CRANFIELD UNIVERSITY

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ANTIVIRAL AND QUALITY EFFECTS OF CHEMICAL ELICITORS AND *CUCUMBER MOSAIC VIRUS* (CMV) INFECTION ON TOMATO PLANTS AND FRUITS

CRANFIELD HEALTH

Plant Science Laboratory

Ph.D THESIS

2012

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Academic Years 2007-2012

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Supervisors: Prof. Leon A. Terry & Dr. Christina Varveri

May 2012

This thesis is submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Abstract

Cucumber mosaic virus (CMV) has emerged as one of the most serious threats to tomato cultivation in Greece. In the present study the effects of Benzothiadiazoles (BTH) and pyraclostrobin against mechanically or aphid-transmitted CMV in tomato plants, of hybrid F1 Clodin, were investigated in greenhouse experiments. BTH was confirmed as capable of inducing systemic acquired resistance (SAR) in tomato seedlings against CMV, while pyraclostrobin was not.

Responses to BTH application and/or CMV inoculation on Spanish tomato hybrid Delos (BTH, BTH+CMV, CMV treatments) were monitored during winter and spring season in Greece. In both seasons the SAR derived from BTH application suppressed CMV. BTH treatment presented increased plant growth, fruit size and marketable tomato yield compared to CMV and BTH+CMV treatments, whereas decreased compared to healthy control. CMV treatment caused the most severe stunting of tomato plants among the examined treatments and resulted in yield loss of marketable fruits, although the total fruit number was higher versus to other treatments.

The nutritional status of tomatoes as defined by nonstructural carbohydrates (NSCs), organic acids and antioxidants content, was not significantly affected by BTH treatment except for lycopene and β-carotene contents which were significantly higher against healthy control fruits. Fruits of CMV treatment ripened significantly later than fruits of all the other treatments and showed significant enhanced antioxidant capacity, ascorbic, lycopene and β-carotene contents compared to healthy control regardless the season of growth. In winter, fruits of BTH+CMV treatment ripened significantly earlier than fruits of healthy control and CMV treatments and had significant reduced fructose and glucose contents and significant increased ascorbic, citric and oxalic acids contents, whereas in spring they had significant reduced citric acid and significant increased antioxidant capacity against healthy control.

The results suggest that repeated foliar application of BTH could be used to reduce CMV incidence in tomato plants. However, the BTH induced resistance showed to affect negatively the tomato plants causing growth inhibition and yield reduction compared to healthy control, and to slightly improve plant growth and fruit size compared to CMV treatment. CMV infected plants produced tomato fruits with remarkable and statistical significant impact upon tomato quality, leading to higher healthful value compared to healthy control.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, *Professor Leon Terry* [Prof. of Plant Sciences, Head of Plant Science Laboratory (PSL) and Head of Food Security and Environmental Health at Cranfield University (CU)], firstly for accepting me into his research group and then for his expertise, understanding and unfailing patience. I am also grateful to my co-supervisor, *Dr. Christina Varveri* [Head of Phytopathology and Researcher in the Laboratory of Virology at Benaki Phytopathological Institute (BPI)] for her useful help, guidance and the assistance she provided to me.

A very special thanks goes out to *Prof. Dimitrios Lydakis* (Emeritus Prof. at the Technological Educational Institute of Crete), without whose motivation I would not have considered of making application to study at CU. I also recognize that this research would not have been possible without the permission of *Dr. Athanasios Alevizatos* (former Head of BPI).

I would like to thank *Msc Rosemary Burns* at CU, *Mrs. Christina Panagiotidi* at BPI, *Mrs. Balwinder Cook* and *Mr.* Allen Hilton for their helpful advice and technical support, as well as the students and staff of PSL for welcoming me and making me feel very comfortable among them. *Dr. Emilia Markellou* at BPI and *Mr. Charles Marshal* at CU are gratefully thanked for statistical advice.

I would also like to extend my sincerest appreciation to *Prof. Naresh Magan*, *Dr. Sarah Morgan* and *Dr. David Aldred* of CU and *Prof. Michael Shaw* of University of Reading for taking time out from their busy schedule to serve as readers and examiners of my 1st year and my final viva respectively.

Last but not least, I am really grateful to my parents *Konstantinos* and *Eleni*, my brother *Zannis* and my sister *Flora* for their tireless support and my best friends for being there for me.

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NOTATION

AA	antimycin A
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACN	acetonitrile
a.i.	active ingredient
AMV	*Alfalfa mosaic virus*
AOX	alternative oxidase
APX	ascorbate peroxidase
ASM	acibenzolar-S-methyl
ATP	adenosine triphosphate
AU	absorbance units
AUDPC	area under disease progress curve
Avr gene	avirulence gene
BABA	*β* Aminobutyric acid
BgMV	*Bottlegourd mosaic virus*
BHT	butylated hydroxytoluene
bp	base pair
BPI	Benaki Phytopathological Institute
BTH	Benzothiadiazoles
BYMV	*Bean yellow mosaic virus*
BYV	*Beet yellows virus*
CaMV	*Cauliflower mosaic virus*
cm	centimeter
CMDV	*Carrot motley dwarf virus*
CMV	*Cucumber mosaic virus*
CN	cyanide
CP	coat protein
CU	Cranfield University
cv.	cultivar
CYDV	*Cereal yellow dwarf virus*
DAA	days after anthesis

DAD	diode array detector
DAS	double antibody sandwich
DDCC	dichlorocyclopropanes
DDPH	2, 2-diphenyl-l-picrylhydrazyl
DCM	dichloromethane
DF	dilution factor
d.f.	degrees of freedom
DHA	dehydroascorbic acid
DNA	deoxyribonucleic acid
dpi	days post inoculation
DTA	days to anthesis
DW	dry weight
EDTA	ethylene-diamine-tetra-acetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector
et al.	and others
FW	fresh weight
GFP	green flurescent protein
GR	glutathione reductase
h	hour
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
HR	hypersensitive response
IC-PCR	immunocapture-polymerase chain reaction
igG	immunoglobulin G
INA	isonicotinic acid
IPM	integrated pest management
ISR	induced systemic resistance
K	potassium
kb	kilo-base
L	litre
LAR	local acquired resistance
LSD	least significant difference

m	metre
min	minute
mg	milligram
mm	millimetre
ml	millilitre
µl	microlitre
ND	non detectable
NDR	natural disease resistance
nm	nanometre
NO	nitric oxide
NSC	non-structural carbohydrate
NSSG	national statistical service of Greece
P	phosphorus
PBS	phosphate buffered saline
PepMV	*Pepino mosaic virus*
PGPR	plant growth promoting rhizobacteria
PMMoV	*Pepper mild mottle virus*
PR proteins	pathogenesis-related proteins
PRSV	*Papaya ringspot virus*
PSL	plant science laboratory
PVP	polyvenylpyrrolidone
PVX	*Potato virus X*
R gene	resistance gene
RdRp	RNA-dependent RNA polymerase
RID	refractive index detector
RIMS	restriction capillary inlet mass spectrometry
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rotations per minute
s	second
SA	salicylate or salicylic acid
SAR	systemic acquired resistance
ScYLV	*Sugarcane yellow leaf virus*

s.d.	standard deviation
s.e.	standard error
SHAM	salicylhydroxamic acid
SL	soluble liquid
SMV	*Soybean mosaic virus*
SOD	superoxide dismutase
SPCSV	*Sweetpotato chlorotic stunt virus*
SPFMV	*Sweetpotato feathery mottle virus*
TCV	*Turnip crinkle virus*
TE	trolox equivalent
TEA	triethylamine
TMV	*Tobacco mosaic virus*
TSWV	*Tomato spotted wilt virus*
TVCV	*Turnip vein-clearing*
TYLCV	*Tomato yellow leaf curl virus*
UK	United Kingdom
UV	ultraviolet
WG	water dispersible granules
WMV2	*Watermelon mosaic virus* 2
w/v	weight by volume
YVMV	*Yellow vein mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

CHAPTER ONE

Introduction

1.1 Project background

Tomato *Solanum lycopersicum* L. Solanaceae is economically one of the most important crops in Mediterranean areas and especially in Greece. On a worldwide scale tomato continues to increase the interest not only for the fresh market but also as component in a variety of processed foods and pharmaceutical products (Rao, 2002; Barba *et al*., 2006; Rao and Rao 2007; Georgé *et al*., 2011). Virus diseases of greenhouse and field tomatoes frequently cause serious damage and large economic loss up to 90% (Balogun, 2008).

Cucumber mosaic virus (CMV) has one of the broadest virus host ranges (Stevenson, 2004) and is one of the most serious pathogens for tomato cultivation in Greece and other countries with a temperate climate (Katis and Avgelis, 1991; Varveri and Boutsika, 1999; Gallitelli, 2000; Kyriakopoulou *et al*., 2000). CMV can result in substantial crop losses, as control of virus diseases is based solely on prevention, and no curative treatment is yet available. Although considerable research has been conducted for inhibitors of virus infection and multiplication, there has not been any result that could offer direct protection to a crop on a commercial scale (Hull, 2002).

Observations made nearly a century ago suggested that plants already infected by a pathogen became more resistant to subsequent infection (Chester, 1933; Ross, 1961 a, b). Today these observations form the basis of an approach to control plant diseases. Plants have evolved many defences that act together to suppress disease. One component is the pathogen-inducible mechanism, systemic acquired resistance (SAR). The induced state is by no means specific, but rather constitutes a more generic increase in plant resistance to various types of pathogen. Moreover, SAR seldom prevents disease from occurring but generally reduces its extent or severity. These characteristics make induced resistance a powerful mechanism to exploit for enhancing the overall resistance in crop plants. Research on SAR using model systems has deepened the understanding of the molecular basis of induced resistance

and promoted the development of synthetic elicitors for use in conventional agriculture.

Elicitors of SAR could potentially revolutionise crop protection. Benzothiadiazoles (BTH), in particular, have been shown to reduce diseases caused by a broad spectrum of pathogens across a diverse range of crops and plant taxa. Regarding viruses, BTH was shown to activate SAR response against *Tobacco mosaic virus* (TMV) in tobacco (Friedrich *et al*., 1996), *Turnip crinkle virus* (TCV) in arabidopsis (Lawton *et al*., 1996), *Tomato spotted wilt virus* (TSWV) in tobacco (Pappu *et al*., 2000; Csinos *et al*., 2001; Momol *et al*., 2001, 2004; Mandal *et al*., 2007, 2008; Nischwitz *et al*., 2008), CMV in tomato (Anfoka, 2000) and in cantaloupe (Smith-Becker *et al*., 2003).

Besides plant activators other compounds have also been shown to suppress disease by inducing SAR. Indeed, strobilurins are an important class of agricultural fungicides with a complex mode of action, which contribute in keeping crops healthy, and improving crop production and quality. There are indications, that pyraclostrobin, a molecule of strobilurins, induces resistance to other pathogens by eliciting plant defence mechanisms. Particularly for viruses, pyraclostrobin has been shown to enhance at some level the resistance of tobacco and tomato against TMV (Herms *et al*., 2002) and CMV (Varveri *et al*., 2006) respectively, when both viruses where mechanically inoculated.

In plants the induced defences are only expressed when and where needed, that is at the site of an infection (Herms and Mattson, 1992). However, systemic induced resistance expresses a form of defence in anticipation of a future pathogen attack, which probably creates a competition or a lack of resource availability. Thus, it has been suggested that the induced resistance in plants may result in an overall cost to their metabolism (Hammerschmidt, 2005). Little information is available on the potential detrimental effect of BTH on plant growth (Terry and Joyce 2000) and even less on fruit quality. In particular there is no information in the literature on the impact of BTH application on sugars or organic acids contents of diverse plants or fruits, except for the ascorbic acid (Skłodowska *et al*., 2010). Little information is available on the effect of BTH on antioxidant capacity in diverse fruits, while more is available concerning vegetation tissues. Hence, most of the published reports about BTH impact on fruit nutritional value have focused on the concentration of potential health-related compounds.

