T × W	1	0.0069	1.41	0.0003	0.03	0.3398	1.85	0.1281	1.04
U × W	1	0.0395	8.03*	0.0000	0.00	0.0014	0.01	0.7585	6.14*
T × U × W	1	0.0023	0.47	0.0001	0.01	0.0087	0.05	0.7490	6.06*
Split-subplot error	8	-	-	-	-	-	-	-	-

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

FIG. 4.2 Photosynthetic parameters in the leaves of one-month-old pea (*Pisum sativum*) plants. **(A)** A_N, **(B)** E, **(C)** g_s, and **(D)** WUE. Other details are the same as in Fig. 4.1

FIG. 4.2

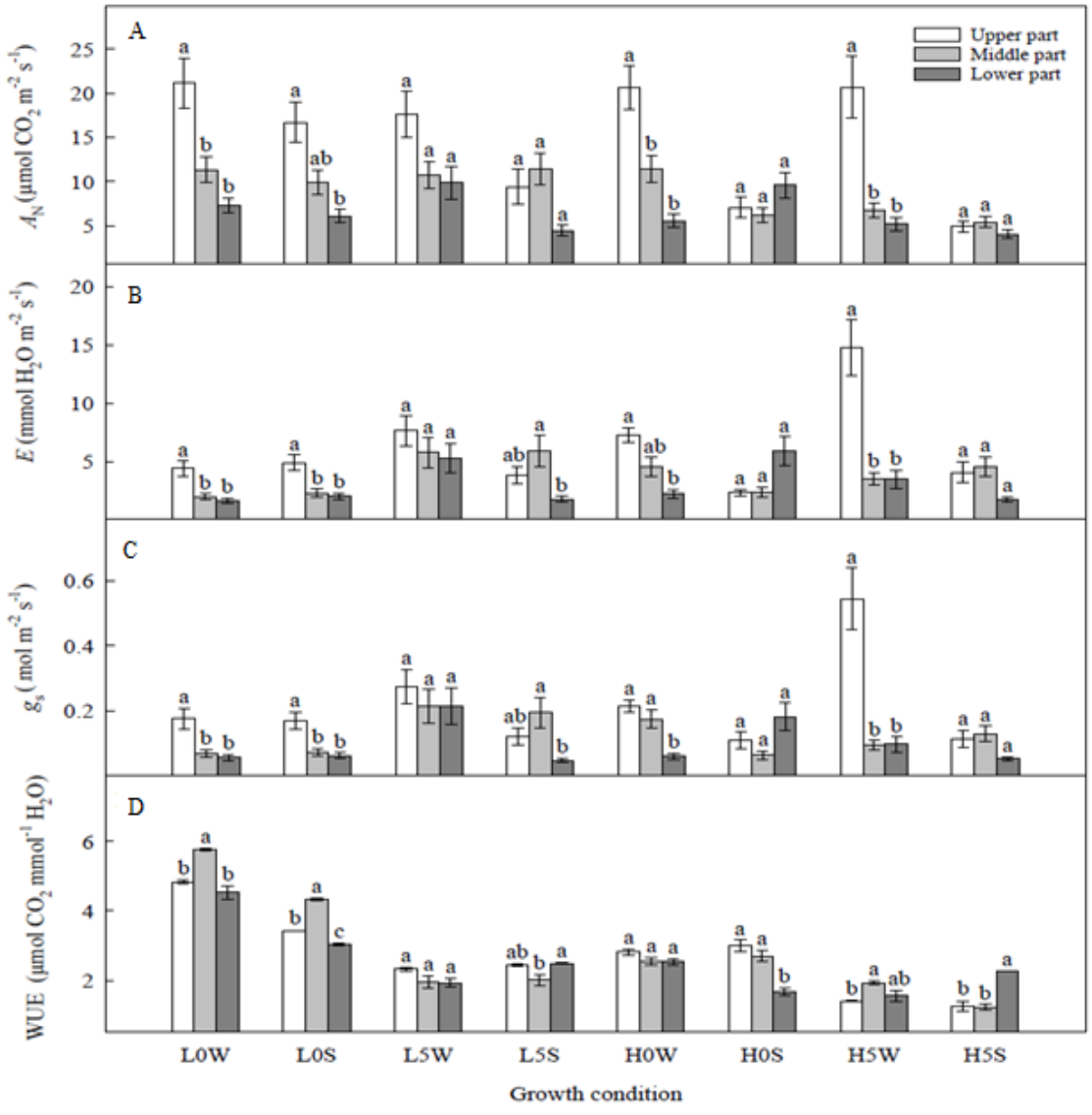
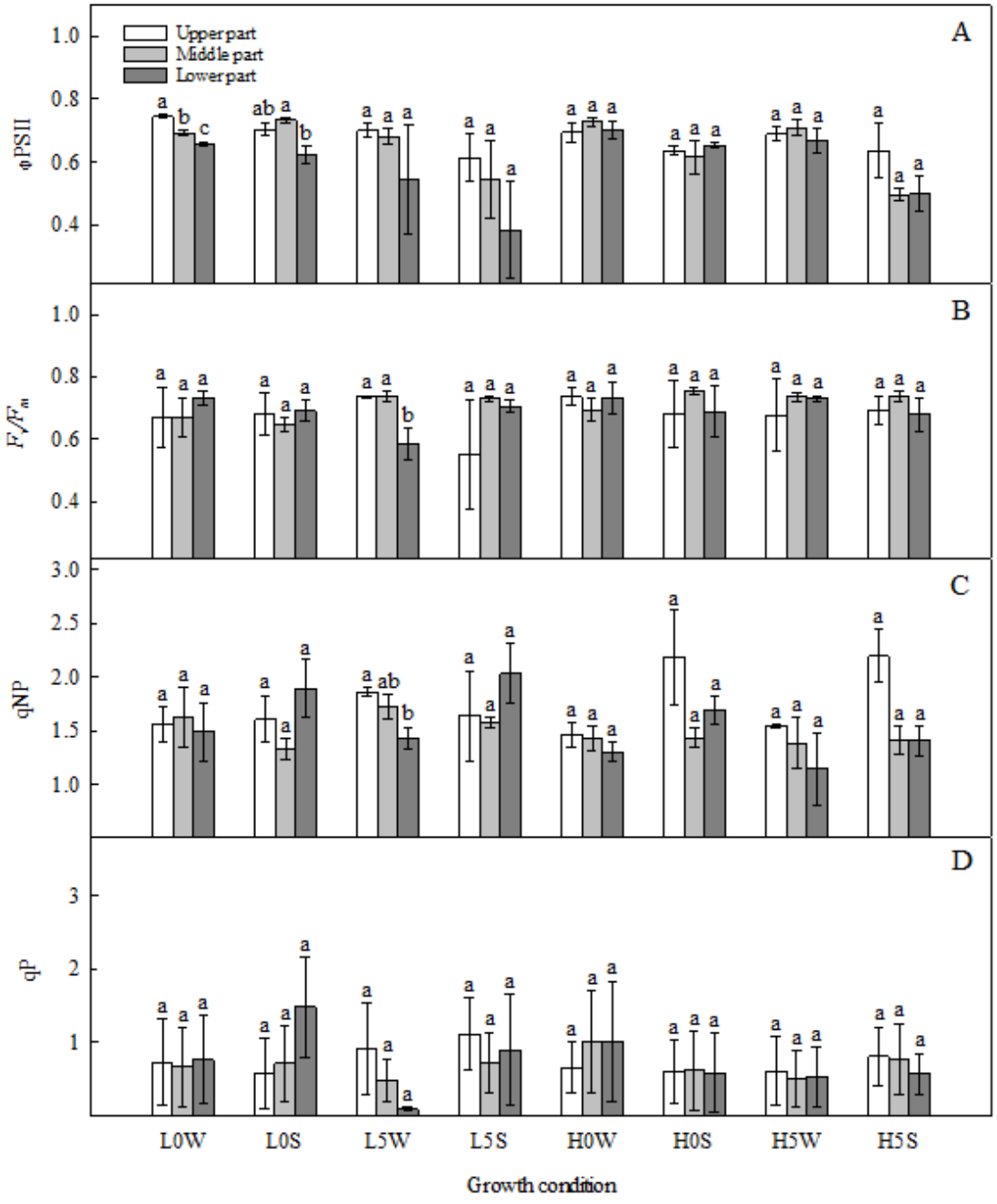

A
B
C
D
Upper part
Middle part
Lower part
A_N (μmol CO_2 m^{-2} s^{-1})
E (mmol H_2O m^{-2} s^{-1})
g_s (mol m^{-2} s^{-1})
WUE (μmol CO_2 $mmol^{-1}$ H_2O)
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

FIG. 4.3 Chlorophyll fluorescence in the leaves of one-month-old pea (*Pisum sativum*) plants. **(A)** ϕPSII, **(B)** F_v/F_m, **(C)** qNP, and **(D)** qP. Other details are the same as in Fig. 4.1.

FIG. 4.3


Upper part
Middle part
Lower part
A
B
C
D
ΦPSII
F_v/F_m
qNP
qP
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

4.4.4 Total chlorophyll

Higher temperature regime, UVB5, and water stress reduced total leaf chlorophyll, which was lower in the lower leaves of the shoot than the upper leaves of the shoot (Table 4.1). Differences in total chlorophyll were significant for the main factors and the two-way and three-way interactions (Table 4.4). On the basis of these interactions, total chlorophyll was highest in the leaves of well-watered plants under lower temperature regime at UVB0, and lowest in the leaves of water-stressed plants under higher temperature regime at UVB5 (Fig.4. 4A).

In all leaf positions, differences in total chlorophyll between all main factors and their two-way and three-way (except for the upper part) interactions were significant (Table 4.5). In all leaf positions, higher temperature regime, UVB5 and water stress reduced total chlorophyll ($P < 0.05$). In the upper position, the two-way interactions revealed that total chlorophyll in the leaves of well-watered plants under lower temperature regime at UVB0 was highest, but lowest in the leaves of water-stressed plants under higher temperature regime at either UVB0 or UVB5. In the middle position, the three-way interaction indicated that total chlorophyll was highest in the leaves of well-watered plants under lower temperature regime at UVB0, but lowest in the leaves of water-stressed plants under lower temperature regime at UVB5. In the lower position, on the basis of three-way interaction, total chlorophyll was highest in the leaves of well-watered plants grown under lower temperature regime at UVB0, similar to that of the middle position, but lowest in the leaves of water-stressed plants grown under higher temperature regime at UVB5 (Fig. 4.4A). Irrespective of growth condition, the upper leaves of plants had higher total chlorophyll than the lower leaves. The opposite trend was found only in the leaves of well-watered plants that were grown under lower temperature regime at UVB0 (Fig. 4.4A).

4.4.5 Flavonoids

Overall, UVB5 decreased leaf flavonoids, although unexpected (Table 4.1). UVB, the two-way interaction between T × U, and T × W, and the three-way interaction significantly affected flavonoids (Table 4.4). As these interactions revealed, the leaves of well-watered plants under lower temperature regime at UVB0 had most flavonoids,

whereas the leaves of water-stressed plants under higher temperature regime at UVB5 had least flavonoids (Fig. 4.4B).

Flavonoids were significantly affected by UVB, watering regime, T × U (middle and lower leaves), T × W (upper and lower leaves), and the three-way interaction (upper and lower leaves; Table 4.5). UVB5 decreased flavonoids in all leaf positions, whereas water stress decreased them in the upper and lower leaves ($P < 0.05$). In the upper position, the two-way and three-way interactions showed that the leaves of well-watered plants under higher temperature regime at UVB0 had most flavonoids, whereas the leaves of water-stressed plants under the same temperature regime at UVB5 had least flavonoids. In the middle position, the T × U interaction revealed that the leaves of plants under lower temperature regime at UVB0 produced most flavonoids, whereas the leaves of plants under the same temperature regime at UVB5 produced least flavonoids. In the lower position, on the basis of three-way interaction, leaf flavonoids were highest in the water-stressed plants under lower temperature regime at UVB0, but lowest in the well-watered plants under lower temperature regime at UVB5 (Fig. 4.4B). The lower leaves of well-watered plants under lower temperature regime at either UVB0 or UVB5, and the upper leaves of water-stressed plants under lower temperature regime at UVB0 or under higher temperature regime at UVB5 produced relatively lower flavonoids than the leaves from other shoot parts (Fig. 4.4B).

4.4.6 Relationship between plant parameters

Pearson's correlation revealed several significant relationships between physiological parameters of plants. For instance, CH_4 emissions from stem was positively correlated with E ($r = 0.463$, $P = 0.023$) and g_s ($r = 0.452$, $P = 0.027$), but negatively correlated with WUE ($r = -0.472$, $P = 0.020$). A_N was positively correlated with E ($r = 0.702$, $P = 0.000$), g_s ($r = 0.725$, $P = 0.000$) and total chlorophyll ($r = 0.659$, $P = 0.000$), but negatively correlated with F_v/F_m ($r = -0.552$, $P = 0.005$). E was positively correlated with g_s ($r = 0.983$, $P = 0.000$), but negatively correlated with WUE ($r = -0.410$, $P = 0.047$) and F_v/F_m ($r = -0.525$, $P = 0.008$). A negative correlation was found between g_s and F_v/F_m ($r = -0.540$, $P = 0.006$). WUE was positively correlated with total chlorophyll

(r = 0.612, P = 0.001) and flavonoids (r = 0.650, P = 0.001). Also, flavonoids had positive correlation with total chlorophyll (r = 0.554, P = 0.005).