Similarly, during pathogen-plant interactions, host metabolism might be strongly altered (Balachandran *et al*., 1997; Shalatin and Wolf, 2000). So quantifying the impact of a plant activator on plant traits is not simple, as the plant's metabolism is also influenced by the pathogen.

1.2 Aim and objectives of project

1.2.1 Aim

The aim of this project was initially to determine the possible *in vivo* antiviral effect of BTH and/or pyraclostrobin in tomato seedlings. The BTH turned out to be the more effective and therefore it was chosen for further study. The overall aim was focused on CMV and especially on the impact of BTH treatment and/or CMV infection on quantity and quality traits of marketable tomato fruits.

1.2.2 Objectives

Research was carried out with three viruses (CMV, TSWV and *Potato virus Y* - PVY), which are different taxonomically, and thus genetically, to examine whether BTH and/or pyraclostrobin have broad or rather limited antiviral effects, depending on the host-virus combination. In particular the objectives were the following:

1) To investigate BTH and pyraclostrobin efficacy against CMV after aphid transmission or mechanical inoculation in tomato plant-seedlings.
2) To examine if BTH and/or pyraclostrobin, under different incubation periods and number of applications, induce resistance in tomato seed plants to mechanically inoculated TSWV and PVY.

Further work was carried out to investigate the effect of a Greek CMV isolate and/or BTH application on tomato plant and fruit of Spanish hybrid Delos. The objectives were the following:

- To investigate BTH efficacy against CMV in produced tomato fruits under greenhouse cultivation.
- To determine the impact of BTH treatment and/or CMV infection on plant growth, fruit morphology and fruit yield production.

- To determine the impact of BTH treatment and/or CMV infection on quality traits of marketable fruits, as regards to nonstructural carbohydrates (NSCs), organic acids, carotenoids and antioxidant capacity.

1.3 Thesis structure

The thesis is arranged into seven chapters. After a brief background and thesis aims and objectives Chapter 2 comprises a review of existing literature. First, tomato plant, its cultivation requirements and the nutritional status of tomato fruit (regarding carotenoids, NSCs, organic acids and tomato's antioxidant capacity) are described. Then some information about the viruses and their control is given, focusing on CMV, TSWV and PVY. Subsequently, SAR is described, followed by the necessary review covering BTH and strobilurins.

Chapter 3 details the materials and methods used in this study. Chapter 4 describes the experiments aimed at determination of possible antiviral effect of BTH and/or pyraclostrobin. Chapter 5 reports the first experiment to investigate the impact of CMV on tomato fruit quantity and quality traits. In this chapter an evaluation of the methods used for the analyses of NSCs, organic acids, carotenoids and antioxidant capacity in tomato fruits takes place. Chapter 6 describes the experiments including BTH treatment and CMV inoculation, giving information about the impact of these treatments on plant and tomato fruit under different cultivation seasons. Chapter 7 is a general discussion which integrates the results from previous chapters.

CHAPTER TWO

Literature review

2.1 *Solanum lycopersicum* L.

Solanum lycopersicum L., formerly known as *Lycopersicon esculentum* Mill., is the common tomato plant (**Figure 2.1**). It belongs to the family Solanaceae (2n = 24) and it is a native of central, south, and southern north America from Mexico to Argentina (Hobson and Grierson, 1993; Olympios, 2001). Tomato is an important agricultural commodity as it is the second most consumed vegetable worldwide, after potato (Georgé *et al*., 2011). The world production came up to 129,649,883 tonnes in 2008 and specifically for Greece totalled 1,338,600 tonnes based on FAO statistics accessed in August 2010.

Tomato fruits are consumed as a fresh crop, or are incorporated, as a major constituent, in many prepared foods as canned, frozen, preserved or dried foods. Cultivated tomatoes vary in size from cherry tomatoes, about 1-2 cm size like the wild tomato, up to beefsteak tomatoes with 10 cm or more diameter. The most widely grown commercial tomatoes tend to be in the range of 5-7 cm diameter (Olympios, 2001). Tomato fruit can be bilocular or multilocular and is comprised of skin, pericarp, columella and locular contents (**Figure 2.2**). The locular cavities are filled with seeds that are surrounded with jelly parenchyma cells. Tomato dry matter normally varies between 5 and 10%, of which about 75% is soluble, and about 1 to 3% consist of skin and seeds (Shi and Le Maguer, 2000). The balance between water content and the various constituents of ripe tomato fruit depends on many factors such as the genotype, the nutritional treatment, the environment where the plant is grown and, to a minor extent, the nature of the postharvest treatment (Hobson and Grierson, 1993). Nearly half of the total dry matter consists of reducing sugars, principally glucose and fructose (glucose 22%, fructose 25% and sucrose 1%), and about 10 to 13% of organic acids with citric as the dominant one (Shi and Le Maguer, 2000; Yin *et al*., 2010).

A diet including tomato is considered to be healthy for several reasons. They are low in fat and calories, cholesterolfree, and a good source of fibre and protein. In addition, tomatoes are rich in vitamins A, C and K, in carotenoids such as lycopene and β-carotene, and in potassium (Hobson and Grierson, 1993, Stahl and Sies, 1996; Shi and Le Maguer, 2000).

Figure 2.1 Tomato plant (source: Olympios, 2001).

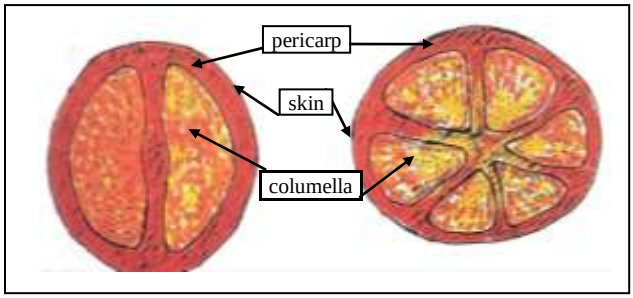

Figure 2.2 Tomato fruits: bilocular (left) and multilocular (right) (source: Olympios, 2001, the labels were inserted). The fruit pericarp was the tissue used for diverse analyses in the current study.

2.1.1 Climatic and soil requirements of tomato plant

The tomato plant as a tropical one needs a sufficiently high temperature to ensure completion of its life cycle and full fruit maturation. The duration of tomato cultivation depends mainly on climatic conditions. Favourable temperatures for its

cultivation are considered the 20-27 °C during the day and 14-20 °C at night (Olympios, 2001). Tomato plants can be grown on many different soil types, but a deep, loamy, well drained, slightly acid soil with a pH of 6.2 to 6.8 and supplied with organic matter and nutrients is the most suitable (Pediaditakis, 1997).

2.1.2 Tomato cultivation in Greece

The tomato was introduced to Greece in 1818, by Friar Francis (Karaoulanis, 1991). The climate of Greece is suitable for successful tomato cultivation, either in field (from spring till autumn) or in greenhouse (during winter). From the geographical areas of Greece, the Peloponnese is the first in tomato cultivation area, whereas Central Greece and Euboea are the first in tomato production.

According to national statistical service of Greece (NSSG) cultivated land with tomato plants was 309,298 acres for the year 2008. From this area, the 128,439 acres were cultivated with industrial tomato and the rest 180,949 with tomato for fresh use. The tomato production of the same year (2008) was 689,011 tonnes of industrial tomato and 649,589 tonnes of tomato for fresh use. According to FAO statistical database the tomato is the fourth most profitable crop in Greece following olive, corn and wheat crops regarding the production and following olive, cotton and vine crops regarding the economic value.

2.2 Carotenoids

Chemically carotenoids are a class of polyunsaturated hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). All carotenoids may be derived from the acyclic $C_{40}H_{56}$ structure having a long central chain of conjugated double bonds by hydrogenation, dehydrogenation, oxidation, cyclization, or any combination of these processes (IUPAC 1974).

Carotenoids are natural pigments that are photosynthesized exclusively by plants and some microorganisms (Shi and Le Maguer, 2000; Rao A. V. and Rao L. G., 2007), so animals need to obtain them from food (Rodriguez-Bernaldo de Quiros and Costa, 2006). Biosynthesis of carotenoids in plants takes place within the plastids, chloroplasts of photosynthetic tissues, and chromoplasts of fruits and flowers (Datta *et al*., 2003). The system of conjugated double bonds lends carotenoids beautiful colours from yellow to red (Graça Dias *et al*., 2007) and also provides them with antioxidant

action by the delocalization of captured free radical species (Baysal *et al*., 2000). Therefore, carotenoids play an important role in human health as they present antioxidant properties which protect the tissues and cells from reactive oxygen species (ROS) and also many carotenoids present provitamin A activity (Baysal *et al*., 2000; Weisburger, 2002; Datta *et al*., 2003; Riso *et al*., 2004; Graça Dias *et al*., 2007). According to epidemiological studies there exists an inverse association between the consumption of foods containing carotenoids and the risk of chronic degenerative diseases such as certain types of cancer, cardiovascular diseases, atherosclerosis, and cataract (Ben-Amotz and Fishler, 1998; Arias *et al*., 2000; Baysal *et al*., 2000; Datta *et al*., 2003).

The majority of carotenoids exhibit absorption between 400 and 500 nm, that is, in the visible region of the spectrum. Moreover, carotenoids obey the Beer-Lambert law, so absorbance measurements can be used to quantify the concentration of a pure carotenoid or to estimate the total carotenoid concentration in a mixture or extract of different carotenoids in a sample (Scott, 2001). Nowadays, carotenoid analysis of food and biological samples is mainly performed by high-performance liquid chromatography (HPLC). It is worth pointing out that these kinds of compounds need a very careful and challenging manipulation due to their chemical lability, as they can easily degrade when exposed to heat, light, and/or oxygen.

2.2.1 Lycopene

Lycopene is the principal pigment responsible for the characteristic red colour of tomatoes (Schofield and Paliyath, 2005). Chemically lycopene is an open-chain hydrocarbon chromophore with eleven conjugated double bonds and has the highest degree of unsaturation among carotenoids (Topal *et al*., 2006; Choudhary *et al*., 2009) (**Figure 2.3**). It occurs in the form of all-trans and cis isomers with the all-trans lycopene being thermodynamically the most stable form (Gupta *et al*., 2010a). In nature, lycopene exists in all-trans form and seven of the eleven bonds can isomerize from the trans form to the mono or poly cis form under the influence of heat, light, or certain chemical reactions (Shi and Le Maguer, 2000). Cis-lycopene is more bioavailable than trans-isomers due to increased solubility in micelles (Karakaya and Yilmaz, 2007).

Lycopene attracts attention due to its biological and physicochemical properties particularly in relation to its effects as a powerful, natural antioxidant that protects against free radical damage of cells (Gupta *et al*., 2010a). Unlike some other carotenoids, lycopene does not have pro-vitamin A properties, but it displays the highest antioxidant capacity compared to them, because it is the most effective on singlet oxygen quenching and on peroxyl radical scavenging (Stahl and Sies, 1996; Shi and Le Maguer, 2000; Rao A.V. and Rao L.G., 2007; Choudhary *et al*., 2009). According to Shi and Le Maguer (2000) there is clinical evidence that characterises lycopene as a micronutrient with important health benefits, moreover, lycopene can be considered as "the vitamin of the twenty-first century" because of its significant physiological effect in the human diet.

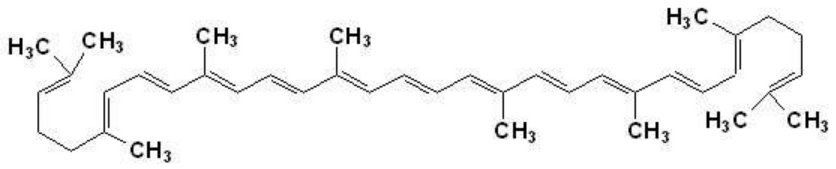

Figure 2.3. Structure of lycopene (source: Rao and Rao, 2007).