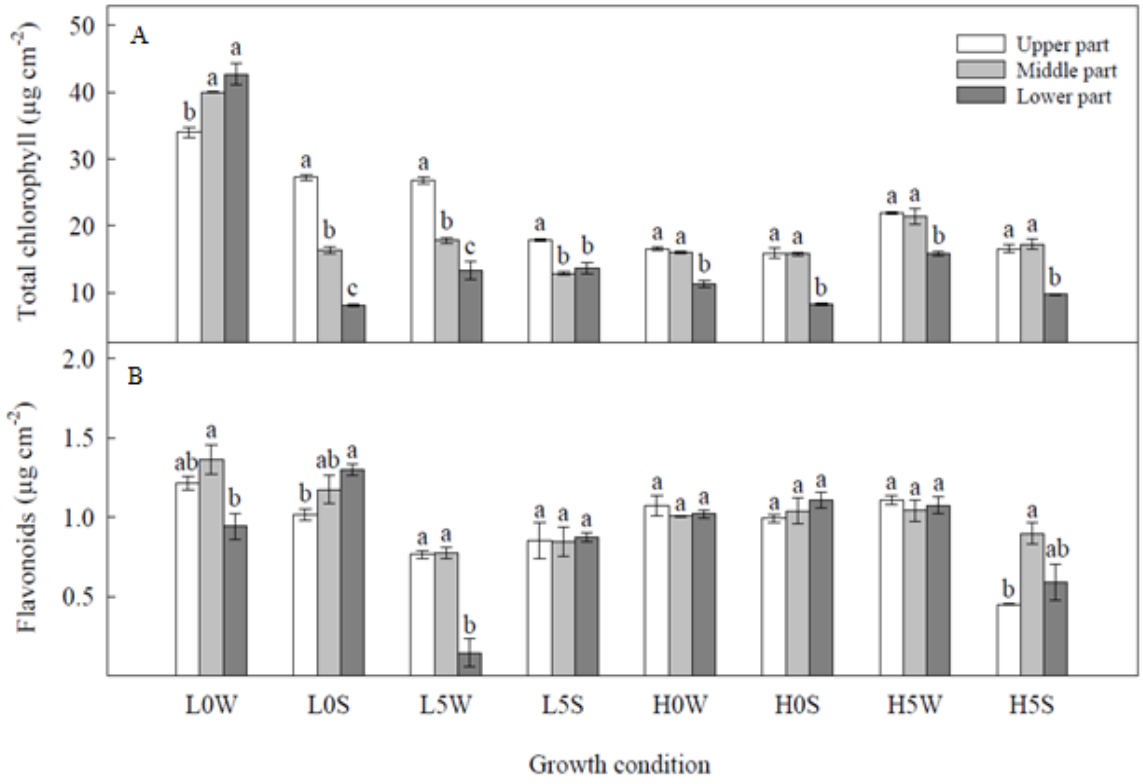
TABLE 4.4 Summary of split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on total leaf chlorophyll and flavonoids in pea (*Pisum sativum*) plants

	Total chlorophyll			Flavonoids		
Treatment	d.f.	MS	*F*	d.f.	MS	*F*
Temperature (T)	1	891.05	1201.10***	1	0.00	1.22
Main plot error	2	-	-	2	-	-
UVB radiation (U)	1	277.34	233.06***	1	1.83	197.21***
T × U	1	900.93	757.08****	1	0.36	38.53**
Subplot error	4	-	-	4	-	-
Watering regime (W)	1	1206.14	1048.89****	1	0.02	0.98
T × W	1	434.68	378.01****	1	0.55	28.50***
U × W	1	196.32	170.72****	1	0.02	1.05
T × U × W	1	496.70	431.95****	1	0.63	32.23***
Split-subplot error	8	-	-	8	-	-

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

FIG. 4.4 Total chlorophyll and flavonoids in the leaves of one-month-old pea (*Pisum sativum*) plants. **(A)** Total chlorophyll, and **(B)** flavonoids. Other details are the same as in Fig. 4.1.

FIG. 4.4


A
B
Upper part
Middle part
Lower part
Total chlorophyll (μg cm-2)
Flavonoids (μg cm-2)
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

4.5 DISCUSSION

In this study, we found that CH_4 emissions from pea plants increased by higher temperature regime and UVB5, and emissions were highest from the upper part of shoot and stem and lower leaves (Table 4.1; Fig. 4.1). The results support our previous findings (Qaderi and Reid, 2009). Higher emissions of CH_4 from the stems than leaves confirms our findings from the hydroponic system (Abdulmajeed *et al*., 2017), suggesting that leaves adapt to environmental stress factors by changing their characteristics, whereas stems might be more sensitive to stress factors than leaves (Leymarie *et al*., 1999; Zhuang *et al*., 2011). Also, the negative correlation of CH_4 emissions from stem with WUE suggests that less water in plants leads to increased aerobic CH_4 emissions. As suggested, increased CH_4 emissions from plants under these stress factors may have been due to the spontaneous breakdown of plant material (Nisbet *et al*., 2009) or the stimulating effects of ROS (McLeod *et al*., 2008). However, in other studies, it has been suggested that CH_4 may come from pectin (a polysaccharide that contains methoxyl group), which is used by plants to build their supporting structures (Keppler *et al*., 2006, 2008). Therefore, it would be logical to assume that stem would produce more CH_4 than younger leaves. Highest CH_4 emissions from the upper part of stem (Table 4.1), suggest that, in this study, shoot upper parts capture more direct light, particularly UVB radiation, than the other parts of plant (Brodersen and Vogelmann, 2007), leading us to suggest that under stress conditions the upper parts of the shoot may emit more CH_4 in the natural habitats.

In this study, all three factors had no effects on CH_4 emissions from leaf and stem when analyzed separately (Table 4.2). As mentioned earlier, our findings indicate that CH_4 emissions were highest from the lower leaves or from the upper part of stem. Highest CH_4 emissions rates from the lower leaves might have been related to their specific characteristics, such as being old, dry, having less nutrients, and being more sensitive to stress factors. Increased CH_4 emissions from the upper part of stem were likely due to its direct exposure to UVB, compared to the lower parts.

Our study revealed that higher temperature regime, UVB5 and water stress decreased WUE (Tables 4.1 and 4.3; Fig. 4.2). It has been shown that WUE can be negatively affected by higher temperatures and enhanced UVB in canola (*Brassica napus*; Qaderi *et*

al., 2010), and by water stress in *Pelargonium* (Nicotra *et al*., 2008) and *Pisum sativum* (Qaderi and Reid, 2009), suggesting that plants under these stress factors probably lost more water than gained CO_2. Also, water stress decreased all parameters of photosynthesis (Tables 4.1 and 4.3; Fig. 4.2). It has been reported that drought affects many plant processes, including activities of photosynthetic apparatus and accumulation of metabolites (Sangtarash *et al*., 2009). Reddy *et al*. (2004*a*) have shown that water stress decreases photosynthesis because of reduced stomatal conductance. In our study, decreased stomatal conductance under water stress supports a report on sunflower (Cechin *et al*., 2010), but does not support some other studies that reported increased g_s under water stress (Zhang *et al*., 2006). Decreased net CO_2 assimilation could have been due to decreased stomatal conductance, likely as the result of stomatal closure to alleviate the effect of higher temperatures and to reduce water loss (Lammertsma *et al*., 2011). We found that higher temperature regime, UVB5 and water stress significantly decreased concentration of total chlorophyll, which might have adversely affected photosynthetic rates (Jones, 2014). As expected, the upper leaves had highest A_N, E and g_s (Tables 4.1, 4.3 and 4.5; Fig. 4.2), which are similar to the results obtained in sugar beet (*Beta vulgaris*; Monti *et al*., 2007) and sunflower (*Helianthus annuus*; Cechin *et al*., 2010), suggesting that younger leaves were less affected by stress factors, due to their high metabolism, compared to older leaves located at the middle and lower parts of shoot. Overall, our study indicated that older leaves were more susceptible to stress factors than younger leaves. Also, it was likely that water stress was more harmful to the plants than other factors, and this stress factor might have reduced plant photosynthetic capacity.

We found that UVB5 and water stress decreased ϕPSII, which was lowest in the lower leaves (Tables 4.1 and 4.3; Fig. 4.3), suggesting that these stress factors inhibit PSII electron transport (Nogués and Baker, 2000), turnover D1 protein in the PSII reaction center (Berry and Björkman, 1980), and decrease photosynthetic activity (Jansen *et al*., 1998). Water stress affected non-photochemical quenching, but none of the main factors affected photochemical quenching. Although qNP and qP were not significantly affected by leaf position, they were relatively higher in the upper leaves, which might have well adapted to the experimental conditions and performed normally. Our findings are similar to those of Shirke (2001) who studied the effects of leaf age on chlorophyll fluorescence

in an evergreen tree (*Prosopis juliflora*). The lower leaves, on the other hand, were relatively stressed during the day but could have recovered when they were exposed to less stress at dark. Overall, photochemical efficiency was lower in these leaves (Tables 4.1 and 4.5; Fig. 4.3).

Our current study also showed that higher temperature regime, UVB5 and water stress decreased total chlorophyll (Table 4.1; Fig. 4.4) as shown in earlier studies (Nilsen and Orcutt, 1996; Qaderi *et al*., 2007; 2010). Overall, the upper leaves had highest chlorophyll compared to other leaves (Table 4.1; Fig. 4.4), and this is similar to the result obtained from the rice plants by Imai *et al*. (2005). This suggests that Rubisco activity could have been high during leaf expansion (Imai *et al*., 2005), usually in the upper leaves, but its activity might have limited after full leaf expansion, which occurred in the lower leaves (Miller and Huffaker, 1982).

In our study, UVB5 decreased flavonoids (Table 4.1; Fig. 4.4), and this indicates that less secondary metabolites, such as flavonoids, have been produced under this condition. These compounds, with antioxidant properties, are important in plant defense against ROS that cause cellular damages (Sharma *et al*., 2012). This finding indicates that the stress factors, used in our study, might have caused damage to chloroplast (see above) or deactivated Rubisco (Ashraf and Harris, 2013). A positive correlation between chlorophyll and flavonoids revealed that changes in these two categories of chemicals occur in parallel. For instance, the water-stressed plants grown under lower temperature regime at UVB5 had lower chlorophyll and flavonoids than the well-watered plants grown under lower temperature regime at UVB0 (Fig. 4.4).

In summary, our study revealed that both higher temperature regime and UVB5 affected CH_4 emissions from pea plants. We found that CH_4 emissions varied with plant organs and shoot parts, as stem emitted more CH_4 than leaf, and the shoot upper part than the lower part. Also, CH_4 emissions were highest from the upper part of stem and lower leaves. These findings support our original hypothesis that the three environmental stress factors, considered in this study, influence the upper parts of the shoot to emit more CH_4 than the other parts. As the mechanism of aerobic CH_4 emissions remains unknown, future studies should focus on exploring its plant source considering both vegetative and reproductive organs of plants growing under multiple environmental factors.

4.6 LITERATURE CITED

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CHAPTER 5

Does Developmental Stage Affect Aerobic Methane Emissions from Pea Plants Grown under Combination of Temperature, UVB Radiation and Water stress?

5.1 ABSTRACT

Many studies have investigated the effect of one or two environmental factors on methane (CH_4) emissions, but the impact that simultaneous application of multiple stress factors may have on emissions has rarely been studied under controlled conditions despite being a more realistic approach. In this study, we determined the effects of temperature, UVB radiation, and watering regime on CH_4 emissions and other physiological parameters of pea plants (*Pisum sativum* L. cv. 237J Sundance) during different vegetative stages, by growing them under controlled conditions: two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two UVB levels [0 and 5 kJ m^{-2} d^{-1}] and two watering regimes (field capacity and wilting point). Measurements were then taken after 10, 20, and 30 days of growth under experimental conditions, after seven days of initial growth under 22/18ºC, before plants reached their reproductive stage. Higher temperature, UVB5, and water stress increased CH_4 emissions and the response was highest in earlier stages of plant growth. Also, all three stress factors increased transpiration (E), higher temperature and UVB5 increased stomatal conductance (g_s), but only higher temperature decreased water use efficiency (WUE). We found that leaves of 37-day-old plants had the highest net CO_2 assimilation (A_N), g_s, and E, compared to leaves of younger plants. Higher temperatures decreased the effective quantum yield of photosystem II (ϕPSII) and non- photochemical quenching (qNP), but increased photochemical quenching (qP), whereas water stress decreased ϕPSII, maximum quantum yield of PSII (F_v/F_m), and qP. The leaves of 17-day-old plants had highest ϕPSII, F_v/F_m, and qP, whereas leaves of older plants (37 days old) had highest qNP. In addition, UVB5 was observed to decrease nitrogen balance index (NBI), but to increase flavonoids. Leaves of younger plants (17-day-old) had highest NBI and total chlorophyll, but had lowest flavonoid content. We concluded that the level of CH_4 emissions decreases with plant age, and that temperature has a greater influence on plant growth compared to UVB and water stress, at the levels tested in this study.

5.2 INTRODUCTION

Methane (CH_4) gas is a significant greenhouse gas, making it one of the major environmental concerns behind global warming. The gas occurs naturally under the sea floor and beneath Earth's crust, and is released into the atmosphere through natural biogenesis and geological activity, but also due to anthropogenic activities (Bousquet *et al*., 2006). Methane ranks as the second most common greenhouse gas after carbon dioxide (CO_2), and its global warming potential (molecule per molecule) is nearly 34 times greater than CO_2 due to its stronger ability to trap heat (Myhre *et al*., 2013). Current atmospheric concentrations of methane are much higher than pre-industrial levels. Levels appeared to be stabilizing in the 1990's and early 2000's, but have increased again over the past 10 years (Myhre *et al*., 2013). Despite a drop in the rate of CH_4 in the atmosphere since the early 1990s, difficulties have been increasing in estimating the general trends in its concentration (Qaderi and Reid, 2009). In research studies, prior to 2006, the global production of CH_4 was attributed to the anaerobic activity of microorganisms (Wood, 2016). However, in 2006, Keppler *et al.* published experimental results suggesting that plants can also produce and emit CH_4 under aerobic conditions with non-microbial sources. Many studies have been done since to investigate this phenomenon under diverse conditions (Qaderi and Reid, 2011). These studies confirmed that CH_4 is emitted from attached (Zhang *et al*., 2014) and detached leaves (Fraser *et al*., 2015) under certain conditions, such as high temperature (Abdulmajeed *et al*., 2017), UVB radiation (Bruhn *et al*., 2014), water stress (Qaderi and Reid, 2011) and elevated CO_2 (Qaderi and Reid, 2011). These environmental conditions cause changes in the phenological, morphological, and physiological characteristics of plants, which appear to lead to formation of CH_4, though the precise physiological mechanisms remain unknown (Qaderi and Reid, 2009). In 2008, a study suggested that the origin of aerobic CH_4 could be the methoxyl groups in plant pectin (Keppler *et al.,* 2006). More recently, other potential sources, including cellulose and lignin (Vigano *et al*., 2009), methionine (Lenhart *et al*., 2015), and leaf surface wax (Bruhn *et al*., 2014) have been suggested.

Temperature is an environmental factor that has been repeatedly linked to the aerobic release of CH_4 from plants in these studies. Temperatures above optimum levels result in a decrease in the level of chlorophyll, specifically chlorophyll *a* and *b*, and a decline in

their associated carotenoid ratio (Cui *et al.,* 2006). Less chlorophyll leads to lower photosynthetic rates (Azoulay-Shemer *et al*., 2015), and higher rates of transpiration (*E*) and stomatal conductance (g_s) (Qaderi *et al.,* 2008). Higher temperature also affects the length of plant developmental stages (Qaderi *et al.,* 2008) through effects on seed germination (Toh *et al*., 2008), seed mass (Roach and Wulff, 1987), leaf area, and total plant biomass (Qaderi and Reid, 2009).

Two other important environmental stressors are UVB radiation and drought, or water stress, both of which are known to have negative effects on plant growth and development. High levels of exposure to UVB can alter the chemical composition within plant cells (Reifenrath and Müller, 2007), which results in shorter plants (Salama *et al*., 2011), changes in leaf anatomy and thickness, and affect developmental rate (Robson *et al*., 2015). Therefore, there are variations in the response to UVB radiation among different vegetative stages **(**Qaderi *et al.,* 2008). Drought has been shown to limit the development of vegetative stages of many plant species, as well as to alter emissions of CH_4 (Yousfi *et al*., 2012). Drought affects gas exchange characteristics (Lenzi *et al*., 2009), chlorophyll *a* content and fluorescence (Guan *et al*., 2015), as well as stem height and diameter (Xu *et al.,* 2008). Environmental stressors that influence plant photosynthesis, growth and development will have different levels of impact as plants age. Thus, we might expect that the impact of the stressors on aerobic CH_4 emissions might also vary with plant life stage. The majority of studies conducted to investigate the effect of environmental stressors on aerobic CH_4 emissions in plants consider only single (McLeod *et al*., 2008) or double environmental factors (Vigano *et al*., 2008). An understanding of the influence of multiple environmental factors is crucial to determining the contribution of plants to the global CH_4 budget. In this study, we investigate the interactive effects of three environmental factors on CH_4 emissions from different vegetative stages of pea plants. Our objective is to determine CH_4 emission rates at different developmental stages of plants grown under two temperatures, two UVB levels, and two watering regimes. We hypothesized that a combination of higher temperatures, UVB5, and water stress would increase CH_4 emissions from pea plants and the rates of emissions would vary at different vegetative stages. The results of this study will

contribute to our understanding of the effects of abiotic stressors on plants as significant sources of atmospheric CH_4.

5.3 MATERIAL AND METHODS

5.3.1 Plant material and growth conditions

Seeds were planted in pots containing a mixture of perlite: vermiculite: peat moss (1:1:2, by volume), and modified Hoagland's solution was used as fertilizer (Zioni *et al*., 1971). After emergence, seedlings were kept for one week under control condition as following: temperature of 22/18ºC, photoperiod 16 h light/8 h dark, photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}, and relative humidity (RH) of ~ 65%.

We used a split plot experimental design, with two temperature regimes (22/18ºC or 28/24ºC), two UVB (UVB_{BE}) radiation regimes (0 and 5 kJ m^{-2} d^{-1}), and two levels of watering (well-watered and water deficit to the point of leaf wilt). Each treatment combination had a minimum of 27 seedlings, one third of which were grown for 10 days, another third for 20 days, and the remaining seedlings were grown for 30 days.

5.3.2 Gas exchange

From each condition, three fully-expanded leaves of each 3 plants were collected to measure net CO_2 assimilation (A_N; µmol CO_2 m^{-2} s^{-1}), transpiration rate (E; mmol H_2O m^{-2} s^{-1}), and stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}). A portable LI-COR photosynthesis system (model 6400XT, LI-COR Inc., Lincoln, NE, USA), calibrated using 400 µmol mol^{-1} of CO_2 with flow rate of 400 mL s^{-1}, was used to perform these measurements. The water use efficiency (WUE; µmol CO_2 $mmol^{-1}$ H_2O) was calculated by using the formula A_N/E (Lambers *et al*., 2008).

5.3.3 Chlorophyll fluorescence

Three fully-expanded leaves of each 3 plants were measured using a portable fluorometer (Fluorpen FP 100, Photon Systems Instruments, Drasov, Czech Republic). Under light conditions, the effective quantum yield of photosystem II (ϕPSII; F_q'/F_m') was determined by measuring the photosynthetic electron transport. For dark-adapted measurements, leaves were clamp for 30 minutes to record the maximum quantum yield of PSII (F_v/F_m),

Non-photochemical quenching (qNP; $(F_m\text{-}F_m')^{-1}$) and photochemical quenching (qP; F_q'/F_v') (Baker, 2008).

5.3.4 Nitrogen balance index, chlorophyll, and flavonoids

Nitrogen balance index (NBI), chlorophyll (μg cm^{-2}), and flavonoid (μg cm^{-2}) content, in three leaf samples under each of the eight conditions applied in this experiment, were estimated by measuring optical absorbance of samples using a Dualex Scientific® System (Dualex Scientific, Force-A, Orsay Cedex, France). Leaf clips were used for this analysis considering their chlorophyll and flavonoid content to be accurate representations for the whole leaf epidermis as projected by Martel and Qaderi (2016).

5.3.5 Statistical analysis

Effects of temperature, UVB radiation, watering regime, vegetative stage and their interactions on CH_4 emissions and other physiological parameters were determined by means of analysis of variance for split-split-split-plot design (SAS Institute, 2011). Other details were provided in Chapter 2.

5.4 RESULTS

5.4.1 Methane emissions

The three-way interaction of UVB (U) × watering regime (W) × vegetative stage (V) significantly affects emissions of CH_4 from plant (Table 5.2). On application of one-way ANOVA, it was observed that higher temperature, UVB5, and water stress increased CH_4 emissions (Table 5.1). However, the amount of CH_4 decreased when 17-day-old plants were grown under a combination of higher temperature and UVB5. It was also observed that UVB5 decreased CH_4 emissions in 27-day-old plants when they received enough water to grow. This is not in concordance with the results of one-way ANOVA, revealed that UVB5 increased CH_4 emissions. Overall, CH_4 emissions was highest during earlier stages of plant growth as compared to other stages.

5.4.2 Photosynthetic parameters

The three-way interaction of U × W × V, significantly affected A_N (Table 5.3). However, none of the main factors had any effect on A_N (Table 5.1). Fig. 5.2A, showed that the 37-day-old plant had highest A_N, regardless of UVB radiation and watering regimes. The four-way interactions of temperature (T) × U × W × V, had significant impact on E. The results of one-way ANOVA showed that the main effect of higher temperature, UVB5, and water stress was to increase the level of *E*, but as shown in Fig. 5.2B, value of *E* did not change under experimental conditions for plants with ages between 17-and 27-day-old. A combination of higher temperatures and UVB5 increased *E* in the well-watered of 37-day-old plants. The four-way interactions also affected water use efficiency (WUE) (Table 5.3). One-way ANOVA indicates that higher temperature increase WUE, however no significant differences were found among experimental conditions (Fig. 5.3D). The two-way interaction of U × W and W × V, revealed that stomatal conductance (g_s) was highest from the well-watered of 37-day-old plant grown at UVB5. One-way ANOVA (Table 5.1) illustrated that higher temperature and UVB5 increased g_s. However, as shown in (Fig. 5.2C) the amount of g_s decreased when plants were grown under higher temperatures at UVB0. Table 5.1 revealed that UVB5 increased g_s; however no differences in g_s values were found in plants grown under lower temperatures regardless of UVB radiation (Fig. 5.2C).

TABLE 5.1 Effects of temperature, UVB radiation, and watering regime on methane emissions and other physiological parameters of pea plants at different vegetative stages of growth under experimental conditions, after one week of initial growth under 22/18ºC. Data are means ± SE of three trials (at least three replicates of each treatment per trial)

	Temperature		**UVB radiation**		**Watering regime**		**Vegetative stages**		
Parameters	**Lower**	**Higher**	**UVB0**	**UVB5**	**Well-watered**	**Water-stressed**	**17 days**	**27 days**	**37 days**
Methane	54.06 ± 3.35B	79.74± 4.62A	59.88 ± 4.01B	73.92 ± 4.81A	60.73 ± 4.18B	73.07 ± 4.73A	72.42 ± 4.11A	67.57 ± 4.38A	60.72 ± 7.54B
A_N	15.28 ± 2.60A	14.58 ± 2.55A	14.54 ± 2.32A	15.32 ± 2.81A	14.75 ± 2.37A	15.11± 2.77A	4.65 ± 0.27B	4.60 ± 0.41B	35.53 ± 1.52A
E	4.09 ± 0.81B	8.68 ± 1.90A	4.77 ± 0.91B	8.00 ± 1.89A	5.53 ± 1.13B	7.24 ± 1.80A	1.99 ± 0.20B	1.65 ± 0.15B	15.52 ± 2.22A
g_s	0.14 ± 0.03B	0.58 ± 0.22A	0.17 ± 0.03B	0.56± 0.22A	0.24 ± 0.07A	0.48 ± 0.21A	0.07 ± 0.01B	0.05 ± 0.01B	0.97 ± 0.31A
WUE	4.00 ± 0.30A	2.20 ± 0.17B	3.42 ± 0.30A	2.78 ± 0.26A	3.08 ± 0.25A	3.12 ± 0.32A	2.57 ± 0.21A	3.23 ± 0.33A	3.49 ± 0.46A
ϕPSII	0.70 ± 0.01A	0.63 ± 0.02B	0.66 ± 0.02A	0.67 ± 0.01A	0.68 ± 0.01A	0.65 ± 0.02B	0.70 ± 0.01A	0.65 ± 0.02B	0.65 ± 0.02B
F_v/F_m	0.75 ± 0.01A	0.73 ± 0.01A	0.73 ± 0.01A	0.74 ± 0.01A	0.75 ± 0.01A	0.72 ± 0.01B	0.77 ± 0.01A	0.70 ± 0.01B	0.74 ± 0.02A
qNP	1.61 ± 0.04A	1.47 ± 0.05B	1.53 ± 0.05A	1.54 ± 0.04A	1.58 ± 0.05A	1.50 ± 0.05A	1.33 ± 0.05B	1.56 ± 0.05A	1.73± 0.05A
qP	0.20 ± 0.01B	0.25 ± 0.01A	0.23 ± 0.02A	0.22 ± 0.01A	0.24 ± 0.02A	0.21 ± 0.01B	0.27 ± 0.02A	0.22 ± 0.02AB	0.18 ± 0.02B
NBI	43.31 ± 2.25A	46.47 ± 1.98A	49.47 ± 2.40A	40.31 ± 1.46B	44.99 ± 2.06A	44.79 ± 2.21A	55.46 ± 2.40A	43.28 ± 1.86B	35.93 ± 1.77C
Total chl.	31.38 ± 0.82A	31.15 ± 0.87A	31.41 ± 0.81A	31.12 ± 0.88A	31.07 ± 0.90A	31.46 ± 0.79A	34.78 ± 0.71A	31.54 ± 0.82B	27.48 ± 0.97C
Flavonoids	0.77 ± 0.02A	0.72 ± 0.03A	0.69 ± 0.02B	0.80 ± 0.02A	0.74 ± 0.02A	0.75 ± 0.03A	0.64 ± 0.03B	0.78 ± 0.03A	0.82 ± 0.02A

TABLE 5.2 Summary of split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, vegetative stage, and their interactions on methane emissions in pea (*Pisum sativum*) plants

		Methane	
Source	d.f.	MS	*F*
Temperature (T)	1	721.82	10.56
Main plot error	2	89.09	1.71
UVB radiation (U)	1	18820.55	263.28**
T × U	1	535.98	7.50
Subplot error	2	84.50	1.62
Watering regime (W)	1	19408.43	233.66***
T × W	1	772.34	9.30*
U × W	1	17911.07	215.63***
Split sub-plot error	4	107.03	2.05
Vegetative stage (V)	2	19112.41	358.14****
T × V	2	578.13	10.83*
U × V	2	18256.83	342.11****
W × V	2	18779.01	351.89****
T × U × W	1	701.58	8.45*
T × U × V	2	333.94	6.26*
T × W × V	2	518.21	9.71*
U × W × V	2	18072.20	338.65****
T × U × W × V	2	177.59	3.33
Split-split-subplot error	8	60.58	1.16

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

TABLE 5.3 Summary of split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, vegetative stage, and their interactions on gas exchange parameters in pea (*Pisum sativum*) plants

		A_N		E		g_s		WUE	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	0.07	0.02	50.70	164.72**	2.12	9.15	0.86	1.64
Main plot error	2	3.05	0.09	0.40	0.03	0.23	0.42	0.53	0.74
UVB radiation (U)	1	864.76	820.96**	127.90	126.24**	4.31	7.15	13.33	36.42*
T × U	1	0.10	0.09	40.16	39.63*	1.31	2.18	1.77	4.85
Subplot error	2	1.05	0.03	1.01	0.08	0.60	1.09	0.36	0.52
Watering regime (W)	1	899.92	292.71****	135.55	61.82**	3.74	1.92	15.02	7.59
T × W	1	0.11	0.03	48.30	22.03**	1.63	0.84	1.16	0.59
U × W	1	620.80	201.92****	42.18	19.24*	47.82	24.59**	1.41	0.71
Split sub-plot error	4	3.07	0.09	2.19	0.18	1.94	3.53	1.98	2.79
Vegetative stage (V)	2	882.21	229.66****	135.55	88.55****	4.02	2.96	14.16	10.85*
T × V	2	2.30	0.60	23.17	15.13**	0.07	0.05	6.91	5.29
U × V	2	810.42	210.97****	115.95	75.74****	7.21	5.31	8.89	6.81*
W × V	2	834.00	217.11****	119.48	78.05****	7.36	5.42*	9.20	7.04*
T × U × W	1	0.00	0.00	42.51	19.39*	1.48	0.76	1.37	0.70
T × U × V	2	5.49	1.43	19.45	12.71**	0.10	0.07	7.35	5.63*
T × W × V	2	2.31	0.6	23.07	15.07**	0.14	0.1	7.05	5.4*
U × W × V	2	837.79	218.09****	132.02	86.24****	3.44	2.53	13.92	10.66*
T × U × W × V	2	5.58	1.45	19.55	12.77**	0.20	0.14	7.15	5.48*
Split-split-subplot error	8	3.84	0.11	1.53	0.13	1.36	2.46	1.31	1.84

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

FIG. 5.1 Methane emissions from different vegetative stages of pea (*Pisum sativum* 237J Sundance). Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes (well-watered and watered-stress) for 10, 20 and 30 days, after one week of initial growth under 22/18ºC. L0W, low temperature-UVB0-well watered; L0S, low temperature-UVB0-water stressed; L5W, low temperature-UVB5-well watered; L5S, low temperature-UVB5-water stressed; H0W, high temperature-UVB0-well watered; H0S, high temperature-UVB0-water stressed; H5W, high temperature-UVB5-well watered; H5S, high temperature-UVB5-water stressed. Bars (mean ± SE) surmounted by different upper-case letters among conditions and by different lower-case letters within vegetative stages are significantly different ($P < 0.05$) according to Scheffé's multiple comparison procedure.