2.2.2 β-carotene

Among the more than 600 different carotenoids that have been isolated from natural sources, β-carotene is well known as a precursor (inactive form) and an important source of vitamin A (Stahl and Sies, 1996; Datta *et al*., 2003; Rao A. V. and Rao L. G., 2007). Chemically β-carotene is classified as a terpenoid and is biosynthesized from lycopene after cyclization (Schofield and Paliyath, 2005). It is made up of eight isoprene units, which are cyclised at each end, and has nine conjugated double bonds (**Figure 2.4**).

β-carotene has important industrial applications because it has a dual influence as a natural colour and nutrient for the food and pharmaceutical products (Ronen *et al*., 2000; Shi and Le Maguer, 2000). In particular, β-carotene is a strongly-coloured red-orange pigment and is the molecule that gives carrots their orange colour (Mills *et al*., 2007). It also has antioxidant properties and is an efficient scavenger of peroxyl radicals, especially at low oxygen tension (Stahl and Sies, 1996). As an antioxidant,

β-carotene has a singlet-oxygen-quenching ability, which is almost half than that of lycopene (Shi and Le Maguer, 2000). Lycopene, as already mentioned above, may be the most powerful carotenoid quencher of singlet oxygen (Choudhary *et al*., 2009). Thus, β-carotene has been used for many years as a food colouring agent, as pro-vitamin A in food and animal feed, as an additive to cosmetics and multivitamin preparations (Ben-Amotz and Fishler, 1998; Ronen *et al*., 2000).

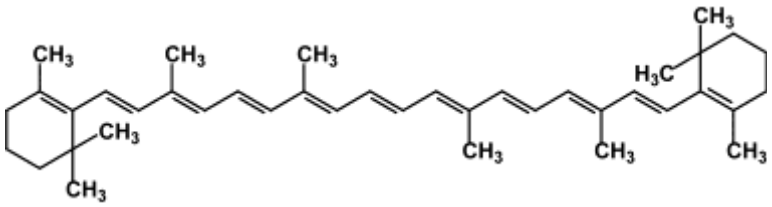

Figure 2.4. Structure of β-carotene (source: Rao and Rao, 2007).

2.2.3 Carotenoids in tomato fruit

There are two main carotenoids that accumulate in ripe tomato fruits lycopene (~90%) and β-carotene (5–10%) (Gould, 1974; Alba *et al*., 2000; Arias *et al*., 2000; Darrigues *et al*., 2008). There is also a small amount of lutein (1-5%) and other carotenoids in trace amounts (< 1%) (Schofield and Paliyath, 2005).

Tomato is the principal model system for studies especially of lycopene assay methods and generally of carotenoid biosynthesis due to many colour changes that occur during fruit development (Arias *et al*., 2000; Choudhary *et al*., 2009). In the early stages the chlorophyll imparts a green colour to fruits. When the ripening process starts, the chlorophyll is degraded and carotenoids are synthesized, mainly consisting of β-carotene, lutein, and violaxanthin. At the next stage of ripening, called the "breaker stage", lycopene begins to accumulate. During this process β-cyclase, the enzyme that converts lycopene to β-carotene, disappears and lycopene concentration increases dramatically between the stage pink and the stage red fruit (Arias *et al*., 2000; Ronen *et al*., 2000).

Undoubtfully, the colour of tomatoes is one of the most important marketing factors that affect the buying decision of the consumer. Lycopene contributes to the characteristic deep-red colour of tomatoes. Additionally, tomatoes and related tomato products, are considered as an important source of dietary lycopene and β-carotene

(Shi and Le Maguer, 2000; Schofield and Paliyath, 2005; Gupta *et al*., 2010a), so their content greatly influences the quality of ripe tomato fruit. The fresh tomato fruits have a wide range of lycopene concentration, which varies from 30 to 200 mg/kg on a fresh basis (FW) and from 430 to 2,950 mg/kg on a dry basis (DW) (Topal *et al*., 2006). Most of the lycopene is attached to the insoluble and fibrous parts of the tomatoes, as the skins can contain about five times more lycopene (540mg/kg on FW) than the tomato pulp (110mg/kg) (Dumas *et al*., 2003; Topal *et al*., 2006). The large variation of lycopene content of tomato indicates that its accumulation depends on many factors, like tomato variety, maturity at the harvesting stage, the environmental conditions under which the fruit was developed and technical processes for extraction and assay (Scott *et al*., 1996; Stahl and Sies, 1996; Datta *et al*., 2003; Dumas *et al*., 2003; Bicanic *et al*., 2005; Georgé *et al*., 2011). Moreover, carotenoid accumulation in ripening fruits is affected by environmental factors both before and after harvest.

Lycopene in fresh tomato fruits occurs mainly in the all-trans form, which is the most prominent isomer (Stahl and Sies, 1996). The main causes of tomato lycopene degradation during processing and storage are isomerization and oxidation. Isomerization of all-trans-isomers to cis-isomers results in an unstable, energy-rich state due to additional energy input (Shi and Le Maguer, 2000). Degradation of lycopene is undesirable because it direct affects the quality and the nutritional status of tomato products.

2.3 Sugars in tomato fruit

Sucrose is a disaccharide molecule with the molecular formula $C_{12}H_{22}O_{11}$, composed of glucose and fructose (**Figure 2.5**). Fructose and glucose are simple monosaccharides ($C_6H_{12}O_6$) found in many plants. Glucose is an important carbohydrate in biology, as cells use it as the primary source of energy and a metabolic intermediate. In particular, glucose is one of the main products of photosynthesis and starts cellular respiration.

In tomato fruit 95% of the sugars content is in the form of glucose and fructose (Davies and Kempton, 1975; Haila *et al.*, 1992; Young *et al.*, 1993; Herrmann, 1998). Glucose and fructose are the breakdown products of sucrose and their levels increase in tomato fruits during the whole period of fruit setting and ripening (Kortstee *et al*., 2007). On the contrary, sucrose is not detected (Loiudice *et*

al. 1995; Hernández Suárez *et al*. 2008 b and c) or sometimes is detected in low amounts (Davies and Kempton, 1975; Haila *et al.*, 1992; Herrmann, 1998; Velterop and Ros, 2001). The sugars concentrations depend on many factors such as the examined tomato variety, the analysed tissue and the stage of fruit ripening or the used quantification method. This is the reason for the observed wide differences in sugars contents in tomato fruit presented in **Table 2.1**.

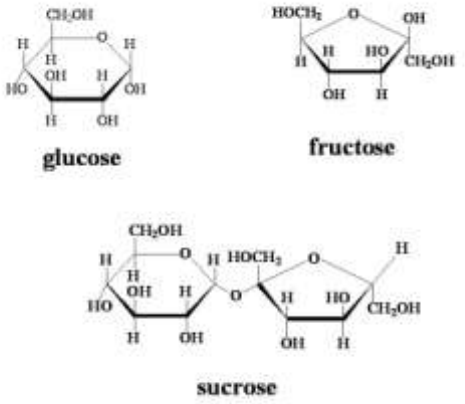

Figure 2.5. Structures of glucose, fructose and sucrose (source: Pinna, 2011).

Table2.1. Sucrose, fructose and glucose contents in fruit of diverse tomato varieties on fresh weight (FW) basis.

Tomato variety	Quantification method	Analysed tissue	Sucrose mg g^{-1}	Fructose mg g^{-1}	Glucose mg g^{-1}	Source
Momotaro	HPLC RID*	whole homogenized ripe tomato	-***	17	15	Islam *et al*., 1996
Naomi F1 cherry	HPLC	whole homogenized ripe tomato	-	17.9	18.6	Raffo *et al*., 2002
Aranca	HPLC RID	whole tomato at breaker stage**	0.008	0.020	0.022	Beullens *et al*., 2006
Climaks	HPLC RID	whole tomato at breaker stage	0.006	0.013	0.012	Beullens *et al*., 2006
Clotilde	HPLC RID	whole tomato at breaker stage	0.004	0.015	0.017	Beullens *et al*., 2006
DRW73-29	HPLC RID	whole tomato at breaker stage	0.004	0.012	0.013	Beullens *et al*., 2006
Cambria	capillary zone electrophoresis	whole homogenized tomato	1.57	12.17	13.82	Galiana-Balaguer *et al*., 2006
FLA7060	capillary zone electrophoresis	whole homogenized tomato	ND****	18.79	19.87	Galiana-Balaguer *et al*., 2006
Genova	capillary zone electrophoresis	whole homogenized tomato	4.35	14.17	13.74	Galiana-Balaguer *et al*., 2006
Tricia	HPLC RID	whole homogenized tomato	ND	2	1.81	Vermeir *et al*., 2007
Bonaparte	HPLC RID	whole homogenized tomato	ND	2.32	2.46	Vermeir *et al*., 2007
Clotilde	HPLC RID	whole homogenized tomato	ND	2.43	2.28	Vermeir *et al*., 2007
Dorothy	HPLC RID	pericarp and seed purée	-	17.96	17.08	Hernández Suárez *et al*., 2008c
Boludo	HPLC RID	pericarp and seed purée	-	21.5	19.7	Hernández Suárez *et al*., 2008c
Dominique	HPLC RID	pericarp and seed purée	-	20.81	19.84	Hernández Suárez *et al*., 2008c
Thomas	HPLC RID	pericarp and seed purée	-	19.7	18.51	Hernández Suárez *et al*., 2008c
Tyna	HPLC RID	pericarp and seed purée	-	23.54	21.86	Hernández Suárez *et al*., 2008c
Daniela	HPLC RID	pericarp and seed purée	-	15.11	12.94	Hernández Suárez *et al*., 2008c
Moneymaker	HPLC	whole homogenized tomato	-	18	15	Luengwilai *et al*., 2010

*RID: refractive index detector
**breaker stage: fruits were about to change colour
***-: no tested
***ND: non detectable

2.4 Organic acids in tomato fruit

Organic acids have functional group -COOH and a great biological value. They are parts of different biological routes, among which the most important is the Krebs cycle. Moreover, organic acids are important because of their influence in organoleptic properties like flavour and aroma (Mato *et al*., 2005) and in fruit pH as well. The major organic acids of tomato fruit are citric and malic, followed by ascorbic, tartaric, oxalic, pyruvic, maleic, fumaric and succinic (Hernández Suárez *et al*., 2008). In the current study only citric, ascorbic and oxalic acids (**Figure 2.6**) were accurately identified using retention times and spectral data of HPLC analysis.

There are many different methods for quantification of organic acids. **Table 2.2** shows results referring to ascorbic, citric and oxalic acids of diverse tomato varieties. The range of values given in literature is wide even for measurements made from the same researcher. According to Dumas *et al*. (2003) the ascorbic acid content range from 0.084 to 0.59 mg g^{-1}, while Hernández Suárez *et al*. (2008a) noted that citric acid's content in tomato fruit range from 2 to 8.5 mg g^{-1}, oxalic's from 0.06 to 0.58 mg g^{-1} and ascorbic's from 0.06 to 0.3 mg g^{-1} on FW basis.

In biochemistry citric acid is important as an intermediate in the citric acid cycle (Krebs cycle) and therefore occurs in metabolism of all living organisms. The citric acid content in tomato fruit increases only slightly during ripening (Picha, 1987; Velterop and Vos, 2001).

The biologically-active isomer of ascorbic acid (vitamin C) is L-ascorbic acid, while D-ascorbic acid (isoascorbic acid) has only 5% of the antiscorbutic effect of ascorbic (Novakova *et al*., 2008). Ascorbic acid is rapidly oxidized to dehydroascorbic acid (DHA) due to the presence of two hydroxyl groups in its structure. Oxidation reactions can be induced by exposure to increased temperatures, high pH and light, presence of oxygen or metals and enzymatic action. This reaction is reversible and a principal step in the antioxidant activity of ascorbic acid. Ascorbic acid cannot be synthesized by the human body, so vegetables and fruits are the major sources of vitamin C for the human diet. The ascorbic content of tomato has been shown to provide a significant contribution to dietary intake. The ascorbate concentration increases, when the tomato ripens, but declines slightly in overripe tomato (Islam *et al*., 1996; Hernández Suárez *et al*., 2008).

Oxalic acid is a dicarboxylic acid and typically in solution occurs as the dihydrate ($C_2O_4H_2.2H_2O$). It is a reducing agent as well as a chelating agent for metal cations, so it forms insoluble salts with calcium and other divalent cations. Thus, from a nutritional point of view, oxalic is an undesirable compound in human nutrition due to its tendency to diminish calcium bioavailability (Guil *et al*., 1996; Hernández Suárez *et al*., 2008). Generally there is a low level of oxalic acid in tomato, so its consumption is not considered as decalcifying (Guil-Guerrero *et al*., 2009).

The soluble solid content in tomatoes is due to reducing sugars and acids. The higher amount of sugars and organic acids a tomato contains, the higher flavour intensity will be perceived (Stevens *et al*., 1977; Bucheli *et al*., 1999; Galiana-Balaguer *et al*., 2006). According to Hernández Suárez *et al*. (2008) the concentration of organic acids in tomato fruit fell with over-ripening, and this fact could be due to the use of organic acids as respiratory substrates or their conversion to sugars during ripening.

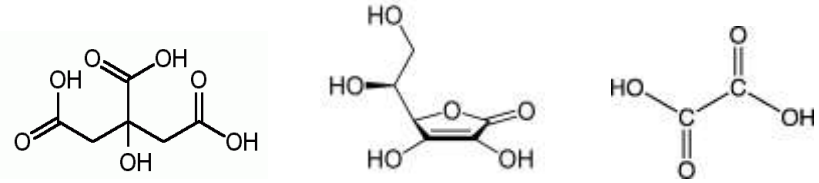

Figure 2.6. Structures of citric, ascorbic and oxalic acids (source: Wikipedia).

2.5 Antioxidant capacity of tomato fruit

The nutrient quality, the colour, even the taste of tomatoes can also depend on their antioxidant contents. The antioxidant capacity of tomato fruit is mainly derived from lycopene, secondly from other carotenes (β-carotene, γ-carotene), vitamins A, C, and E and lutein, phytoene, phytofluene and phenolics (flavonoids, hydroxycinnamic acids) (Wang *et al*., 1996; Martinez-Valverde *et al*., 2002; Dumas *et al*., 2003; Chang and Liu, 2007).

The antioxidant content of tomatoes depends on genetic, on environmental factors (temperature, light, water availability, nutrient availability), on agricultural techniques (for instance the use of plant growth regulators, date of harvest, etc) (Dumas *et al*., 2003) and on the post-harvest storage conditions (Odriozola-Serrano *et al*., 2009). Giovanelly *et al*. (1997) reported that post-harvest ripened tomatoes are richer in antioxidants than vine-ripened tomatoes.

Table2.2. Citric, oxalic and ascorbic acids contents in fruit of diverse tomato varieties on fresh weight (FW) or dry weight (DW) basis.

Tomato variety	Quantification method	Analysed tissue	Basis	Citric mg g^{-1}	Oxalic mg g^{-1}	Ascorbic mg g^{-1}	Source
Momotaro	HPLC UV-VIS	whole homogenized tomato	FW	-**	-	0.17	Islam *et al*., 1996
Naomi F1 cherry	HPLC	whole homogenized tomato	FW	6.7	0.5	0.11	Raffo *et al*., 2002
Allflesh	HPLC	peeled homogenized tomato	DW	21.94	0.37	5.40	Rotino *et al*., 2005
UC82	HPLC	peeled homogenized tomato	DW	14.34	0.36	4.46	Rotino *et al*., 2005
Ri4	HPLC	peeled homogenized tomato	DW	22.71	0.36	4.37	Rotino *et al*., 2005
Ri5	HPLC	peeled homogenized tomato	DW	18.37	0.23	3.74	Rotino *et al*., 2005
Aranca	HPLC DAD	whole tomato at breaker stage*	FW	0.011	-	-	Beullens *et al*., 2006
Climaks	HPLC DAD	whole tomato at breaker stage	FW	0.018	-	-	Beullens *et al*., 2006
Clotilde	HPLC DAD	whole tomato at breaker stage	FW	0.019	-	-	Beullens *et al*., 2006
DRW73-29	HPLC DAD	whole tomato at breaker stage	FW	0.015	-	-	Beullens *et al*., 2006
Cambria	capillary zone electrophoresis	whole homogenized tomato	FW	2.79	0.24	-	Galiana-Balaguer *et al*., 2006
FLA7060	capillary zone electrophoresis	whole homogenized tomato	FW	1.81	0.39	-	Galiana-Balaguer *et al*., 2006
Genova	capillary zone electrophoresis	whole homogenized tomato	FW	4.34	0.27	-	Galiana-Balaguer *et al*., 2006
HyPeel45 conv	UV-VIS spectrophotometer	raw tomato juice	DW	-	-	1.19	Burret *et al*., 2007
Rogers 1570	UV-VIS spectrophotometer	raw tomato juice	DW	-	-	1.74	Burret *et al*., 2007
Bos315	UV-VIS spectrophotometer	raw tomato juice	DW	-	-	0.86	Burret *et al*., 2007
HM830	UV-VIS spectrophotometer	raw tomato juice	DW	-	-	1.37	Burret *et al*., 2007
Tricia	HPLC DAD	whole homogenized tomato	FW	0.48	-	-	Vermeir *et al*., 2007
Bonaparte	HPLC DAD	whole homogenized tomato	FW	0.36	-	-	Vermeir *et al*., 2007
Clotilde	HPLC DAD	whole homogenized tomato	FW	0.55	-	-	Vermeir *et al*., 2007
Dorothy	HPLC	whole homogenized tomato	FW	3.4	0.25	0.15	Hernández Suárez *et al*.,2008a
Boludo	HPLC	whole homogenized tomato	FW	3.89	0.26	0.16	Hernández Suárez *et al*.,2008a
Dominique	HPLC	whole homogenized tomato	FW	3.53	0.25	0.14	Hernández Suárez *et al*.,2008a
Thomas	HPLC	whole homogenized tomato	FW	3.22	0.29	0.16	Hernández Suárez *et al*.,2008a
Dunkan	HPLC	whole homogenized tomato	FW	3.51	0.24	0.15	Hernández Suárez *et al*.,2008a
Overall	HPLC	whole homogenized tomato	FW	3.54	0.26	0.15	Hernández Suárez *et al*.,2008a

*breaker stage: fruits were about to change colour
**-: no tested

2.6 Plant viruses and their control

2.6.1 Plant viruses

Plant virus disease incidence can range from a few scattered plants in a field to total crop failure (loss 90-100%), depending on many factors (Balogun, 2008). Some of these factors are the particular virus or combination of viruses present, the virulence of the virus strains, the presence of other diseases and the susceptibility of the plant variety. Moreover, the timing of infection and the age of the plant at infection time, the abundance of insect vectors, and environmental conditions play a crucial role in virus disease incidence (Matthews, 1991; Taiwo and Akinjogunla, 2006; Balogun, 2008).

Viruses are transmitted from plant to plant by vectors or by mechanical means. Once established in a susceptible host, viruses propagate outwards from inoculated cells to all parts of the plant. There are two distinct phases of virus movement; slow cell-to-cell movement via plasmodesmata in inoculated leaves, and faster long-distance movement from leaf to leaf through the vasculature, usually the phloem (Carrington *et al*., 1996; Nelson *et al*., 1998). Both movement processes utilize existing plant transport and communication routes as well as the assistance of host factors (Lucas, 1995). So, a plant defence strategy aimed at viruses could conceivably target any one or all of these processes: replication, trans-plasmodesmatal movement or transport through the vasculature.

Viral diseases affect tomato production to some degree nearly every year and the majority of them survive only in living plants or briefly in insects. CMV is one of the most economically important, and is a common virus observed all over Greece (Varveri and Boutsika, 1999).

2.6.2 Control of diseases caused by viruses

To this day, virus diseases cannot be controlled once the plant is infected, as no curative treatment is available. Although considerable effort has gone into searching for inhibitors of virus infection and multiplication that could be used to give direct protection to a crop against virus infection, there has been no successful control on a commercial scale (Hull, 2002). Therefore, every effort should be made to prevent introduction of virus diseases into the plants. There is a range of control measures

aimed at trying to mitigate losses caused by viruses. Sanitation is the primary means of controlling virus diseases, for example seed procurement from reputable sources, decontamination of tools etc. Moreover, primarily infected plants should be removed and destroyed immediately to prevent spread of the pathogens. Furthermore, perennial and annual weeds, which may serve as alternate hosts, should be controlled. Finally, insecticides should be used early in the season to reduce initial infection and spread by controlling insect-vector populations.

In particular, control of viruses in tomato should be based on an integrated pest management (IPM) approach on an annual basis. Growers should be aware of the precautions necessary to reduce virus incidence and ward off to a certain extent the danger of virus diseases being a routine problem in tomato plantings. They should rotate tomato crops with small grain, corn, or pasture, and avoid planting tomatoes close to established cucurbits, potato, tobacco, eggplant, and pepper crops that suffer from the same virus pathogens.

Even if sources of infection are available, and the vectors are active, there is one more kind of control measure available termed “cross-protection” or “mild strain protection” (Hull, 2002). It has been demonstrated that infection of a plant with a pathogen may protect it from a following infection (Chester 1933; Ross 1961; White 1979). Thus, plants might be purposely infected with a mild strain of a virus as a protective measure against severe strains. This kind of protection in essence emerged from SAR, but it is not to be recommended as a general practice, for two main reasons. Firstly, a serious disease may result from mixed infection when an unrelated virus is introduced into the crop and secondly, the genome of the mild strain may recombine with that of another virus, leading to the production of a new virus (Urban *et al*., 1990; Lecop, 1998). There is research investigating different methods of SAR application, by chemicals that activate the natural defences of plants, as an alternative way to prevent plant virus diseases.

2.6.3 *Cucumber mosaic virus* (CMV)

2.6.3.1 Taxonomy

CMV belongs to the family Bromoviridae, genus *Cucumovirus* and is an important crop pathogen. It was firstly reported in *Cucumis sativus*, from the U.S.A, by Price (1934). It is widespread virus existing in numerous strains and it is now distributed worldwide.

2.6.3.2 Morphological and physicochemical characteristics

CMV is a (+) sense RNA plant virus with a tripartite genome of about 8,650 nucleotides. It has three functional pieces of single-stranded RNA (ssRNA), packaged in three icosahedral, isometric, not enveloped particles about 28-30 nm in diameter (**Figure 2.7**) without a conspicuous capsomere arrangement (Francki *et al*., 1979; Palukaitis *et al*., 1992; Conti *et al*., 1996; Agrios, 2005). All three components of its genome, called RNA1, RNA2 and RNA3, must be present to cause infection. The capsid is composed of 180 copies of a single protein species of about 24 kDa (Hull, 2002). The molecular weight of CMV ranges between 5.8 and 6.7 × 10^6 Da, of which 18% is RNA and 82% protein (Francki *et al*., 1979; Agrios, 2005).

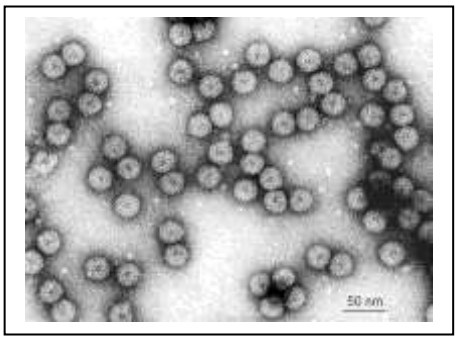

Figure 2.7. CMV particles (source: virology team of Scottish Crop Research Institute).