FIG. 5.1

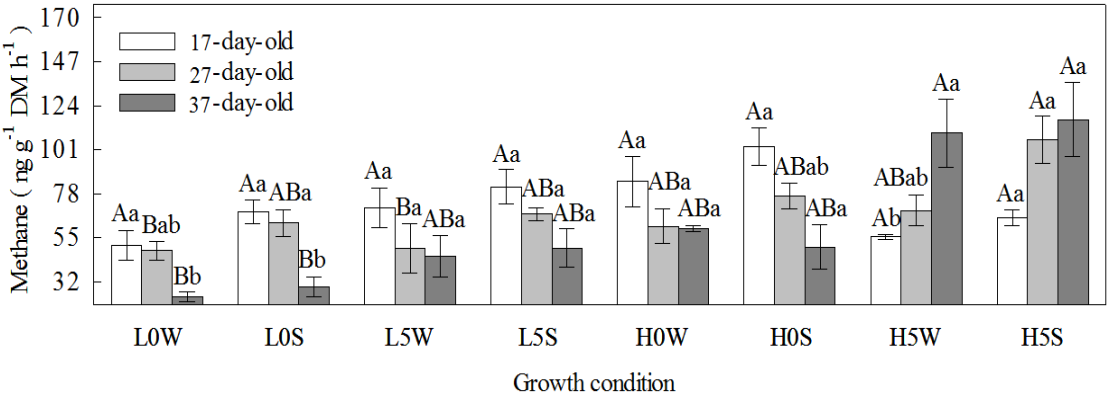
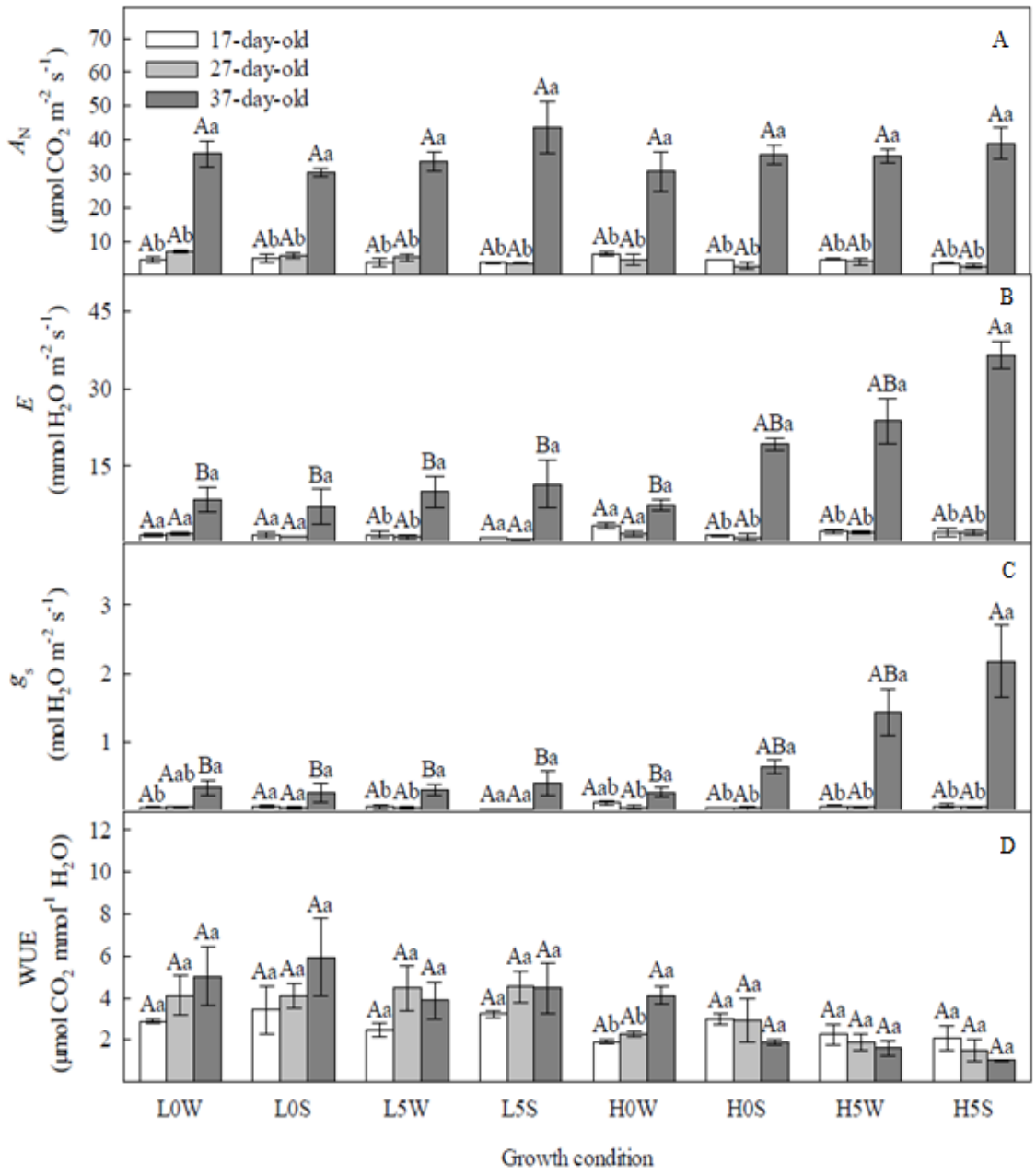

Methane (ng g-1 DM h-1)
170
147
124
101
78
55
32
17-day-old
27-day-old
37-day-old
Aa
Bab
Bb
Aa
ABa
Bb
Aa
Ba
ABa
Aa
ABa
ABa
Aa
ABa
ABa
Aa
ABab
ABa
Ab
ABab
Aa
Aa
Aa
Aa
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

FIG. 5.2 Photosynthetic parameters from different vegetative stages of pea (*Pisum sativum* 237J Sundance). Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes (well-watered and watered-stress) for 10, 20 and 30 days, after one week of initial growth under 22/18ºC. (**A**) A_N, net CO_2 assimilation (**B**) E, transpiration, (**C**) g_s, stomatal conductance, and (**D**) WUE, water use efficiency. Other details are the same as in Fig. 5.1.

FIG. 5.2


17-day-old
27-day-old
37-day-old
A
B
C
D
A_N (μmol CO_2 m^{-2} s^{-1})
E (mmol H_2O m^{-2} s^{-1})
g_s (mol H_2O m^{-2} s^{-1})
WUE (μmol CO_2 mmol^{-1} H_2O)
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

5.4.3 Chlorophyll fluorescence

On the basis of one-way ANOVA, it was observed that higher temperatures decreased ϕPSII and qNP while increasing qP values. On the other hand, water stress decreased ϕPSII, F_v/F_m, and qP values (Table 5.1). However, only the two-way interaction, of U × W, had significant effect on ϕPSII, F_v/F_m, and qNP values, whereas the interactions, of U × W, U × V, and W × V, affected qP only. The two-way interaction, of U × W, had significant effect on ϕPSII. Higher temperature and UVB5 were observed to decrease ϕPSII values. However, as shown in Fig. 5.3A, the levels of ϕPSII did not change under higher temperatures regardless of UVB levels and watering regimes for 27-day-old plants. Also the same interaction had significant effect on F_v/F_m, where one-way ANOVA revealed that water stress decreased F_v/F_m values (Table 5.1). No differences in the levels of F_v/F_m were observed among different conditions, while within plant ages: F_v/F_m was lowest from the well-watered plant of 27-day-old plant grown under lower temperature (Fig. 5.3B).

In case of qNP, higher temperatures were observed to decrease the level of qNP (Table 5.1). The two-way interaction of U × W, had significant effect on qNP (Table 5.4). However, a combination of lower temperature, UVB0 and water stress affected qNP in plants of different ages with highest qNP values from 37-day-old plants and lowest from 17-day-old (Fig. 5.3C).

The two-way interactions of U × W, U × V, and W × V, affected qP (Table 5.4). The one-way ANOVA revealed that higher temperatures increased qP and water stress decreased it (Table 5.1), However, no significant differences were found in the values of qP regardless of the four main factors among conditions. Furthermore, qP decreased with increasing ages of plants grown under L0W and H5S (Fig. 5.3D).

5.4.4 Nitrogen balance index

UVB radiation, watering regimes, and vegetative stage of plants as well as the three-way interactions among them had significant effects on nitrogen balance index (NBI) values (Table 5.5). One-way ANOVA revealed that only water stress decreased NBI, but temperature or watering regimes did not exhibit this response (Table 5.1). This is not corroborated by the results given in Fig. 5.4A, that show no differences existed in the

levels of NBI from 27- and 37-day-old plant among experimental conditions. A combination of UVB5 radiation and water stress decreased NBI from 17-day-old plants.

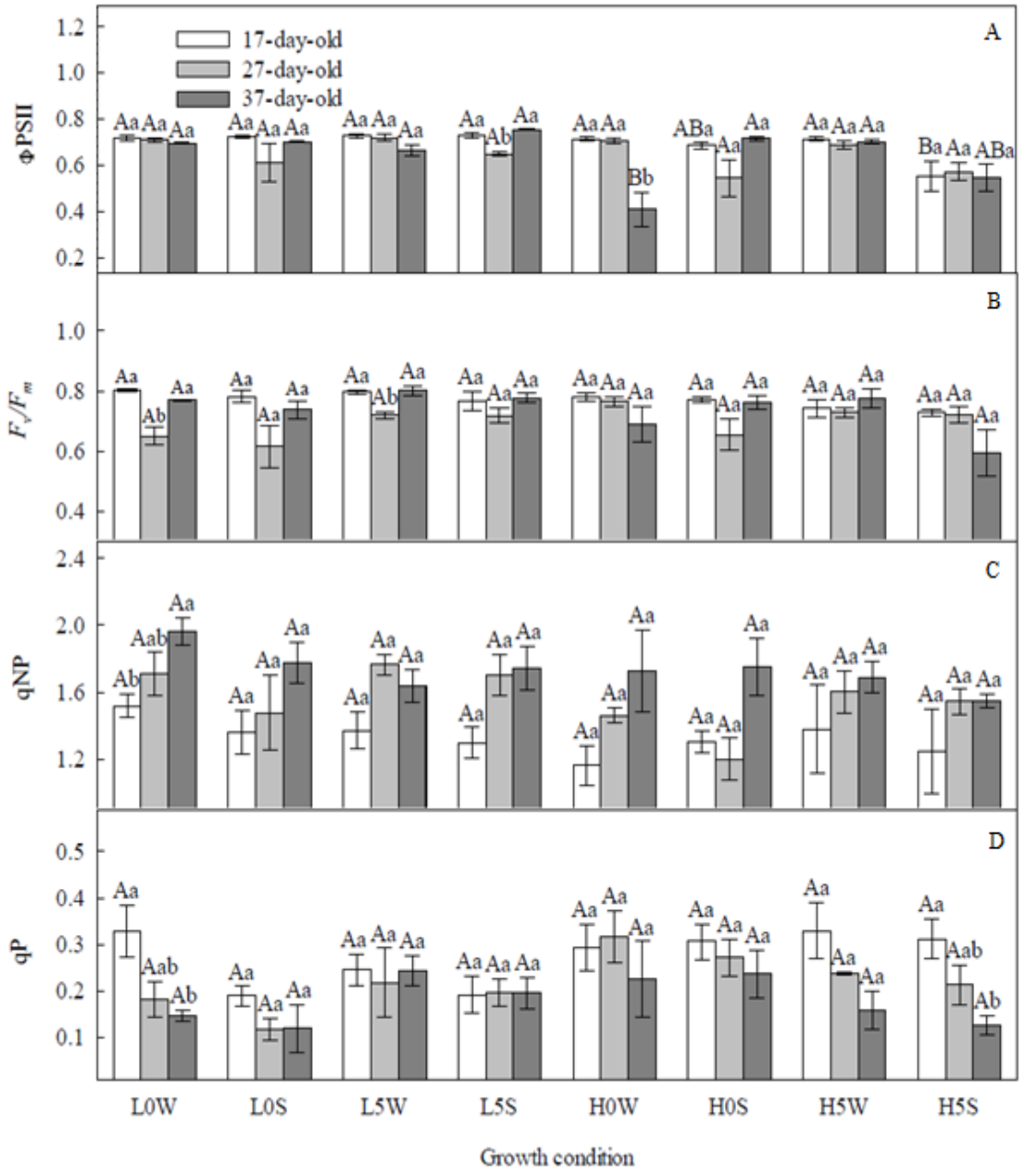
TABLE 5.4 Summary of split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, vegetative stage, and their interactions on chlorophyll fluorescence parameters in pea (*Pisum sativum*) plants

		ϕPSII		F_v/F_m		qNP		qP	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	0.68	1.64	0.76	1.95	0.59	1.69	0.91	2.18
Main plot error	2	0.41	1.09	0.39	1.02	0.35	0.90	0.33	1.08
UVB radiation (U)	1	2.40	5.48	2.02	4.43	0.09	0.2	6.21	14.97
T × U	1	0.26	0.60	0.31	0.68	0.16	0.37	0.44	1.07
Subplot error	2	0.44	1.15	0.46	1.20	0.45	1.15	0.45	1.00
Watering regime (W)	1	1.95	0.99	1.61	0.82	0.23	0.12	5.54	2.83
T × W	1	0.41	0.21	0.48	0.24	0.37	0.19	0.59	0.3
U × W	1	40.74	20.71*	39.11	19.94*	20.45	10.59*	53.79	27.47**
Split sub-plot error	4	1.97	5.19	1.96	5.17	1.93	4.99	1.75	6.01
Vegetative stage (V)	2	2.17	1.70	1.81	1.41	0.15	0.11	5.87	4.56
T × V	2	0.75	0.58	0.67	0.52	0.84	0.64	0.54	0.42
U × V	2	4.67	3.65	4.14	3.23	0.11	0.08	9.58	7.44*
W × V	2	4.77	3.73	4.22	3.29	0.11	0.08	9.80	7.6*
T × U × W	1	0.34	0.18	0.40	0.20	0.28	0.15	0.51	0.26
T × U × V	2	0.69	0.54	0.62	0.49	0.86	0.65	0.48	0.37
T × W × V	2	0.81	0.63	0.73	0.57	0.89	0.67	0.62	0.48
U × W × V	2	1.77	1.39	1.45	1.13	0.16	0.12	5.12	3.98
T × U × W × V	2	0.65	0.51	0.59	0.46	0.76	0.57	0.49	0.38
Split-split-subplot error	8	1.28	3.37	1.28	3.38	1.33	3.43	1.06	3.08

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 5.3 Chlorophyll fluorescence from different vegetative stages of pea (*Pisum sativum* 237J Sundance). Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes (well-watered and watered-stress) for 10, 20 and 30 days, after one week of initial growth under 22/18ºC. (**A**) ϕPSII, quantum yield of PSII, (**B**) F_v/F_m, maximum quantum yield of PSII, (**C**) qNP, non-photochemical quenching, and (**D**) qP, photochemical quenching. Other details are the same as in Fig. 5.1.

FIG. 5.3


17-day-old
27-day-old
37-day-old
A
B
C
D
ΦPSII
F_v/F_m
qNP
qP
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

5.4.5 Total chlorophyll

UVB radiations, watering regimes, and vegetative stages as well as the three-way interactions among them had significant effects on total chlorophyll (Table 5.5). However, none of these main factors were observed to affect chlorophyll content on the basis of one-way ANOVA. This explains the results obtained in Fig. 5.4B that shows no differences in the amount of chlorophyll among experimental conditions in plants of all ages, while chlorophyll content decreased with increasing plant ages when they were grown under a combination of higher temperature, UVB0, and water stress (Fig. 5.4B).