2.6.3.3 Host range and virus transmission

CMV has an extensive host range, maybe the largest of any plant virus, infecting over 1,200 species in more than 101 plant families (Stevenson, 2004). Actually, CMV disease affects a number of important horticultural crops including principally solanaceous, cucurbits and legumes. Furthermore, CMV affects ornamentals and many weeds (Huang *et al*., 1987; Chabbouh and Cherif, 1990; Freeman and Horsham, 2006).

The basic vectors of CMV are aphids, which are insects of the order Hemiptera, suborder Homoptera, and family Aphididae. More than 75 aphid species are capable of transmitting the virus in the non-persistent manner. Generally, the virus is acquired by the aphid within one minute of feeding on an infected plant, and without any latency period the virus is transmitted in less than one minute to other healthy plants in the same way, but the aphid's ability to transmit the virus quickly declines and is lost within several hours (Francki *et al*., 1979). The main species reported as vectors are: *Myzus persicae* (**Figure 2.8**), *Acyrthosiphon pisum*, *Aphis gossypii*, *A. craccivora*, *A. fabae*. Transmission efficiency varies with aphid species, virus strain, host plant species, environmental conditions, and time of year. CMV is also transmitted by mechanical means. This virus cannot withstand drying or persist in the soil and in crop debris for long. It is not seed-borne in tomato but can be carried in the seed of 19 other plant species (Sikora, 2004).

Figure 2.8. M*yzus persicae* measures about 1.5 to 2.0 mm in length (source: Holopainen). This species was used for virus transmission in the current study.

2.6.3.4 Symptomatology of CMV on tomato plants

Certain strains of CMV cause partial or total loss in crop depending on tomato variety, and there are strains that have been reported as specific for tomato. Tomatoes infected with CMV develop a slight yellowing mosaic and mottling (intermingling of

dark green, light green and yellow tissue) leaves (**Figure 2.9**). Expanding leaves typically become twisted and curl downward, and leaflets are often much narrowed and develop a "shoestring" appearance as a result of a restriction of the leaf surface around the midrib of the leaf. Diseased tomato plants are often bushy (shortened internodes), stunted, and dwarfed. The fruits produced mature unevenly, are few, usually small, misshaped, covered with bumpy protrusions, or have brown necrotic areas in their interior side (**Figure 2.10**) (MacNab *et al*., 1983; Conti *et al*., 1996; Šutić *et al*., 1999; Gallitelli, 2000; Cerkauskas, 2004; Malathrakis *et al*., 2007). However, the severity of disease may range from non obvious symptoms, to death of tomato plant.

Figure 2.9. Tomato plant infected with CMV showing the characteristic mosaic on leaves (source: Malathrakis *et al*., 2007).

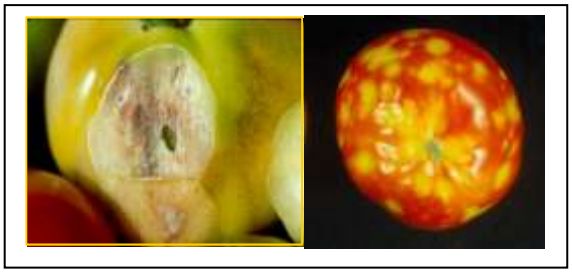

Figure 2.10. Tomato fruits infected with CMV (source: Zitter and Murphy, 2009).

2.6.3.5 Biochemical changes in CMV infected tomato plants and fruits

There is little information in the literature about the impact of CMV infection exclusively on biochemistry of tomato plants and fruits. Afeal *et al*. (1996) investigated the biochemical changes in tomato plants that had been infected with CMV. The infected plants did not show any change in the moisture content, but they showed significant differences in other measured parameters. The levels of reducing sugars, free amino acids and proteins were significantly higher in CMV infected tomato plants compared to the control. They also noticed that the longer the plants were infected, the steeper increase in these parameters was observed. Akanda *et al*. (1998) showed that leaves of CMV infected tomato plants had reduced concentration of chrorophyll and β-carotene versus healthy leaves.

Another research study was conducted by Georgieva *et al*. (2000) to examine the metabolic changes in tomato fruits infected with CMV. They found that peroxidase activity and glucose-6-phoshate dehydrogenase activity were enhanced by CMV infection. These results were expected by the authors, as the former enzyme is an important component of plant defence responses, and the latter is necessary for the synthesis of the virus's RNA.

2.6.4 *Tomato spotted wilt virus* (TSWV)

2.6.4.1 Taxonomy

TSWV belongs to the family Bunyaviridae, genus *Tospovirus*. It was first observed in Australia in 1915 (Brittlebank, 1919) and was later shown to have viral etiology (Samuel *et al*., 1930). Subsequently this virus was reported from many other regions and is now world-wide distributed.

2.6.4.2 Morphological and physicochemical characteristics

TSWV is a pleomorphic (–) sense RNA plant virus. Its genome consists of three single-stranded, linear RNA molecules (ssRNA) encapsulated by a multi-protein coat, with total size approximately 17,200 nucleotides. All three RNAs, called L, M and S are required for infectivity, and their molecular weights are 3.1, 1.6 and 0.9 × 10^6 Da respectively (Conti *et al*., 1996). TSWV has quasi-spherical enveloped virions particles, about 80-120 nm in diameter, rounded in profile without a conspicuous

capsomere arrangement. The virions consist of 5% nucleic acid, 70% protein, 20% lipid and also 5% carbohydrate (Scott, 2000).

2.6.5 *Potato virus Y* (PVY)

2.6.5.1 Taxonomy

PVY belongs to the family Potyviridae, and is the type member of the *Potyvirus* genus. It was firstly reported in *Solanum tuberosum* from the U.K. by Smith (1931) it occurs worldwide and apart from potato it can cause serious diseases in other solanaceous and leguminous crops.

2.6.5.2 Morphological and physicochemical characteristics

PVY is a (+) sense RNA plant virus, and its unipartite genome consists of single-stranded, linear RNA (ssRNA), with total size 9,700 nucleotides (Makkouk and Gumpf, 1974; Hinostroza-Orihuela, 1975; Conti *et al*., 1996). It has filamentous, not enveloped, flexuous virion particles, with a clear modal length of 680-740 nm × 11-15 nm wide. The particles contain approximately 5% nucleic acid and 95% protein (Leiser and Richter, 1979; Hollings and Brunt, 1981).

2.7 Systemic acquired resistance (SAR)

2.7.1 Systemic acquired resistance as a plant natural resistance mechanism

Plant natural resistance to potential parasites is regulated by two fundamental mechanisms: the "nonhost" which is triggered by a general inherent capability of the plant to act as nonhost and the "gene-for-gene" (Flor, 1971) resistance which is triggered after some interaction between the products of plant and pathogens genes. For the scope of this study, only the latter case of "gene-for-gene" response is relevant, when a cultivar resistance (R) gene product recognizes an avirulence one in the attacking pathogen and triggers an array of biochemical reactions that halt the pathogen around the site of attempted invasion (Richberg *et al*., 1998; Gozzo, 2003).

Natural disease resistance (NDR) is activated after lesion formation, and is a variety of biochemical responses that often lead to an enhanced, acquired resistance around the sites of attempted penetration. There are three forms of NDR: the local acquired resistance (LAR) in the tissue surrounding the primary infection site, the systemic acquired resistance (SAR) in formerly uninoculated organs and the induced systemic resistance (ISR) associated with the colonization of plant roots by certain plant growth promoting rhizobacteria (PGPR) (van Loon *et al.*, 1998; Herms *et al*., 2002; Terry and Joyce, 2004a; Vallad and Goodman, 2004). SAR is distinguished from other disease resistance responses, because it is the process of a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens, and it is dependent on endogenous salicylic acid (SA) (Ryals *et al*., 1996; Oostendorp *et al*., 2001; Gozzo, 2003; Vallad and Goodman, 2004).

SAR in plants is analogous to the innate immunity in animals and was first described by Chester in 1933, who referred to it as "acquired physiological immunity". Consequently, Ross in 1961 and later White in 1979 clearly described the concepts of LAR and SAR from results of experiments using TMV to sensitize tobacco (*Nicotiana tabacum* L.) against subsequent "challenge" inoculations of TMV on infected leaves or on distal uninfected leaves, respectively (Hull, 2002; Vallad and Goodman, 2004). These studies led to the development of the classic SAR models during the 1980s in many other plants demonstrating that SAR was conserved across diverse plant families (reviewed in Sticher *et al*., 1997). SAR has been shown in at

least 20 plant species from at least six different plant families, and is effective against a broad range of pathogens (Sticher *et al*., 1997; Lucas, 1999).

Indeed, many pathogens can induce SAR, especially, but not only, those that cause tissue necrosis, and the resistance observed following induction of SAR is effective against a wide range of pathogens, which is why SAR resistance is sometimes called "broad spectrum". Results of lab and field studies show that SAR is effective as a tool for controlling plant pathogens and parasites, including fungi, bacteria, viruses, nematodes, insect herbivores and against parasitic weeds (Vallad and Goodman, 2004).

Once established, SAR may last for a certain period of time (from weeks to months), during which any attempted invasion by a virulent pathogen is hampered as the pathogen were an avirulent one (Gozzo, 2003). Summarily, in SAR, plant defence is preconditioned by prior infection or treatment which results in resistance (or tolerance) against subsequent challenge by a pathogen or parasite. The timing of this defence response is critical and can be the difference between being able to cope or not with the challenge of a pathogen or parasite.

2.7.2 The SAR signal transduction pathway

In induced resistance processes, more than one biochemical pathway appears to be activated, based on the requirement of different signal transduction pathways depending on SA for SAR or jasmonic acid and ethylene for ISR (Stricher *et al*., 1997; Vallad and Goodman, 2004). SAR may also have practical value in agriculture, as well as being an interesting paradigm for signal transduction. Thus, understanding of the biochemical changes leading to the resistance state could enable the development of either genetically engineered plants with enhanced disease resistance or novel mode of action plant protection chemicals that act by stimulating the inherent disease resistance mechanisms of plants (Ryals *et al.*, 1995; Ryals *et al.*, 1996; Vallad and Goodman, 2004).

Mechanisms by which SAR is induced and established are not thoroughly known. The classic form of SAR can be triggered by exposing the plant to virulent, avirulent, and nonpathogenic microbes or artificially with chemicals. The oxidative burst, which produces high levels of ROS, comes before the hypersensitive response (HR), and plays a substantially beneficial role in defeating the pathogen attack. Nitric

oxide (NO) and hydrogen peroxide (H_2O_2) influence the hypersensitive cell collapse. Despite its typical occurrence in gene-for-gene resistance, HR may not be an absolute requirement for the induction of SAR. In the hours and days following after the formation of a necrotic lesion, either as a part of the HR or as a symptom of disease, SA begins to accumulate, antioxidants are activated and after all these the SAR pathway is activated (Ryals *et al*, 1996; Sticher *et al*., 1997; Murphy *et al*, 1999; Gozzo, 2003; Hafez *et al*., 2004).

SAR induced by the exposure of root or foliar tissues to biotic or abiotic elicitors, is dependent on the phytohormone SA and is associated with the accumulation of pathogenesis related (PR) proteins as illustrated in the left scheme of **Figure 2.11** (Vallad and Goodman, 2004). SA initially accumulates at the site of infection or application of the abiotic elicitor and later in distal parts of the plant. Moreover, enhancement of several enzyme activities has been observed during the first stages of the infection process, such as phytoalexins, *β*-1,3-glucanase, chitinase, peroxidase, lipoxygenase, and catalase (CAT), which may contribute, directly or indirectly, to pathogenesis resistance (Lebeda *et al*., 2001).

PR proteins called the proteins synthesized *de novo* by plants during infection with various pathogens and have been extracted mainly from dicotyledonous plants. Some of them show properties related to the above mentioned enzymes (van Loon *et al.*, 1998). Genes encoding these proteins were shown to be induced at the onset of the SAR (Kessmann *et al.*, 1994).

The scheme of **Figure 2.12** (Murphy *et al*., 1999) shows a possible model of the SAR pathway, in which depletion of SA (due to the expression of *nahG* gene that catalyzes the conversion of free SA to catechol, which is inactive in SAR) causes a breakdown of both SAR and gene-for-gene resistance. Afterwards, the plant defence signal transduction pathway separates downstream of SA into two branches. One branch leads to the induction of resistance to the replication or long-distance movement of viruses, whereas the other leads to the induction of PR proteins and fungal and bacterial resistance (Chivasa *et al*., 1997).

This model is based on the evidence that in tobacco, the SA-induced resistance to TMV and *Potato virus X* (PVX) replication and to CMV systemic movement can be inhibited by salicylhydroxamic acid (SHAM), which is an inhibitor of alternative oxidase (AOX) and a negative regulator of ROS production (see **Figure 2.12**). However, SHAM is not found to inhibit SA-induced synthesis of PR proteins.

Similarly, SA-induced resistance to a fungal pathogen (*Botrytis cinerea*) or a bacterial pathogen (*Erwinia carotovora*) is not blocked by SHAM (Chivasa *et al*., 1997; Chivasa and Carr, 1998; Naylor *et al*., 1998; Murphy *et al.* 1999; Hull, 2002). So the model of SA-induced resistance is consistent with the emerging view of defence signal transduction as a web of converging and branching signaling pathways.

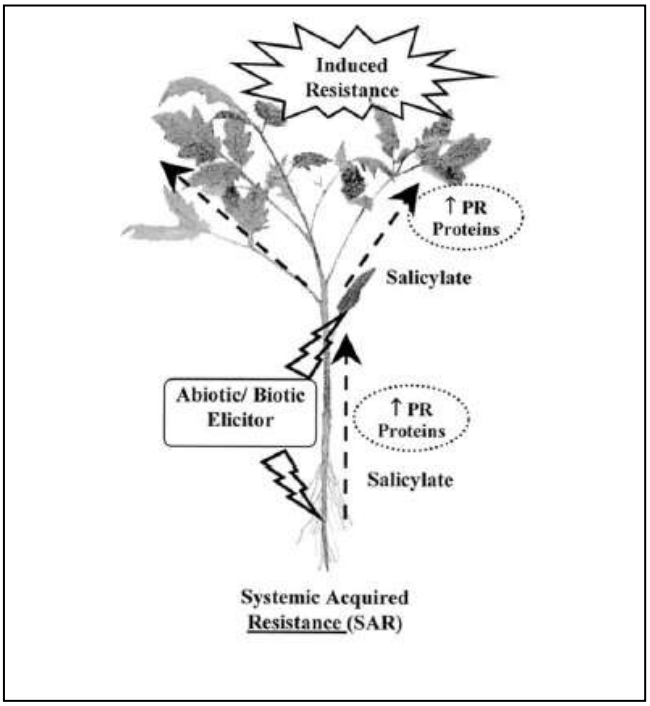

Figure 2.11. Depiction of Systemic Acquired Resistance (source: Vallad and Goodman, 2004).

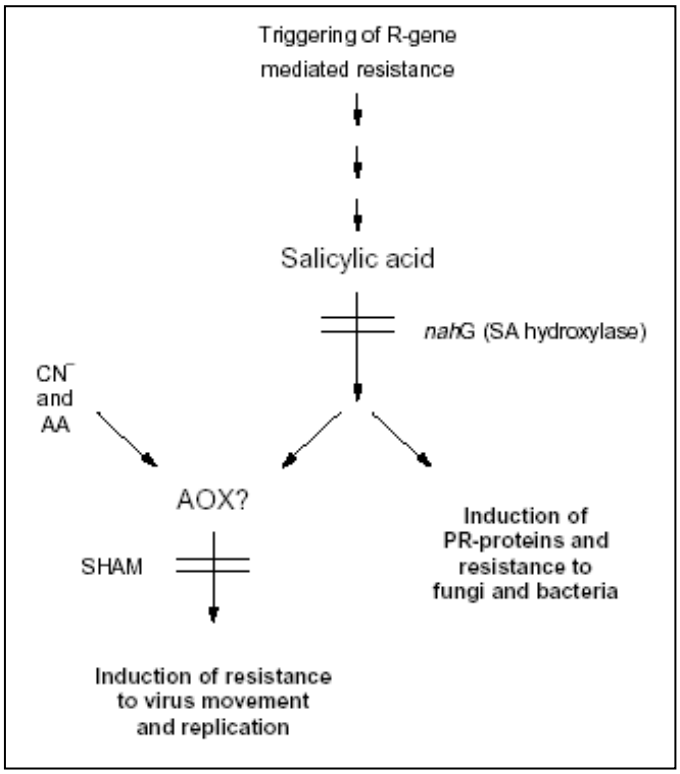

Figure 2.12.: Possible model to explain the induction of resistance to several pathogens. Activation of resistant genes after the attack of a pathogen causes localised death and the activation of a defence signal transduction pathway. That pathway includes salicylic acid (SA). Downstream of SA, the pathway appears to be divided to a branch (right) that induces the production of pathogenesis related (PR) proteins (conferring resistance to fungi and bacteria) and to another branch (left) which is salicylhydroxamic acid (SHAM) sensitive and induces resistance to viruses (source: Murphy *et al.*, 1999).

2.7.3 Effect of salicylic acid (SA) on viruses

SA is shown to have a role of wide significance in the plant kingdom as being one of the key components of defence signal transduction. In the case of viruses, SA could target virus replication or trans-plasmodesmatal movement or transport through the vasculature in the viral infection cycle by decreasing the expression of plant genes that encode factors upholding replication and virus spread. Alternatively, SA might induce accumulation of substances that inhibit virus replication and movement. There

is evidence that SA can induce interference with at least two of these processes: replication and systemic movement (Murphy *et al*., 1999; Murphy and Carr, 2002).

Experiments with TMV engineered to express green fluorescent protein (GFP), showed that SA-induced resistance to TMV results from the inhibition of virus replication and virus movement through the plasmodesmata linking neighbouring cells (Murphy and Carr, 2002; Singh *et al*., 2004). Moreover, SA treatment decreases the RNA accumulation of TMV and PVX in tobacco (Chivasas *et al*., 1997; Naylor *et al*., 1998). However, exogenous SA application may be phytotoxic (van Leeuwen, 2007).

On the other hand, there are viruses for which SA inhibits neither replication nor cell-to-cell movement. For example, CMV accumulated in directly inoculated leaves of SA-treated tobacco (Murphy *et al*., 1999; Murphy and Carr, 2002). The ability of CMV to escape from SA-induced inhibition of replication and/or local movement appears to be due to a specific viral gene product, the CMV 2b protein, that also functions as an inhibitor of RNA interference (RNAi) induction (Ji and Ding, 2001). Despite this, SA treatment or induction of SAR can inhibit the systemic spread of CMV through the plant, by inhibiting the long-distance movement of this virus through the phloem (Naylor *et al*., 1998; Singh *et al*., 2004).

All the above results suggest that SA is able to trigger resistance to viral replication (e.g. TMV), gene expression (e.g. TMV or PVX), cell-to-cell movement (e.g. TMV), or long-distance movement (e.g. CMV).

Of course, among viruses there may be varying degrees of sensitivity to the effects of SA. For example, it has been observed that SA can inhibit long-distance movement of *Alfalfa mosaic virus* (AMV) in tobacco, but has no effect on the replication of that virus (Naylor *et al*., 1998). This disagrees with experiments with cowpea protoplasts showing that AMV replication is inhibited by SA (Hooft van Huijsduijnen *et al*., 1986). Taken into account these results concluded that the same virus might be targeted differently in different plant species.

It has been demonstrated that SA can increase AOX activity and induce members of the *Aox* gene family. This evidence encouraged some researchers to test if AOX activity or gene expression may be connected with pathogen resistance. This suggests that the induction of resistance to viruses may be regulated through a disruption of mitochondrial redox homeostasis (Singh *et al*., 2004).

2.7.4 Role of alternative oxidase (AOX) in the induction of virus resistance

AOX is the terminal oxidase of the cyanide-resistant alternative respiratory pathway, in the mitochondrial electron flow in plants (Vanlerberghe and McIntosh, 1997). Adenosine triphosphate (ATP) is not generated due to its activity (Moore *et al*., 2002).

The evidence that resistance to viruses is promoted by inducers or inhibited by an inhibitor of AOX activity supports the hypothesis that AOX is involved in defence signal transduction (Murphy *et al*., 1999). As has already been mentioned, application of exogenous SA or increase of endogenous SA induces transcription of A*ox* genes and activates the alternative respiratory pathway. On the other hand SHAM is a relatively competitive inhibitor of AOX (Laties, 1982).

Moreover, researchers have observed that *Aox* gene expression and AOX protein accumulation are increased in plant tissues expressing the HR, or expressing resistance to viruses. This finding suggests an association between AOX and pathogen resistance (Lennon *et al*., 1997; Chivasa and Carr, 1998; Lacomme and Roby, 1999; Simons *et al*., 1999; Singh *et al*., 2004).

Further evidence that confirms the existence of a virus-specific branch of the defence signaling pathway has come from studies using metabolic inhibitors. Cyanide (CN^-) and antimycin A (AA) inhibit electron transfer in the cytochrome pathway, motivate electrons into the alternative pathway, and raise *Aox* gene induction (Vanlerberghe and McIntosh, 1992). For example, in tobacco and *Arabidopsis*, resistance to several viruses, including CMV, can be activated using non-fatal concentrations of AA and CN^-, without PR gene expression (Wong *et al*., 2002; Mayers *et al*., 2005; Gilliland *et al*., 2006).

According to the above data the SHAM-sensitive pathway is induced by SA and potentially by AOX. The virus-specific branch (see left branch **Figure 2.12**) can be activated by CN^- and AA (Chivasa and Carr, 1998) or inhibited with SHAM (Chivasa *et al*., 1997), independently of the other branch. These observations have led to the suggestion that AOX activity might play a role in the induction of virus resistance, but without a direct interaction between AOX and a virus component.

It has been demonstrated by Carr (unpublished results) that SA-induced resistance to TMV is antagonized by SHAM in tomato (Murphy *et al*., 1999). So it would appear that the SHAM-sensitive pathway might be the same among

solanaceous plants. On the other hand, Mayers *et al*. (2005) examined plants of different families and found that the SA-induced resistance to CMV is similar in tobacco and *A. thaliana* but different in squash (*Cucurbita pepo*). So, one cannot assume that SA-mediated pathways are the only means of responding against CMV (Ryu *et al*., 2004; Sekine *et al*., 2004). These results show that different host species may use significantly different approaches to resist infection by the same virus. Thus, a virus that is inhibited in one way by plants of one species may be inhibited in another host species using a different mechanism induced by a distinctly different signaling pathway. All the above suggest that it is difficult to attempt to construct general rules governing signaling in plant-pathogen interactions based on work in a restricted number of model plants.

2.8 Benzothiadiazoles (BTH) as chemical activators of SAR

2.8.1 Induced resistance in plants by chemical compounds

The induced state is not specific, but constitutes a more general increase in plant resistance to different types of pathogens. Particularly, it rarely prevents disease from occurring but generally reduces its extent or severity (Hammerschmidt *et al*., 2001). These characteristics make induced resistance a powerful mechanism for enhancing the overall resistance in crop plants, as the plant acquires a sort of aspecific immunization against challenge infections. From the practical point of view, the most important fact is that acquired resistance can also be induced, or enhanced, by exogenous application of SA or synthetic compounds that may have more powerful effects.