5.4.6 Flavonoid content

The two-way interaction, of U × W, affected flavonoid content (Table 5.5). On the basis of one-way ANOVA, UVB5 was observed to increase flavonoid content (Table 5.1). However, no differences were observed in the amounts of flavonoids among different experimental conditions (Fig. 5.4C). Among plants of various ages, flavonoid content increased with increasing plant age under a combination of lower temperature and UVB0 regardless of watering regimes (Fig. 5.4C).

5.4.7 Relationship between plant parameters

Pearson's correlation coefficients were significant for several relationships of physiological parameters. For instance, CH_4 emissions was negatively correlated with WUE (r = – 0.713, P = 0.000), and qNP (r = – 0.482, P = 0.017), but positively correlated with g_s (r = 0.465, P = 0.022).

5.5 DISCUSSION

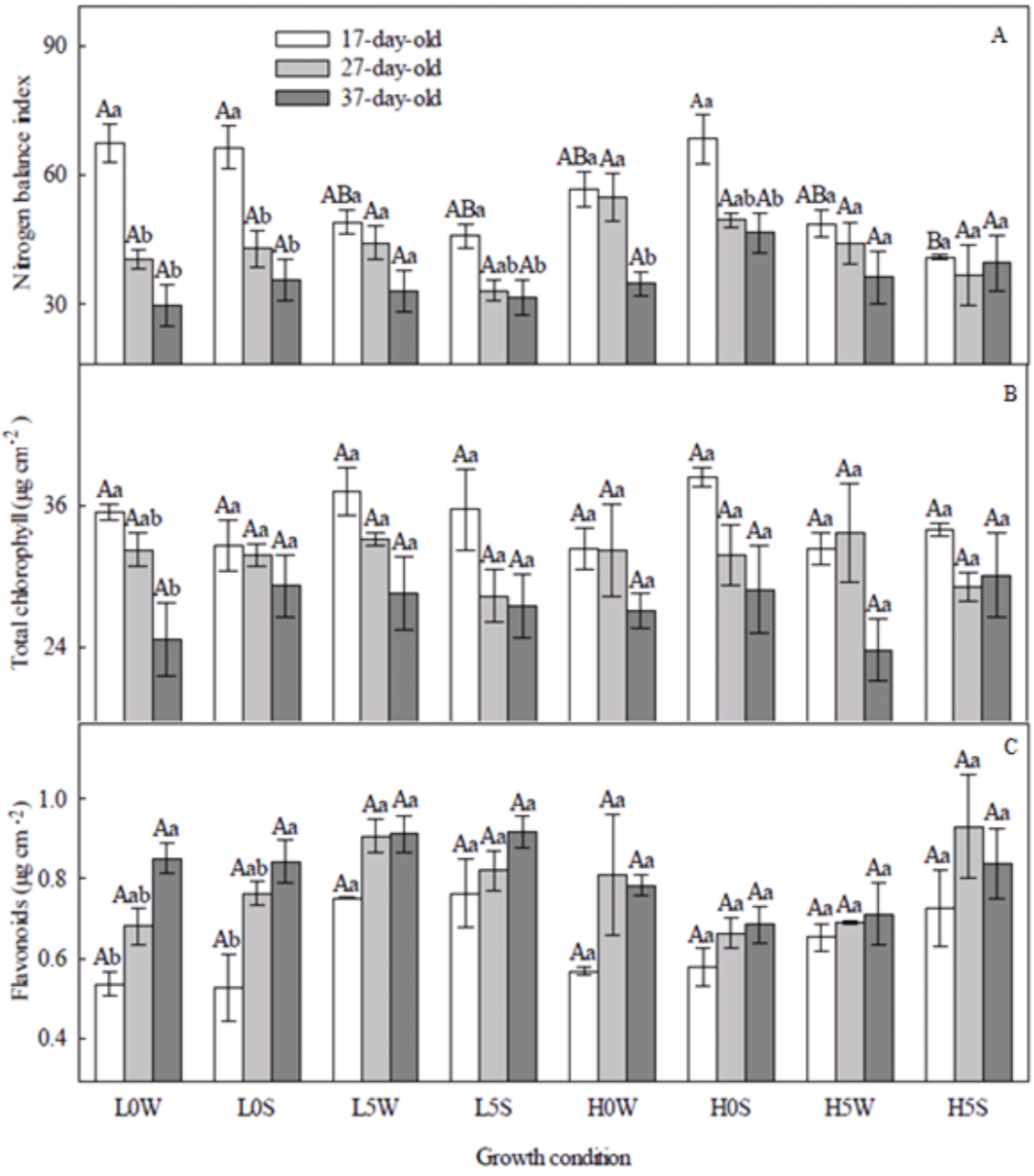
Methane is the primary end-product of anaerobic mineralization process. This mineralization may take place naturally; for example, natural wetlands contribute 20-39% CH_4 to the global occurrence of this gas through a process called *methanogenesis* (Zhang *et al*., 2016). However, recent studies show that plants are a significant source of aerobic CH_4 emissions (McLeod *et al*., 2008). Plants produce CH_4 under both normal (Keppler *et al*., 2006) and stressed conditions (Bruhn *et al*., 2014).

TABLE 5.5 Summary of split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, vegetative stage, and their interactions on nitrogen balance index, total chlorophyll and flavonoids in pea (*Pisum sativum*) plants

		Nitrogen balance index		Chlorophyll		Flavonoids	
Source	d.f.	MS	*F*	MS	*F*	MS	*F*
Temperature (T)	1	139.70	16.36	12.43	8.4	0.75	2.12
Main plot error	2	8.54	0.46	1.48	0.41	0.35	0.93
UVB radiation (U)	1	8470.75	954.35**	3978.11	4797.89***	1.76	3.67
T × U	1	84.90	9.57	1.75	2.11	0.30	0.62
Subplot error	2	8.87	0.48	0.83	0.23	0.48	1.26
Watering regime (W)	1	8746.00	1449.81****	4114.37	1965.93****	1.38	0.7
T × W	1	152.78	25.33**	14.63	6.99	0.48	0.24
U × W	1	7780.04	1289.68****	3473.31	1659.62****	37.95	19.41*
Split sub-plot error	4	6.03	0.32	2.09	0.58	1.95	5.12***
Vegetative stage (V)	2	8607.40	514.82****	4045.75	1197.57****	1.56	1.22
T × V	2	87.47	5.23	1.00	0.3	0.68	0.52
U × V	2	8179.47	489.22****	3816.77	1129.79****	3.78	2.93
W × V	2	8413.96	503.25****	3926.54	1162.28****	3.85	2.99
T × U × W	1	131.28	21.76**	9.73	4.65	0.40	0.2
T × U × V	2	26.19	1.57	0.00	0.00	0.63	0.49
T × W × V	2	70.35	4.21	0.00	0.00	0.74	0.57
U × W × V	2	8143.67	487.08****	3830.88	1133.97****	1.24	0.96
T × U × W × V	2	0.00	0.00	0.00	0.00	0.59	0.46
Split-split-subplot error	8	16.72	0.90	3.38	0.93	1.29	3.37**

FIG. 5.4 Nitrogen balance index, total chlorophyll and flavonoids from different vegetative stages of pea (*Pisum sativum* 237J Sundance). Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes (well-watered and watered-stress) for 10, 20 and 30 days, after one week of initial growth under 22/18ºC. (**A**) NBI, nitrogen balance index, (**B**) total chlorophyll, and (**C**) flavonoids. Other details are the same as in Fig. 5.1.

FIG. 5.4


17-day-old
27-day-old
37-day-old
A
B
C
Nitrogen balance index
Total chlorophyll (μg cm-2)
Flavonoids (μg cm-2)
90
60
30
36
24
1.0
0.8
0.6
0.4
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

A combined application of the four environmental factors in this study indicated that the 37-day-old water-stressed plants grown under higher temperatures at UVB5 had highest level of CH_4 emissions, whereas the 37-day-old well-watered plants grown at lower temperatures at UVB0 had lowest level of CH_4 emissions. This has been corroborated in previous research, which reports that plants under stress conditions emit more CH_4 as a result of plant material being broken down (Nisbet *et al.,* 2009). Specifically, higher temperatures harm a plant by damaging its DNA or through the manufacture of reactive oxygen species (ROS) (Stapleton and Walbot, 1994), which then causes the release of CH_4 from the pectin substance as suggested in research by McLeod *et al.* (2008) and Messenger *et al.* (2009).

In this study, we found that CH_4 emissions decreases with increase in plant's age. It is, therefore, true that the 17-day-old plants emitted more CH_4 than the 37-day-old plants. Martel and Qaderi (2017) argued that the results that they found in sunflower (*Helianthus annuus*) and chrysanthemum (*Chrysanthemum coronarium*), might be related to plant acclimation over time and an increased organic carbon sources that, in turn, decreases the tissues undergoing metabolic processes. They further assert that physical injury, such as removal of leaves, accelerates the emissions of CH_4 gas, regardless of plant age or other controllable variables, such as light intensity. Additionally, Sano and Kawashima (1982) reported that older plants had higher activity of proline synthesis in tobacco plants, a substance that allows plants to withstand water deficit, which is an explanation for older plants being less affected by stress conditions.

All three environmental factors increased *E*. A higher *E* in plants usually translates into lower WUE as it showed in our result in this study, and supported the study by (Bacon, 2009). We also found that none of the main factors affected A_N, however, an earlier study has shown that g_s increased as a result of stress conditions, such as UVB radiation, and this increase was caused by an increase in A_N (Qaderi *et al.,* 2008), which is not in agreement with our finding (Table 5.1). Overall, differences in photosynthetic capacity are different based on plant species. In our study, older pea plants 37-days-old experienced highest A_N, g_s, and *E* when they were exposed to stress conditions (35.53 ± 1.52, 0.97 ± 0.31, and 15.52 ± 2.22, respectively), compared to younger plants (20 days old; 4.60 ± 0.41, 0.05 ± 0.01, and 1.65 ± 0.15, respectively).

This may be attributed to the fact that old plants are acclimated with the stress condition over 37 days period of their life, which is enough for them to cope with a new condition. However, these observations are completely opposing to Field and Mooney's ideas (1983) who stated that photosynthetic capacity and g_s decreased with leaves age in chaparral shrub (*Lepechinia calycina*). Reifenrath and Müller (2007) also reported that in Brassicaceae, young leaves had higher level of photosynthetic activity than old leaves, whereas Jaikumar *et al*. (2016), on contrary, stated that photosynthetic activity was higher in older plants than in younger plants. Jaikumar *et al*. (2016) also found that there was a similar level of photosynthetic activity in plants of different ages depending upon the differences in water status. Grulke and Miller (1994) revealed that the seedlings of conifers had lower photosynthetic rate than the young trees, suggesting that older plants had lower overall risk of death and may have increased stress tolerance as they grew, which is similar to our results. Furthermore, the decline in A_N in younger plants in our experiment might be due to stomatal closure that decreases g_s (Davis and McCree, 1978) and *E* values (Caird *et al*., 2007). These results suggest that early developmental stages of plants are expected to be more sensitive to climate change than adult stages (Donohue *et al*., 2010), which influence them much easily to emit higher CH_4 emissions.

Chlorophyll fluorescence is variably influenced by the environmental factors studied here. This parameter is used to screen plants for heat tolerance (Guan *et al*., 2015). Higher temperatures reduced ϕPSII and qNP, which is supported other studies (Huxman *et al*., 1998). Furthermore, our results revealed that higher temperature increased F_v/F_m and qP that is similar to (Linkosalo *et al*., 2014). Water stress decreased ϕPSII, F_v/F_m and qP, as expected from most influence factor, such as drought (Qaderi *et al.,* 2013). On the basis of data from different vegetative stages, younger plants had highest ϕPSII, F_v/F_m, and qP values, but had lowest qNP. This suggests that stress conditions, such as higher temperature or water stress, induce stomatal closure to maintain optimal leaf water status, which leads to decreased CO_2 concentration inside the leaf. All these processes in the cell thus negatively affect the photochemical activity of PSII leading to decreased chlorophyll fluorescence activity. Plants with short-term exposure, 10 days, still have a good state of ϕPSII, F_v/F_m and qP, that decreased with the time of exposure. Not only the duration of the exposure that affects chlorophyll flurecence, the temperature level also

affects plants. Our results supported the work done by Mauromicale *et al*. (2006), who revealed that the effect of plant age on chlorophyll fluorescence is varied based on differences in temperature; they found that plants are able to grow under 18°C to 26°C. On the other hand, thylakoid components in wheat are destroyed when plants were grown at high temperatures up to 35°C leading to a decrease in PSII activity (Harding *et al*., 1990).

With respect to age or vegetative stages of plants, our results revealed that the 17-day-old plants had the highest chlorophyll content, but it started to decrease with plant age, which is in concordance with the results of previous research (Day *et al.,* 1996; Mauromicale *et al*., 2006). It has been suggested that chlorophyll reduces, as the plant gets older due to diminishing nutrients. Furthermore, in our study UVB5 radiation was observed to decrease NBI (Table 5.1), which is correlated to the leaf N content and can be used to predict the status of nitrogen nutrition on plant (Agati *et al*., 2013). We found that chlorophyll content increased significantly with increasing nitrogen rates but decreased with plant age. Our results are similar to the work of Mauromicale *et al*. (2006) in potato crops, suggesting that increase in nitrogen supply stimulates the photosynthetic capacity of leaves via an increase in the stromal and thylakoid protein contents in the leaves (Evan, 1989).

UVB5 increased flavonoids and since these chemicals act as UV-absorbents, thus protect plants from radiation damage (Stapleton and Walbot, 1994; A-H-Mackerness, 2000). This finding is again in agreement with previous studies (Gerhardt *et al*., 2008). The increase in flavonoids suggests that plants grown at supplemental UVB are influenced to accumulate secondary metabolites, such as flavonoids, to absorb extra radiation. Long-term exposure to stress conditions, such as 30 days, influences plants to accumulate higher level of flavonoids than short-term exposure (Kakani *et al.,* 2003; Reddy *et al.,* 2004; Reifenrath and Müller, 2007).

In conclusion, release of aerobic CH_4 from living plants was acknowledged and published in research in early 2006. Since that time, many studies have confirmed this finding, using different species of plants or growing plants under different environmental conditions. However, these studies have largely dealt with biochemical mechanisms and the sources of this gas. Our work has confirmed that emissions of CH_4 from plants is

significant and that it increases when plants are exposed to stressful conditions. Moreover, CH_4 emissions from the leaves of pea plants decreases with plant age. Our finding is a significant step forward in the research field of under consideration because it provides researchers with a pattern and path for CH_4 emissions from plants at their different vegetative stages. However, further studies are required to gather more information regarding the patterns in other plant species or their different developmental stages.

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CHAPTER 6

Variation in Aerobic Methane Emissions at the Reproductive Stages of Pea Plants Exposed to Environmental Stress Factors

6.1 ABSTRACT

Plants emit methane (CH_4) under normal aerobic conditions, but CH_4 increases when plants are exposed to environmental stress factors. Previous research has measured CH_4 emitted from plants under single or double factors, while few studies have dealt with multiple stressors simultaneously. In this study, we determined the interactive effects of temperature, UVB radiation, and watering regime on CH_4 emissions from different reproductive stages of pea, such as fully opened flower and pods at different ages (1, 5, and 10 days). Plants (*Pisum sativum* L. cv. 237J Sundance) were grown under controlled conditions: two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two UVB levels [0 and 5 kJ m^{-2} d^{-1}], and two watering regimes (field capacity and wilting point) after seven days of initial growth under 22/18ºC. Measurements of the emitted gas were taken after 30 days of growth under experimental conditions, when plants had completed transition into reproductive stage and had started to produce flowers, which in turn would produce pods. Our results revealed that temperature significantly affected CH_4 emissions from flowers, whereas UVB radiation and watering regimes affected pea pods only at different ages. Higher temperatures increase CH_4 emissions from flowers. One-day-old pods were observed to have highest CH_4 emissions as compared to 5- and 10-day-old pods. Our result also showed that a combination of the three factors, temperature, UVB radiation and watering regimes, would reduce the negative effects of individual factors. In summary, CH_4 emissions levels varied among reproductive stages and emissions levels decreased with pod age.

6.2 INTRODUCTION

Global warming is considered as one of the major environmental issues of the 21st century. Inefficient environmental management practices, particularly those of agricultural and waste management, and intensive industrialization are the core drivers of global warming (Lashof and Ahuja, 1990). These practices raise the levels of greenhouse gases, such as carbon dioxide (CO_2) and methane (CH_4), in the atmosphere creating gaseous layers that traps heat thus leading to increase in temperatures over time (Collins *et al.,* 2010). Carbon dioxide is the most harmful greenhouse gas, which can last in the atmosphere for extended duration up to several centuries (Lashof and Ahuja, 1990). This causes it to have long-term effects by continuously compounding the amount of trapped heat around the Earth (Collins *et al.,* 2010).

The impact of CH_4 in influencing global warming has been highlighted (Lashof and Ahuja, 1990). Myhre *et al*. (2013) reported that CH_4 has a global warming potential of up to 34 times higher than CO_2, which makes the impact of CH_4 on global warming very crucial. Methane enters the atmosphere as a fossil fuel that originates from garbage dumps (Calzadilla *et al.,* 2013), wetlands, coal mines, and rice paddies (Howarth *et al.,* 2011). Recently, plants were reported as an important source of CH_4 in the atmosphere (Van Groenigen *et al.,* 2011). This phenomenon was first proposed and discussed by Keppler *et al.* (2006). The study suggested that terrestrial plants produce CH_4. Subsequently, many studies have used different approaches to investigate aerobic CH_4 emissions from terrestrial plants. For example, Dueck *et al.* (2007) used laser-based measuring techniques in conjunction with stable isotope ^{13}C to measure aerobic CH_4 emissions from terrestrial plants but concluded that the emissions levels were not substantial. The amount of gas produced from plants differs depending on the environmental conditions (Lashof and Ahuja, 1990). Methane emissions from plants is actually one of the many physiological processes, which plants exhibit in response to environmental factors, such as exposure to stressful temperature, UVB radiation, and water-availability levels.

Temperature has a critical effect on the growth patterns of plants (Abdulmajeed and Qaderi, 2017). Rising global temperatures cause heat stress and affect agricultural production (Warrag and Hall, 1983). Jiang *et al.* (2015) reported that heat stress affects

pollen production per flower and also leads to lessened reproductive success in leguminous plants, such as *Pisum sativum*. Temperature also reduces the viability of pollen and reduces the pod set (Teramura and Sullivan, 1994). Overall, temperature affects the vegetative (Jiang *et al.,* 2015) and reproductive (Konsens *et al.,* 1991) stages of plant growth. However, the level of temperatures imposes different temperature stresses (Petkova *et al.,* 2009). For example, temperatures up to 45ºC affect the reproductive processes of pea but those below do not (Jiang *et al.,* 2015). Similarly, according to Sousa-Majer *et al.* (2004), high temperatures cause reduction in the mass and number of seeds in *Pisum sativum* pod.

UVB radiation is another important stress factor that brings out numerous physiological changes in the plant. Grammatikopoulos *et al.* (2001) found that fluctuations in UVB radiations have effect on plant growth. Furthermore, flowering, pollination, and seed production affected by UVB radiation (Tevini and Teramura, 1989). UVB radiations delays flowering time and change the lifetime of flower production (Sammpson and Cane, 2000). Water availability in the form of insufficient access to water might leave plants under drought stress. Sousa-Majer *et al.* (2004) applied deficiency of water to *Pisum sativum* to find out that there was a significant reduction in the number of seeds produced by the plant in the presence of drought; the mass of the pods also reduced. The implication is that water stress has a substantial impact on the vegetative and reproductive aspects of the *Pisum sativum* plants (Rodiño *et al.,* 2008). The results will be a valuable contribution toward determining the link between global warming and emissions of CH_4 by plants. It will also help to estimate the impact of various stressful conditions created by global warming on plant growth and reproduction.

In this study, our objective is to investigate the effects of multiple environmental factors on CH_4 emissions from the reproductive parts of a plant, the flower and the pod. We hypothesize that a combination of higher temperature, supplemental UVB radiation, and water stress will increase CH_4 emissions, and the level of emissions would vary with reproductive organs.

6.3 MATERIAL AND METHODS

Plant material and growth conditions

Pea seeds were planted in pots that had a mixture of perlite: vermiculite: peat moss (1:1:2, by volume). Modified Hoagland's solution was used as a fertilizer (Zioni *et al*., 1971). After emergence, seedlings were kept for one week under controlled conditions: temperature of 22/18ºC, photoperiod 16 h light/8 h dark, photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}, and relative humidity (RH) of ~ 65%. Following this, each of the growth chambers with either lower (22/18ºC) or higher (28/24ºC) temperature regimes were supplied with two levels of biologically effective UVB (UVB_{BE}) radiation (0 and 5 kJ m^{-2} d^{-1}) and two levels of watering regimes (well-watered and water deficit to the point of leaf wilt). At least 24 seedlings were placed under each of the eight conditions and divided into four groups of different growing periods. Plants started to produce flowers after 30 to 35 days of growth under experimental conditions (see Appendix V). Methane emissions were measured from fully-opened flowers as well as from 1-, 5-, and 10-day-old pods. Other details about measuring CH_4 emissions were provided in Chapter 2.

6.3.1 Statistical analysis

Effects of temperature, UVB radiation, watering regime, reproductive stage and their interactions on CH_4 emissions and dry mass were determined by means of analysis of variance for split-split-plot (flower) or split-split-split-plot design (pod) (SAS Institute, 2011). Other details were provided in Chapter 2.

6.4 RESULTS

6.4.1 Flower and pod dry mass

Our results from one-way ANOVA and under a combination of the three factors revealed that higher temperatures decreased flower dry mass (Table 6.1, Table 6.2 and Fig. 6.1). On the contrary, only the two-way interaction between W (watering regime) × D (dry mass) had significant effect on dry mass of pod (Table 6.3). However, the one-way ANOVA revealed none of the main factors had any effect on the dry mass of pods (Table

6.1), except for stage variation: 10-day-old pods showed highest CH_4 emissions compared to other ages in most experimental conditions (Table 6.1 and Fig. 6.3).

6.4.2 Methane emission from flower and pod

Only the interaction between U (UVB level) × W (watering regime) had significant effect on CH_4 emissions from flowers (Table 6.4). However, the one-way ANOVA revealed that higher temperature increased CH_4 emissions from flowers (228.45 ± 3.35 ng g^{-1}DM h^{-1}), whereas lower temperatures decreased CH_4 emissions (161.69 ± 4.62 ng g^{-1} DM h^{-1}) (Table 6.1). However, the three-way interaction was not significant, but it was obsereved that under a combination of these three factors water stress increased CH_4 emissions from flower, regardless of UVB levels and temperatures (Fig. 6.1). This was not in agreement with plants' response under lower temperatures at UVB0, where CH_4 emissions decreased when plant received water stress (Fig. 6.2). Overall, flowers of the water-stressed plants grown under higher temperatures at UVB0 had highest CH_4 emissions (260.88 ± 22.01), whereas flowers of the well-watered plants grown under lower temperatures at UVB5 had lowest CH_4 emissions (122.97 ± 23.82) (Fig. 6.1).

The two-way interaction of W × P, had significant effects on CH_4 emissions from pods (Table 6.5). However, none of the main factors affected CH_4 emissions on the basis of one-way ANOVA (Table 6.1). As shown in Fig. 6.4, no differences on CH_4 levels were observed within pods age from 1 and 10-day-old. Whereas there was a variation in CH_4 emissions, only in the 5-day-old pods with highest levels of CH_4 emitted from the well-watered plants grown under lower temperatures at UVB0 and lowest from the water-stressed plants grown under lower temperatures at UVB5. Overall, highest CH_4 emissions was from 1-day-old pods 208.05 ± 13.15 ng g^{-1} DM h^{-1}, and lowest emissions from the 10-day-old pods 31.28 ± 0.93 ng g^{-1} DM h^{-1} (Table 6.1).

6.5 DISCUSSION

Higher temperatures during flowering stage significantly increased CH_4 emissions, but decreased flowers dry mass (Table 6.1). Our results are corroborated by Devasirvatham *et al*. (2012), who reported that heat stress affects flower development in chickpea by reducing flower size thus causing yield loss. This suggests that higher temperatures cause reduction in pollen production per flower thus reducing the number of pods per plants.

Our results revealed that flowers developed under a combination of higher temperature, UVB0, and water stress had highest CH_4 emissions as a response to stress conditions. This might be a way for the plants to mitigate the harmful effects of these factors as reported by Qaderi and Reid (2011). Furthermore, the flower is directly affected by photoperiod, temperature (Truong and Duthion, 1993) and even by UVB radiation (Saile-Mark *et al*., 1996). During the flowering development phase, plants produced a large number of aborted or incompletely developed flowers when grown under higher temperatures (Sato *et al*., 2001). Furthermore, heat stress (33/30°C day/dark temperatures) causes immediate abortion of reproductive organs in pea (Guilioni *et al*., 1997). Results from this study also illustrate that the CH_4 emission rates decreased with increased pod size and age (Fig. 6.2). Suggesting that pods are having fewer anti-oxidant compounds, such as flavonoid (Tomas-Barberan *et al*., 1991), sugar content in beach pea (Chavan *et al*., 1999), and chlorophyll content (Grover and Sinha, 1985) in early developmental stage and these compounds increase with pods maturation. On the other hand, lowest CH_4 emissions was observed from the 10-day-old pods suggesting that older pods would have acclimated to the stress condition as well as had more protective compounds, such as flavonoids and chlorophyll. Overall, the 10-day-old pods were less stressed compared to the 1- and 5-day-old pods leading them to emit less CH_4.

Findings from this investigation have led us to conclude that temperature regime significantly affected CH_4 emissions from flowers, whereas UVB radiation and watering regime had significant effects on CH_4 emissions from pea pods on the basis of one-way ANOVA. However, the effect of a combination of the three factors, temperature, UVB radiation, and watering regime, on flowers and pod development does not show a clear pattern, suggesting that one stress factor might have alleviated the effects of other factor. Moreover, our study revealed that CH_4 emissions decreased with pod age. Further studies are required to measure CH_4 emissions from pods of different ages, such as pods at the stage of maturity or harvesting time. Also, conducting similar experiments on different species would help us reach a firm conclusion about the effects of stress factors on plant reproductive parts and CH_4 emission from them.

TABLE 6.1 Effects of temperature, UVB radiation, and watering regime on methane emissions,and flowe and pod dry mass from different reproductive parts of pea plants grown under experimental conditions, after one week of initial growth under 22/18ºC. Data are means ± SE of three trials. Means followed by different upper-case letters within each parameter and condition are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure

	Temperature		UVB radiation		Watering regime		Reproductive stages (pod)		
Parameters	**Lower**	**Higher**	**UVB0**	**UVB5**	**Well-watered**	**Water-stressed**	**1-day-old**	**5-day-old**	**10-day-old**
CH_4 from flowers	228.45 ± 3.35A	161.69 ± 4.62B	212.47 ± 4.01A	177.67 ± 4.81A	189.34 ± 4.18A	200.80 ± 4.73A	--	--	--
Flower DM	19.80 ± 2.47A	11.23 ± 0.57B	17.37 ± 2.57A	13.64 ± 1.05A	17.40 ± 2.57A	13.64 ± 1.08A	--	--	--
CH_4 from pods	97.42 ± 11.26A	98.89 ± 11.23A	97.99 ± 11.38A	98.47 ± 11.25A	100.13 ± 18.99A	96.18 ± 11.52A	208.05 ± 13.22A	55.13 ± 2.84B	31.28 ± 0.93C
Pod DM	49.82 ± 3.84A	46.41 ± 3.43A	48.32 ± 3.67A	47.91 ± 3.63A	47.87 ± 3.85A	48.36 ± 3.43A	14.59 ± 1.14C	49.08 ± 2.31B	80.79 ± 2.62A

DM: Dry mass

TABLE 6.2 Split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on dry mass of pea (*Pisum sativum*) flowers

		Dry mass	
Source	d.f.	MS	*F*
Temperature (T)	1	882.37	14.41*
Main plot error	5	61.23	0.96
UVB radiation (U)	1	0.16	0.00
T × U	1	46.02	0.57
Subplot error	10	81.01	0.75
Watering regime (W)	1	169.50	2.06
T × W	1	25.81	0.31
U × W	1	52.50	0.64
T × U × W	1	8.67	0.11
Split-subplot error	20	82.13	0.56

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 6.3 Split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, pod age, and their interactions on dry mass of pea (*Pisum sativum*) pods

		Dry mass	
Source	d.f.	MS	*F*
Temperature (T)	1	0.03	0.00
Main plot error	2	41.21	0.24
UVB radiation (U)	1	274.85	12.37
T × U	1	0.51	0.02
Subplot error	2	22.26	0.13
Watering regime (W)	1	39392.10	1104.61****
T × W	1	21.91	0.61
U × W	1	205.26	5.76
Split sub-plot error	4	35.66	0.21
Pod dry mass (D)	2	34057.76	1048.51****
T × D	2	9.77	0.30
U × D	2	125.95	3.88
W × D	2	33699.26	1037.47****
T × U × W	1	1.81	0.05
T × U × D	2	0.94	0.03
T × W × D	2	14.02	0.43
U × W × D	2	85.10	2.62
T × U × W × D	2	0.49	0.02
Split-split-subplot error	16	32.48	0.19

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 6.4 Split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, and their interactions on methane emissions from pea (*Pisum sativum*) flowers

		Methane	
Source	d.f.	MS	*F*
Temperature (T)	1	53493.25	10.16*
Main plot error	5	5262.55	2.83
UVB radiation (U)	1	14536.18	3.12
T × U	1	142.11	0.03
Subplot error	10	4651.89	2.83
Watering regime (W)	1	1574.93	0.34
T × W	1	14896.40	3.23
U × W	1	24248.97	5.25*
T × U × W	1	7430.36	1.61
Split-subplot error	20	4617.89	1.45

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

TABLE 6.5 Split-split-split-plot analysis of variance for effects of temperature, UVB radiation, watering regime, pod age, and their interactions on methane emissions from pea (*Pisum sativum*) pods

		Methane	
Source	d.f.	MS	*F*
Temperature (T)	1	111.34	0.20
Main plot error	2	555.44	0.37
UVB radiation (U)	1	472.03	0.93
T × U	1	161.26	0.32
Subplot error	2	505.12	0.34
Watering regime (W)	1	143555.33	267.91****
T × W	1	0.00	0.00
U × W	1	647.46	1.21
Split sub-plot error	4	535.83	0.35
Pod age (P)	2	133868.34	250.70****
T × P	2	63.15	0.12
U × P	2	775.41	1.45
W × P	2	132459.20	248.07****
T × U × W	1	265.25	0.50
T × U × P	2	29.46	0.06
T × W × P	2	33.73	0.46
U × W × P	2	984.88	1.84
T × U × W × P	2	259.10	0.49
Split-split-subplot error	16	533.95	0.36

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

FIG. 6.1 Flower dry mass of plants grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes for 30-35 days, after one week of initial growth under 22/18ºC. L0W, low temperature-UVB0-well watered; L0S, low temperature-UVB0-water stressed; L5W, low temperature-UVB5-well watered; L5S, low temperature-UVB5-water stressed; H0W, high temperature-UVB0-well watered; H0S,high temperature-UVB0-water stressed; H5W, high temperature-UVB5-well watered; H5S, high temperature-UVB5-water stressed. Bars (mean ± SE) surmounted by different upper-case letters are significantly different ($P < 0.05$) according to Scheffé's multiple comparison procedure.