These compounds act on the endogenous defence pathways of the plant and they provide broad-spectrum disease control (Lucas, 1999). The classic form of SAR can be triggered artificially with various inorganic and organic chemicals compounds called "plant activators". A chemical in order to be considered as a true activator or elicitor of plant defence reactions in crop protection, or SAR inducer, should fulfill the following three criteria (Kessman *et al*., 1994; Friedrich *et al*., 1996; Ryals *et al*., 1996; Stricher *et al*., 1997; Brisset *et al*., 2000):

1. show no direct antimicrobial activity
2. protect against a range of pathogens without specificity
3. activate host defence mechanisms which are similar to those induced systemically after biological activation of SAR, even in tissues not treated with the SAR inducer.

Most published literature has shown positive effects of chemical elicitors in inducing SAR. However, like all technologies, there are benefits and drawbacks that need to be considered. On the one hand synthetic elicitors provide a way to control disease without exerting direct selective pressure on pathogen populations. This happens because the plant activators are non-toxic and have no direct effect on microbes. So probably they work on a number of systems, making resistance more difficult to be acquired by pathogens and their use seems to be mild for the environment relatively to current pesticides with direct modes of action. These

characteristics make SAR an attractive approach for managing crop pests in a sustainable manner within the scope of a conventional agriculture system.

On the other hand, there are biological limitations that may hinder the practical use of chemical elicitors. The efficacy of SAR is also dependent on the pathogen, and some pathogens do not respond to elicitors. Thus, several elicitors of SAR failed to reduce symptoms and in some cases worsened them, for example symptoms of late leaf spot caused by *Phaeoisariopsis personata* of peanut *Arachis hypogaea* L., (Zhang *et al*., 2001). Moreover, depending on the plant and elicitor, a set period of time is required for the establishment of SAR that corresponds to the time required for the activation of biochemical processes for the coordinated accumulation of PR proteins (and transcripts) and SA throughout the plant (Ward *et al*., 1991; Uknes *et al*., 1992; Cameron *et al*., 1994; Godard *et al*., 1999; Resende *et al*., 2002; Soylu *et al*., 2003; Mandal *et al*., 2008). This lag time between treatment and expression of resistance that occurs, may limit the practical application of disease resistance inducers. Furthermore, the efficacy of the inducers depends on the part of the plant that will be treated, so the way the inducers should be applied in order to optimize their action is an important factor to be considered (Rocha and Hammerschmidt, 2005). Hence, some reports may obscure the fact that in some plant-pathogen systems or environments SAR elicitors are relatively ineffectual (Terry and Joyce, 2004a).

Synoptically, there are many unresolved questions about the use of this kind of chemical compound, such as optimal timing and method of application on different crops, integration with other types of pesticide, and interactions with the physiology of the plant. These chemicals are, by definition, altering plant gene expression and metabolism, so, when they are used may have side effects on growth or yield characteristics. There also appear to be differences in the efficacy of chemical defence activators on different crop species. SAR elicitors may not be commercially viable in some plant-pathogen systems (Terry and Joyce, 2004a). Understanding the biochemical interactions occurring between plants, pathogens and the chemical inducers will provide information that may be useful for the optimization of this approach on disease control.

2.8.2 Benzothiadiazoles (BTH)

The Benzo (1,2,3)-thiadiazoles (BTH) (**Figure 2.13**) form a class of chemicals which stimulate the plant's own defence mechanisms (Kunz *et al*., 1997). Their mode of action is the SAR activation downstream of SA. A derivative of BTH, acibenzolar-S-methyl (ASM) (**Figure 2.13** note **1**) was discovered in 1989 by Ciba Geigy (Novartis) and commercialized under the trade name actigard in the United States (Syngenta Crop Protection Inc., Greensboro, NC, formerly Novartis Crop Protection) and Bion in Europe (Syngenta Ltd., Basel, Switzerland) (Mandal *et al*., 2008).

BTH is suitable for use in field or greenhouse because of its low toxicological risk, rapid degradation in plant tissues, and its low impact both on humans and environment (Soylu *et al*., 2003; Cao *et al*., 2011). According to Soylu *et al*. (2003) BTH did not exhibit *in vitro* antimicrobial activity even at concentrations exceeding the levels effective in plants. BTH can be translocated systemically in plants. For instance Scarponi *et al*. (2001) demonstrated that BTH was rapidly transferred to apical leaves of tomato plants. Moreover, BTH can take the place of SA in the natural SAR signal pathway, inducing the same spectrum of resistance and the same set of molecular markers. In field-grown tomatoes, BTH was found to increase the expression level of the pathogenesis-related gene, P4 (equivalent to PR-1 of tobacco and *A. thaliana*), extending findings from lab-based experiments to the field (Thaler *et al*., 1999).

Benzothiadiazoles also activate a very wide spectrum of resistance under practical field conditions that includes fungal, bacterial and viral pathogens (Oostendorp *et al*., 2001). As regards viruses, BTH can induce some degree of resistance to them (Anfoka, 2000; Pappu *et al*., 2000; Csinos *et al*., 2001; Momol *et al*., 2001; 2004; Mandal *et al*., 2007; 2008; Nischwitz *et al*., 2008), even in plants that do not possess a corresponding resistance gene and therefore would be completely susceptible, in normal circumstances (Friedrich *et al*., 1996).

Many experiments have evaluated a range of BTH applications and have reported a balance between effective disease control and phytotoxic effects or reduced plant productivity (Cole, 1999; Louws *et al*., 2001; Abbasi *et al*., 2002; Perez *et al*., 2003). In several of the field experiments using BTH crop yield was reduced, but often these reductions were statistically insignificant (Louws *et al*., 2001; Romero *et al*., 2001). On the other hand there are articles showing that BTH had no negative

effect on fruit production. So, in two field trials, the cantaloupe production of BTH treated plants was unaffected in the absence of disease pressure (Smith-Becker *et al*., 2003).

An inherent difference between monocotyledons and dicotyledons exists in terms of the longevity of the induced resistance elicited by BTH. While single applications of BTH were generally sufficient to induce resistance over the life of a monocotyledon crop, such as wheat, dicotyledon crops required repeated applications of BTH to extend protection over time (Görlach *et al*., 1996; Cole, 1999; Morris *et al*., 1998; Louws *et al*., 2001; Romero *et al*., 2001). Scarponi *et al*. (2001) showed that BTH totally disappeared from tomato leaves three days after its application.

The timing of BTH treatment and the developmental stage of the plant may be as important in determining BTH's efficacy (Terry and Joyce, 2004a; 2004b) as the time between BTH application and the challenge with the pathogen (Godard *et al*., 1999; Resende *et al*., 2002; Soylu *et al*., 2003; Mandal *et al*., 2008). As mentioned above, this happens because a certain lag-time is required for activation of the plant defence mechanisms. So, BTH has to be applied protectively or at an early stage in the disease progression (Ruess *et al*., 1995). In addition, according to Tally *et al*. (2000) BTH is regarded more effective when used as a preventative prophylactic treatment.

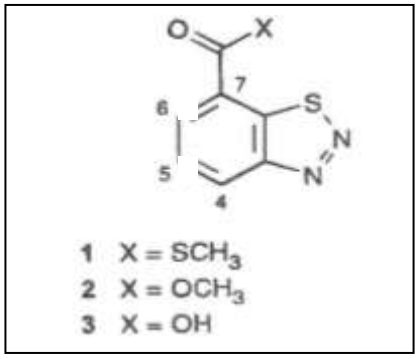

Figure 2.13. The Benzo (1,2,3) thiadiazoles (BTH) lead structure (source:Kunz *et al*., 1997). (**1**): S-methyl benzothiadiazole-7-carbothioate (ASM), (**2**): Methyl benzothiadiazole-7-carboxylate, (**3**): Benzothiadiazole-7-carboxylic acid.

2.8.3 Response of BTH treated tomato plants against diverse pathogens

BTH proved to be an efficient activator of several plant defence mechanisms and a useful tool for induced resistance in tomato and other plant species (Soylu *et al*., 2003). Benhamou and Bélanger (1998) were the first who revealed that BTH induces SAR in tomato plants. Specifically, they demonstrated that BTH treated tomato plants were protected against the root rot disease caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

Later, Tally *et al*. (2000) showed that BTH applications on tomato plants activated resistance against late blight, caused by *Phytophthora infestans*. Additionally, repeated applications of BTH reduced early blight on tomato, caused by *Alternaria alternata*, while others found that BTH offered little protection against the combined effects of late blight and early blight of tomato (Inbar *et al*., 1998; Louws *et al*., 2001).

Achuo *et al*. (2004) demonstrated that soil or leaf treatment with BTH induced resistance against *B. cinerea* in tomato, but neither soil nor foliar application of BTH had any effect on the infection of tomato by *Oidium neolycopersici*. Similarly, Inbar *et al*. (1998) and Hennin *et al*. (2001) earlier had reported that BTH did not significantly reduce powdery mildew (*O. neolycopersici*) infection of tomato.

According to literature, the eliciting activity of BTH on tomato plants is quite effective against bacterial pathogens as well. Hence, in a series of field experiments repeated applications of BTH were effective at reducing the severity of bacterial spot (caused by *Xanthomonas axonopodis* pv. *vesicatoria* or *X. campestris* pv. *vesicatoria*) and bacterial speck (caused by *Pseudomonas syringae* pv. *tomato*) on foliage and fruit of tomato, in the same way with standard anti-bacterial treatments including copper hydroxide. Moreover, BTH was effective at controlling field epidemics of bacterial speck and spot when copper resistant strains of *P. syringae* and *X. axonopodis* predominated (Louws *et al*., 2001). Obradovic *et al*. (2005) showed that BTH completely prevented the occurrence of typical symptoms of bacterial spot (caused by *X. campestris*) in greenhouse experiments with tomatoes.

Herman *et al*. (2008) also provided evidence that BTH effectively reduced bacterial speck (*P. syringae* pv. *tomato*) incidence and severity in greenhouse grown tomatoes, both alone and in combination with an ISR-inducing product. They demonstrated that BTH application led to elevated activation of SA and ethylene-

mediated responses, based on real-time polymerase chain reaction analysis of marker gene expression levels.

Most literature on BTH plant activator reports that its treatments positively suppress disease, although there are some studies which demonstrate that BTH derivatives are relatively ineffective in some plant-pathogen systems and/or environments, as mentioned above for powdery mildew (Inbar *et al*., 1998; Hennin *et al*., 2001; Achuo *et al*., 2004).

2.8.4 Antiviral activity of BTH

Little is known about the ability of BTH to trigger SAR in different plant species against virus diseases, as most of these studies referred to tobacco plant (Friedrich *et al*., 1996; Csinos *et al*., 2001; Mandal *et al*., 2007; 2008; Momol *et al*., 2001; 2004; Nischwitz *et al*., 2008). BTH was reported to suppress accumulation of viral RNA of TMV in tobacco (Friedrich *et al*., 1996). Treatment of *Arabidopsis* with BTH resulted in resistance to *Turnip crinkle virus* (TCV). This resistance was characterized by a decrease in necrotic symptoms, which was apparently due to inhibition of viral replication as viral RNA could not be detected in the plant (Lawton *et al*., 1996).

Anfoka (2000) first proved that BTH triggers SAR and protects tomato plants against aphid transmitted CMV-Y using the cotton aphid (*Aphis gossypii* Glover). BTH application as a drench, seven days before transmission of CMV-Y, protected plants against the necrosis caused by CMV-Y. The resistance was evident as a decreased disease incidence and severity. The disease incidence in BTH-treated plants did not exceed 12.5% whereas 91.7% of control plants were severely infected. The induced resistance in tomato plants markedly reduced the replication of viral RNA. According to Anfoka one hypothesis for these observations may be that the activation of SAR genes in BTH-treated tomato plants results in modifications of the structure of the plasmodesmata which reduce the rate of virus movement.