FIG. 6.2 Methane emissions from flowers of plants grown under eight experimental conditions. Other details are the same as in Fig. 6.1.

FIG. 6.1

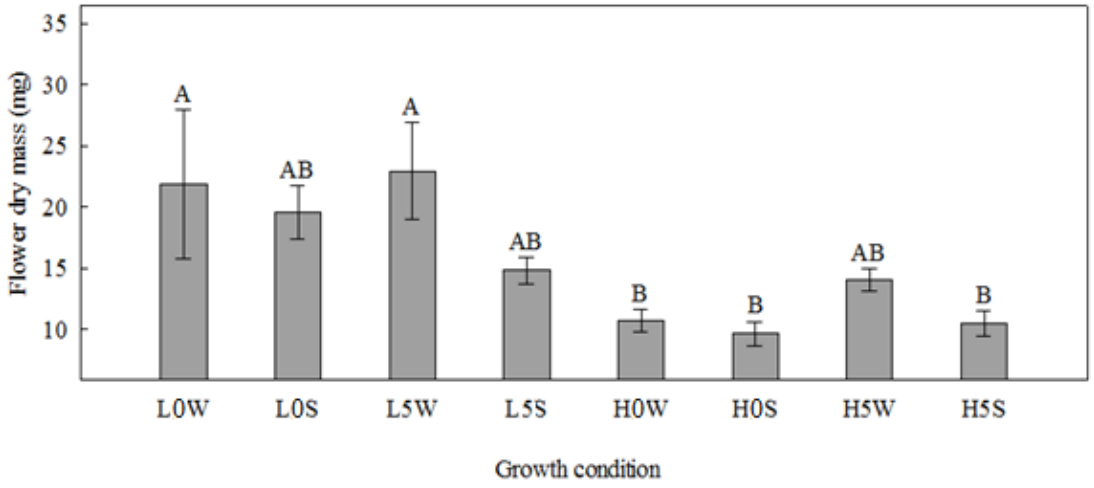

Flower dry mass (mg)
35
30
25
20
15
10
A
AB
A
AB
B
B
AB
B
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

FIG. 6.2

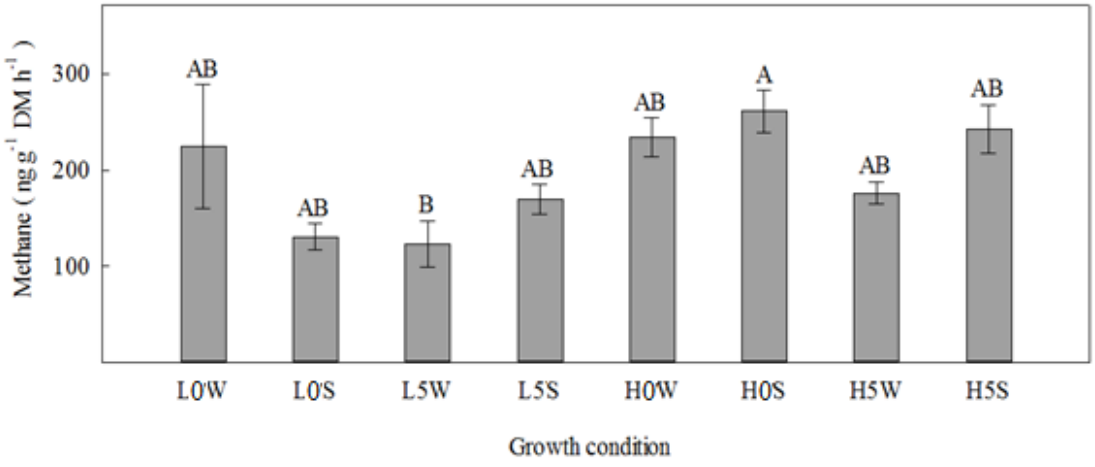

Methane (ng g-1 DM h-1)
300
200
100
AB
AB
B
AB
AB
A
AB
AB
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

FIG. 6.3 Pod dry mass at different ages (1-, 5-, and 10-day-old) of plants grown under eight experimental conditions. Other details are the same as in Fig. 6.1.

FIG. 6.4 Methane emissions from different ages of pod (1, 5, and 10-day-old) of plants grown under eight experimental conditions. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h day/8 h dark), two levels of UVB radiation (0 and 5 kJ m^{-2} d^{-1}) and two watering regimes, after one week of initial growth under 22/18ºC. Bars (mean ± SE) surmounted by different upper-case letters among pod age or by different lower-case letters within pod ages are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. Other details are the same as in Fig. 6.1.

FIG. 6.3

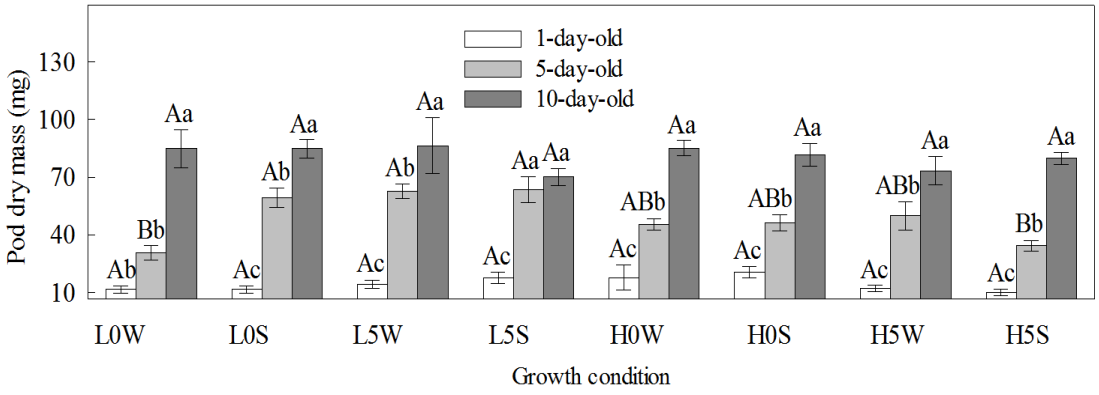
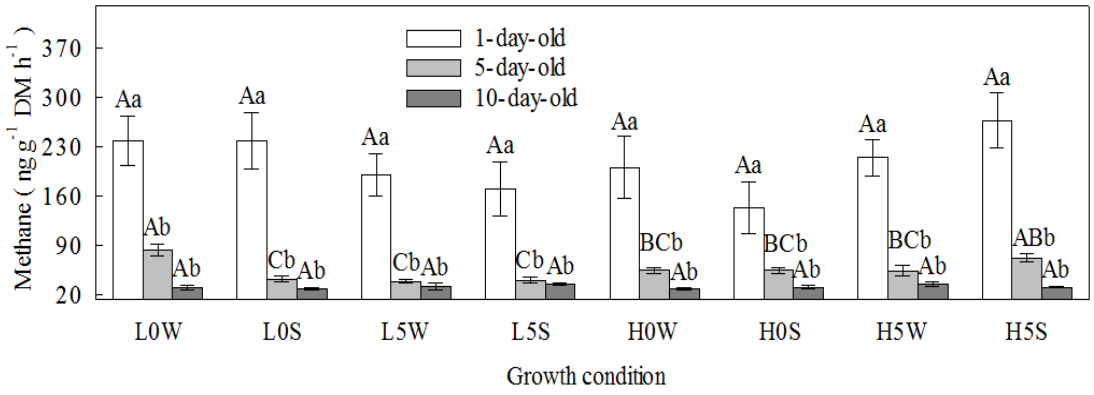

1-day-old
5-day-old
10-day-old
Pod dry mass (mg)
130
100
70
40
10
Aa
Bb
Ab
Ac
Aa
Ab
Ac
Aa
Ab
Ac
Aa
Aa
Ac
Aa
ABb
Ac
Aa
ABb
Ac
Aa
Bb
Ac
Aa
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

FIG. 6.4


1-day-old
5-day-old
10-day-old
Methane (ng g-1 DM h-1)
370
300
230
160
90
20
Aa
Ab
Ab
Aa
Cb
Ab
Aa
Cb
Ab
Aa
Cb
Ab
Aa
BCb
Ab
Aa
BCb
Ab
Aa
BCb
Ab
Aa
ABb
Ab
L0W
L0S
L5W
L5S
H0W
H0S
H5W
H5S
Growth condition

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CHAPTER 7
Conclusion

7.1 Conclusion

Environmental stress factors negatively affect growth and physiological processes of plants. The negative impacts faced by plants manifest as altered phenological, morphological, chemical, and physiological characteristics. High temperature disrupts structural and functional properties of chloroplasts, reduce chlorophyll *a* and chlorophyll *b*, and alters the ratio of chlorophyll/carotenoids, which leads to reduced photosynthesis and, in turn, to decreased biomass. Enhanced UVB radiation can harm living organisms by damaging their proteins, DNA, lipids, and membranes. Drought has similar negative influence on many biological processes; it particularly decreases CO_2 assimilation rates, mainly due to reduction in stomatal conductance (Reddy *et al*., 2004). Furthermore, drought limits photosynthesis because of stomatal closure (Felxas and Medrano, 2002) and decreases chlorophyll content, plant height, stem diameter, total dry mass, relative leaf expansion rate, and leaf elongation (Kirnak *et al*., 2001).

Environmental stress factors, such as UVB radiation, temperature and drought, may cause plants to emit aerobic methane (CH_4) (Qaderi and Reid, 2009; 2011). For the first time, Keppler *et al*. (2006) reported that besides anaerobic microbial processes (Frenzel and Rudolph, 1998), living plants are also a natural source of CH_4 that can emit the gas under natural aerobic conditions. Such a finding may have important implications on the global CH_4 budget. Methane is considered a very potent greenhouse gas, which contributes significantly to global warming (Nisbet *et al*., 2009). Methane is the second most important greenhouse gas after CO_2 and has a global warming potential (GWP) of 34 times that of CO_2 on a 100-year timescale (Myhre *et al*., 2013). Many studies on CH_4 emission from plants have dealt with the effect of a single (Bowling*et al*., 2009) or two (Vigano *et al*. 2008) environmental factors. However, few studies have considered the combined effects of various factors on plants (Qaderi and Reid, 2009; 2011). Our study provides a relatively realistic approach to investigate the effects of co-occurring environmental stress factors, such as temperature, UVB and drought, on CH_4 emissions from plants.

Anaerobic production of CH_4 from microbial sources, particularly from wetlands, isacknowledged as the most prominent source of CH_4 accumulation in the atmosphere

despite wetlands being localized in some regions of the world only. Different research groups from all over the world have independently proved the emission of CH_4 from plants under aerobic conditions and its increase under stress. Natural aerobic production of CH_4 from vegetation is a rather recently discovered phenomenon that exists globally and is not localized. However, since the mechanism by which plants emit CH_4 is not clear, the findings from one plant species cannot be generalized to all species. Recent studies have focused on the origin of CH_4 emission from plants. Pectin was suggested as the source of CH_4 emission from plants because of the large amount of methoxyl groups that it contains (Keppler *et al.,* 2006; Bruhn *et al.,* 2009). Both pectin and lignin are considered precursors of CH_4 in plants because of their unique carbon isotope signatures of methyl groups that associate them with CH_4 emission (Keppler *et al.,* 2008). Vigano *et al.* (2009) reported that in addition to pectin, cellulose and lignin may also be sources of CH_4 emissions from plants, while Bruhn *et al*. (2014) suggested that emissions may result from wax.

The main work that is described in this thesis is concerned with aerobic CH_4 production from plant matter. Methane emission from plants is still a new aspect of study since Keppler and his colleagues published their findings in 2006. The number of papers published in this area until now is around 70 or so thus there is quite some room for many studies to be done to fill the gap in this field of study. Here, the introductory chapter gives the reader an idea about the origin of aerobic CH_4 emissions from plants (Chapter 1). In the first research chapter, our main purpose was to choose specific pea varieties to serve as models for further experiments (Chapter 2). We selected pea (*Pisum sativum* L.) plants because they were one of the best CH_4 emitters among five other crops, including faba bean, sunflower, canola, barley and wheat, used in previous studies (Qaderi and Reid, 2009; 2011). After choosing pea, we tested ten pea varieties – 231E Cascadia, 231C Oregon giant, 237J Sundance, 237M Legacy, UT234 Lincoln, 238A Knight, 236A Paladio, 422 Ho Lan Dow, 234 Lincoln, and 234B Bolero – for CH_4 emission rates. Our finding from this project is that higher temperature and water stress together significantly increase CH_4 emissions from pea and the extent of these emissions varies with variety. 237J Sundance was observed to have highest CH_4 emission, whereas 422 Ho Lan Dow had lowest CH_4 emission. The ten varieties in this investigation have been chosen as

common pea varieties from Nova Scotia, Canada and came from different categories (Table 1, Chapter 1). Having higher rates of CH_4 emission means that the plant is experiencing stress conditions and is more sensitive than other varieties because plants emit CH_4 to alleviate the negative effect of stress factors. The variety 422 Ho Lan Dow was observed to have less CH_4 emission, which means less stress, thus it was hypothesized that 422 Ho Lan Dow should have higher stem height and diameter, leaf area and numbers, total dry mass, transpiration rate, effective quantum yield of PSII, and wax content as compared to 237J Sundance.

Moreover, we were able to characterize CH_4 emission rates from different plant organs (leaf, stem, and root; Chapter 3), different shoot parts (upper, middle, and lower; Chapter 4), as well as different vegetative (10, 20, and 30 days; Chapter 5) and reproductive (pod and flower; Chapter 6) stages. We were very enthusiastic to know the results when we began to address these points. Our initial findings are compiled in Chapters 3 and 4. We found the plant organ that was affected by stress conditions the most, was stem. This is because pea stem is probably low in several protective properties, such as waxy layer, pectin, and chlorophyll content. On the other hand, the presence of these traits in leaves, helps to function as a defense system and facilitate acclimation to the new/stress condition better than stems. In the experiment in hydroponic system, plants were grown under two common stress factors, temperature and UVB radiation (see Chapter 3). As per the results, only higher temperatures increased CH_4 emissions from pea plants, considering one-way ANOVA. However, when UVB5 exposure was combined with higher temperatures, CH_4 emission increased even further (Fig. 3.2). No differences were observed in most of the phenological and morphological parameters studied here under experimental conditions regardless of plant organs. This is attributed to plants receiving enough water, when grown hydroponically, which might have alleviated the negative effects of higher temperatures and UVB5 on plant growth and development.

Experiments with plant shoot revealed that higher temperatures and UVB5 influence plants to emit CH_4 and the rates of emission vary from different plant parts (Chapter 4). A combined application of the three stress factors led to CH_4 emissions being higher from stem as compared to leaf. This result supported our previous findings from the

hydroponic system (see Chapter 3). Also, CH_4 emissions were observed to be highest from the upper parts of the stem as was expected because the shoot's upper parts capture direct light, particularly UVB radiation. It was assumed that upper parts of the plants were more stressed, thus emitted more CH_4. Furthermore, the rate of CH_4 emission was highest in the lower leaves, which could be related to their specific characteristics, such as being old, drier, having less nutrients, and being more sensitive to stress factors. However, no differences were found in other parameters, such as A_N, E, g_s, and chlorophyll fluorescence, among all experimental conditions. Whereas the three-way interaction, T × W × V, was significant for WUE and qP. This interaction showed that higher temperature, UVB5, and water stress decreased WUE, while no changes were observed for qP. Also, the three-way interaction was significant for total chlorophyll and flavonoid content with their values being higher under lower temperature and UVB0 exposure, regardless of watering regime.

In experiments during vegetative stages, CH_4 emissions were measured from plants at diffrent ages (17, 27, and 37 days; see Chapter 5). The results showed that higher temperature, UVB5, and water stress increased CH_4 emissions, on the basis of one-way ANOVA. Methane emission was highest in the earlier stages (17 and 27 days) of plant growth and started to decrease with plant age. Older pea plants (37-days-old) had highest A_N, g_s, E, F_v/F_m, and flavonoid content, when they were exposed to stress conditions as compared to younger plants. This is attributable to old plants being better acclimated to stress conditions over 37-day period of their life, which is enough for them to cope with a new condition and, in turn, release less CH_4. Nonetheless, all these experiments confirm the emission of aerobic CH_4 from plants. Since we were recording CH_4 rates from different vegetative stages of plants, it led us to address questions about the rate of CH_4 emission from different reproductive organs (flower and pod) as well.

Methane emissions from the reproductive organs were addressed in our last experiment (Chapter 6), where the aim was to investigate the rates of CH_4 emission at different reproductive stages: flower (fully opened flower) and pod (1, 5, and 10 days). Pea plants of 237J Sundance variety usually reach the reproductive stage between 30 to 40 days of planting, depending on the growth condition. We found that lower

temperatures increased CH_4 emissions from flowers, whereas none of the main factors had significant effect on CH_4 emissions from pods on the basis of one-way ANOVA. Our results also revealed that one-day-old pods had higher CH_4 emission, whereas 5 and 10-day-old pods had lower. Under a combination of the three factors, CH_4 emissions increased from flowers of plants grown under higher temperatures and water stress, regardless of exposure to UVB radiation. Within pod ages, there were no differences in CH_4 emissions under experimental conditions.

Findings from all these experiments contribute to the field of aerobic CH_4 emissions from plants. Further studies are required to answer the following questions:

1. What are the precursors of aerobic CH_4 in plants?
2. Is the level of aerobic CH_4 emission from plants large enough to be included in the calculation of total global CH_4 budget?
3. What is the most powerful environmental factor that influences plant aerobic CH_4 emission?
4. Is CH_4 emitted through stomata, the epidermis of the shoot, or pores and gaps of the wax cuticle?

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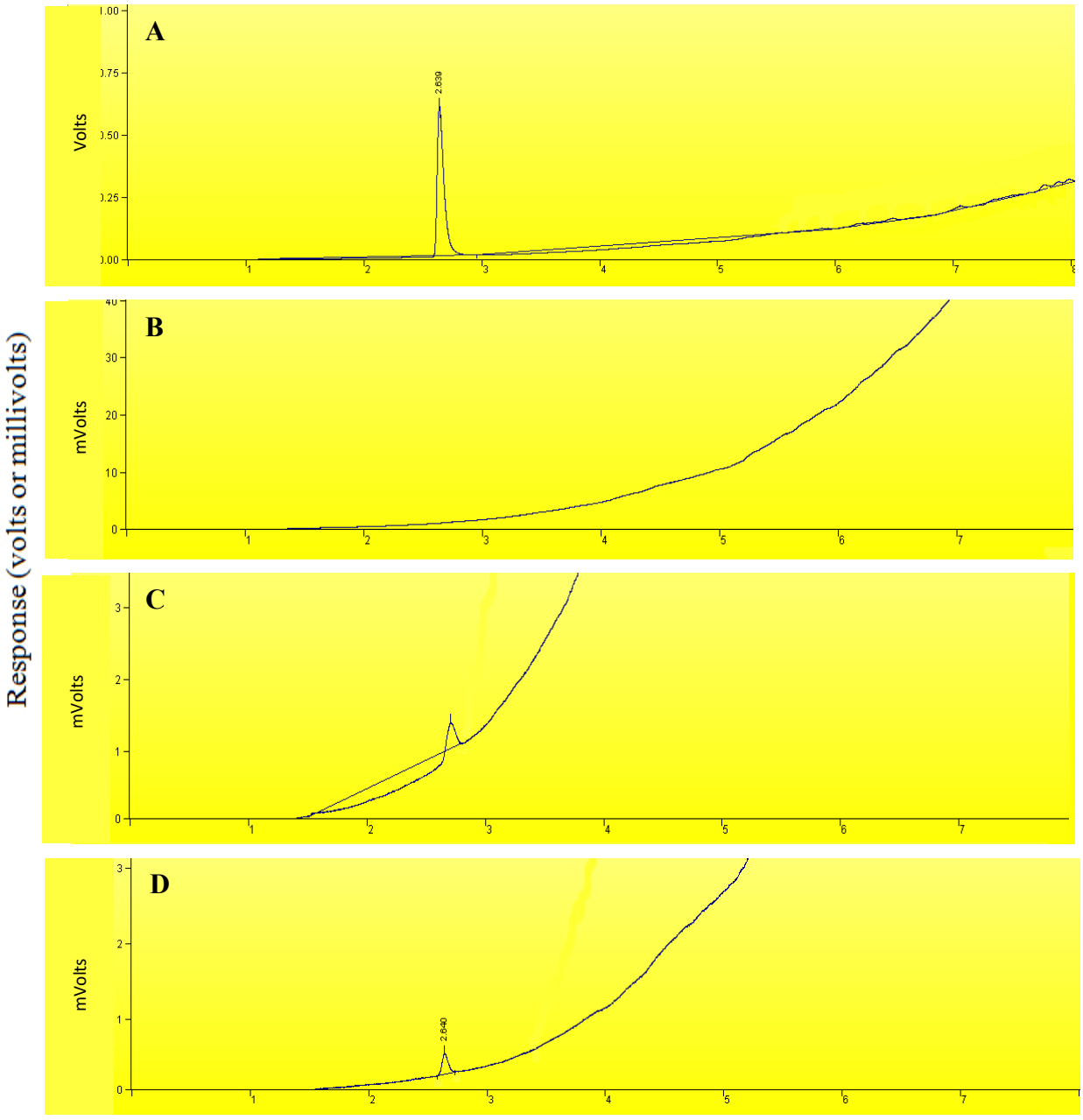
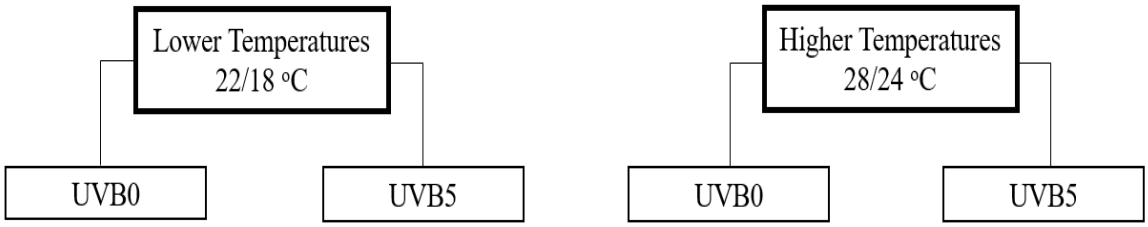
Appendix I

GC-FID (gas chromatography-flame ionization detector) chromatograms. **A**, 5 µml CH_4 gas as standard; **B**, blank injection (no gas was injected); **C**, ambient air from surrounding area in which leaves were incubated in syringes; and **D**, gas from plant sample inc ubated



Appendix II

Experimental design: plants were grown in controlled-environment growth chambers under two temperature regimes (22/18 and 28/24ºC) and two UVB levels (0 (zero), and 5 (ambient) kJ m^{-2} d^{-1}).

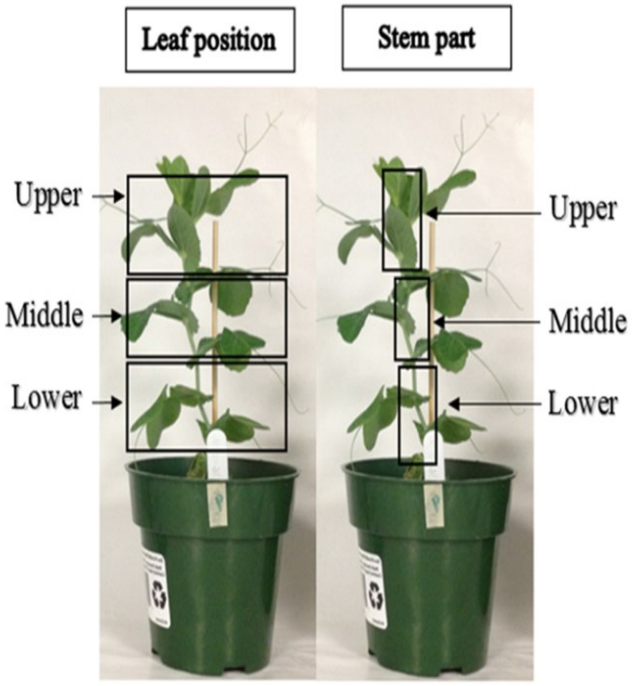


Appendix III

Experimental setup in the hydroponic system. Plants were grown under 22/18ºC for one week before being transferred under experimental conditions.

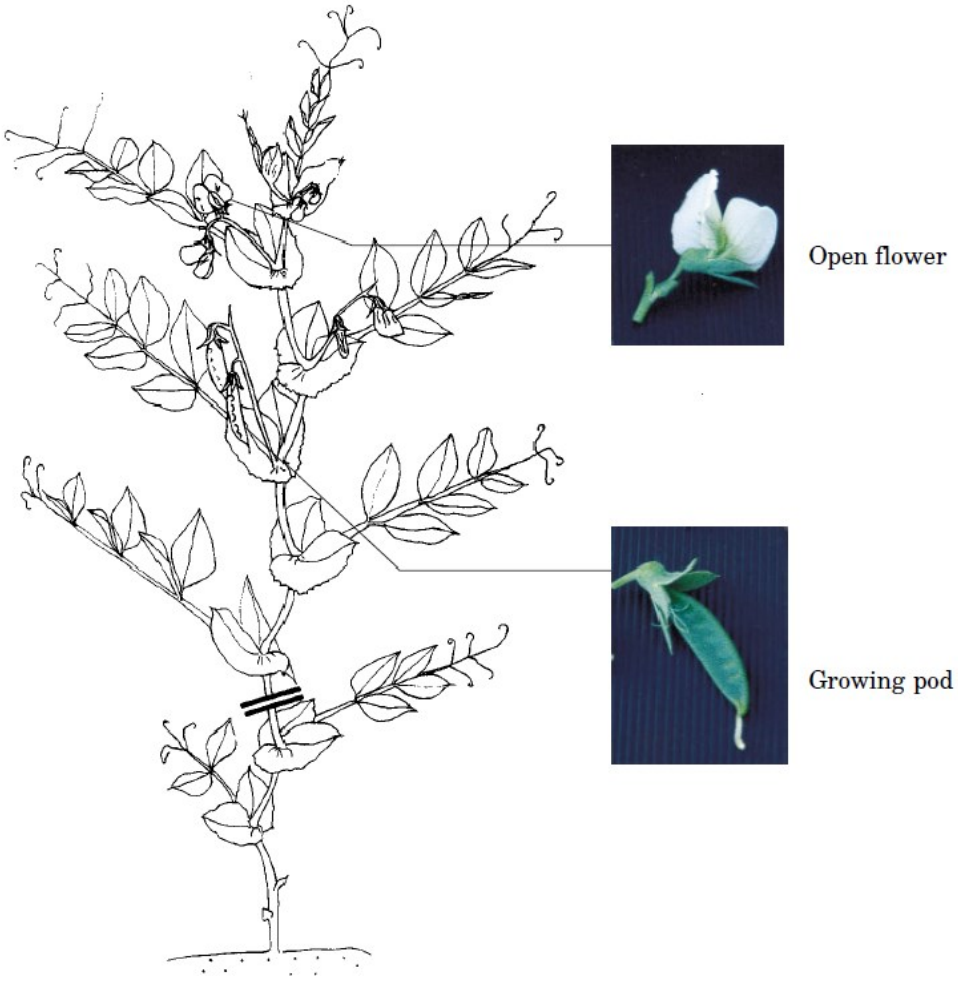
Appendix IV

Picture of one-month-old pea (*Pisum sativum*) plants. Methane emissions were measured from the leaves and stems at three shoot parts (upper, middle, and lower).



Appendix V

Illustration of an open flower and a growing pod in pea (*Pisum sativum*) Guilioni *et al*., 1997.



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Appendix VII

Copyright agreement letter 2

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