Another reference for BTH treatment against CMV is that of Smith-Becker *et al*. (2003), where one application of BTH induced the systemic accumulation of chitinase, and effectively delayed the spread of CMV in cantaloupe in greenhouse trials. Foliar applications of BTH were tested in replicated field trials singly and in combination with imidacloprid for their effect on reducing the impact of TSWV in

tobacco. Results showed that BTH significantly reduced final disease incidence in two trials, whereas imidacloprid, when applied alone, suppressed disease incidence only in one out of four trials. Significant reduction in incidence occurred at three of the four locations when both were applied together (Pappu *et al*., 2000).

Benzothiadiazole was also found to protect tobacco plants against the effects of the thrip-vectored TSWV in field-grown tobacco, as assessed by a reduction in the number of symptomatic plants (Csinos *et al*., 2001). Studies on the effect of BTH on TSWV incidence on tomato and tobacco showed a significant reduction in incidence in combination with other treatments such as reflective mulches and insecticides. The metalized mulch was most effective in reducing disease incidence. BTH reduced incidence of TSWV on the standard black mulch but not on metalized mulch. The regimen of metalized mulch, BTH and insecticides reduced TSWV by as much as 76% (Momol *et al*., 2001; 2004).

Mandal *et al*., (2007) determined if transplant age and preplant applications of BTH and imidacloprid influence TSWV incidence in flue-cured tobacco. They found that 14-week-old tobacco plants had fewer local lesions per plant and significantly fewer systemic infections when mechanically inoculated in the greenhouse than the 6- or 10-week-old tobacco plants. In a more recent study they demonstrated that treatment of flue-cured tobacco with BTH activated high levels of resistance against a severe isolate of TSWV and the expression of the PR proteins inversely correlated with the induction in the number of local lesions caused by this virus (Mandal *et al*., 2008). Pretreatment with BTH and imidacloprid decreased infection caused by TSWV in flue-cured tobacco, as it reduced the percentage of symptomatic plants, and increased yields in four of five field trials over four years, compared with the nontreated controls (Nischwitz *et al*., 2008).

2.9 Pyraclostrobin a strobilurin fungicide

2.9.1 Strobilurins

Strobilurins are one of the most important classes of agricultural fungicides and were first launched in 1996 (Bartlett *et al*., 2002). Their invention was inspired by a group of fungicidally active natural products, isolated and identified from the mycelium of *Strobilurus tenacellus* and *Oudemansiella mucida*. The simplest of the natural strobilurins are strobilurin A and oudemansin A, which belong to natural *β*-methoxyacrylates (Bartlett *et al*., 2001; 2002). Many pesticide companies were able to discover synthetic analogues that are more efficacious and more stable than the natural ones. Till 2007 there were about eight synthetic strobilurins in the fungicides market (Balba, 2007).

Strobilurin fungicides have become an integral part of disease-management programmes on a wide range of crops in many countries of the world. One of the key reasons for the outstanding commercial success of strobilurins is that they have broad-spectrum activity, as they give control of fungi from all four classes of plant pathogenic fungi, namely the Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes. Other major reasons for the success of strobilurins are the control of fungal isolates resistant to other fungicide modes of action, the low use-rates and the excellent yield and quality benefits.

The fungicidal activity of the strobilurins stems from their ability to inhibit mitochondrial respiration by binding at the so-called Q_o site of cytochrome b and block electron transfer between cytochrome b and cytochrome c1. This disrupts the energy cycle within the fungus by halting the production of ATP (Sauter *et al*., 1999; Bartlett *et al*., 2001; Herms *et al*., 2002). Because of this disruption of energy production, spore germination and zoospore mobility, which are highly energy-demanding stages of fungal development, are particularly sensitive to strobilurins. So, strobilurins are best applied prior to infection or in the early stages of disease development in order to use their potent effects against spore germination and zoospore mobility. Although the strobilurins effects on plants have been studied for many years, there is no evidence of any direct interaction between strobilurins and enzymes of receptor systems other than mitochondrial respiration.

Usually the fungicides have focused on control of phytopathogens with the purpose of reducing the inoculum level. For strobilurins the concept of disease control gained other perspectives, especially when considering the advantages obtained by the action of positive physiological effects on the plants (Venancio *et al*., 2003). After intense research on the fungicidal properties of strobilurins, the evidence of their direct influence in physiological processes of plants not infected or threatened by pathogens was strengthened. This activity was named "physiological effect" or "greening effect". Moreover, apart from direct activity with the pathogen, strobilurins are also known to trigger the host defence responses (Herms *et al*., 2002; Sudisha *et al*., 2005).

The "greening effect" is associated with the ability of strobilurins to maintain the green leaf area, therefore to delay chlorophyll loss and hence leaf senescence, thereby increasing the photosynthetic duration of the crop, with resultant yield benefits (Bartlett *et al*., 2002; Kleven *et al*., 2003). For instance, in Europe (UK), strobilurins have been shown to increase yields by up to 15% in wheat and barley in comparison to conventional triazole fungicides (Kleven *et al*., 2003). The increase in biomass and production, obtained by application of strobilurins, even in plants not affected by fungi, is of special interest for agricultural practices.

One hypothesis to explain this phenomenon indicates that a variety of physiological processes are directly affected by strobilurins, including:

- the carbon dioxide compensation point,
- 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and thereby ethylene biosynthesis,
- leaf senescence,
- chlorophyll content,
- photosynthetic activity,
- stomatal aperture,
- water consumption,
- plant antioxidant enzyme activity,
- endogenous levels of abscisic acid (ABA) and other plant hormones, and
- nitrate reductase activity.

Indeed the strobilurins proved to inhibit the biosynthesis of ethylene through reduction of the activity of ACC-synthase in tissue of wheat buds (Grossmann and

Retzlaff, 1997). This was related to the delay in the senescence of leaves and, as a result, to the prolonged photosynthetic activity of green tissue and a better management of stress (Grossmann and Retzlaff, 1997; Grossmann *et al.*, 1999). Moreover, the NADH-nitrate reductase that catalyzes the first stage of nitrate assimilation, is considered as the relevant target for the effect on production caused by strobilurins (Köehle *et al*, 2003), because this brings on greater assimilation of nitrogen for more complex metabolisms, which leads to an increase in biomass.

A second hypothesis presented for the "greening effect" has been that strobilurins prevent the spores of pathogenic, non-pathogenic and saprophytic fungi from germinating and thereby stop the elicitation of energy-demanding host-defence responses, whereas other fungicides do not (Bertelsen *et al*., 2001).

It is possible that elements of both hypotheses contribute to these "unexpectedly good" yield benefits with strobilurins in wheat and barley. There are a number of published studies on grain, fruit, tuber and bulb quality following disease control with a strobilurin fungicide in a wide range of crops other than wheat and barley. Especially for tomatoes, there is evidence that strobilurins increased soluble sugars and extended their shelf life (Siviero *et al*., 2001 reviewed in Bartlett *et al*., 2002).

Finally, strobilurins have a number of environmental advantages, as they show few signs of specific toxicity related to their pesticidal mode of action. This class of fingicides presents minimal risk to human health, and is generally of low toxicity. The commercialised strobilurins are considered safe to birds, mammalian wildlife, bees, earthworms and beneficial insects at limit doses. They are relatively readily degraded, they have a half-life of 4-6 weeks in soil, and they are completely mineralised by microbes and light.

2.9.2 Induction of resistance to viruses by strobilurins

Since the bc1 complex persists in all eukaryotae, at least one partial inhibition in the transportation of electrons must also be expected in plant cells after absorbing the strobilurin fungicides (Venancio *et al*., 2003). A transitory influence in the plant mitochondria does not necessarily result in phytotoxicity, because the toxicity at the organism level is determined by the dose of a compound and there is no toxicity for plants at limit doses of strobilurins.

Several studies in plants refer to the involvement of mitochondria in the defence response induced by pathogens. Such a model of induction of resistance to viruses, including strobilurins, was suggested by Singh *et al*. (2004) (**Figure 2.14**). According to the authors, SA can stimulate resistance to viruses using at least two pathways of transduction. It can stimulate increase in the transcription of RNA-dependent RNA polymerase (*RdRp1*) (Xie *et al*., 2001; Yu *et al*.; 2003). Alternatively, SA can inhibit respiration in the chain of transportation of electrons (Xie and Chen, 1999), leading to an increase in the ROS in mitochondria. Other inhibitors of respiration in the transportation of electrons such as AA, CN^- (Chivasa and Carr, 1998) or strobilurins type fungicides (Herms *et al*., 2002) can also cause an increase in ROS. The amplitude and duration of this increase in mitochondrial ROS will be under negative regulation through AOX.

Although not shown in this diagram (**Figure 2.14**), the expression of the *Aox* gene in the nucleus is transitory and stimulated by inhibition of the chain of electron transportation, leading to increase in the accumulation of AOX in the mitochondria. Then AOX controls the buildup of mitochondrial ROS, and signals of ROS can be detected by a protein sensor that is still unidentified, by means of a mechanism of thiol/disulfite change (Dutilleul *et al*., 2003). Subsequent signals are proposed to the induction of nuclear genes that affect movement and viral replication.

The effects described above can explain the induction of resistance against viral attack. Strobilurins' effect on resistance was investigated by one of the best models of the system for studying interactions, specifically interaction between TMV and tobacco plants. Therefore, the strobilurin group, in addition to serving as potent fungicides, probably also protects plants by increasing their inherited capacity to activate a cellular defence response.

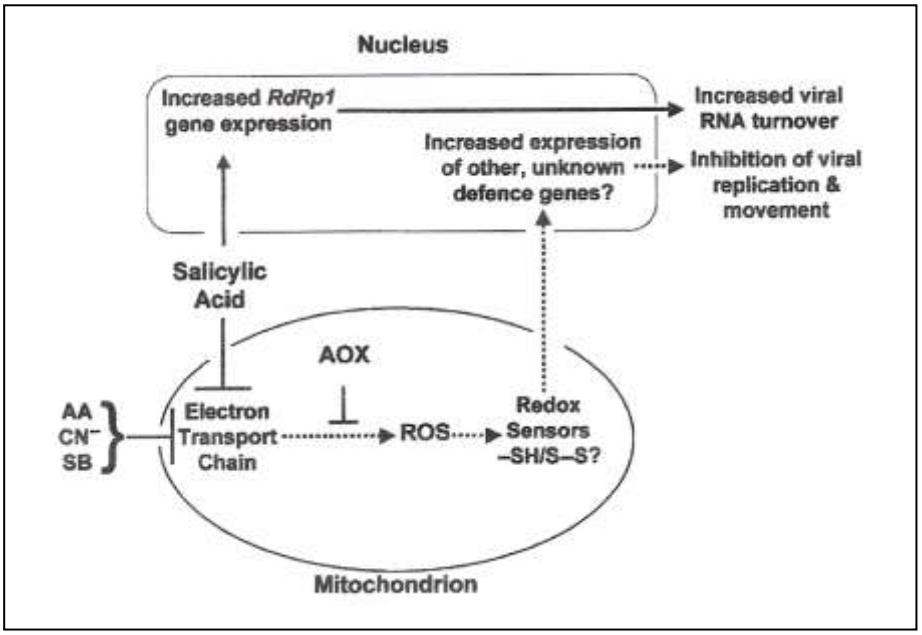

Figure 2.14. A model of induction of resistance to viruses by inhibitors of respiratory electron transport chain such as salicylic acid (SA), antimycin A (AA), cyanide (CN^-) or strobilurin-type fungicides (SB). SA can also stimulate increased transcription of RNA-dependent RNA polymerase (*RdRp1*) which is thought to enhance the turnover of viral RNA via RNA interference (RNAi) based mechanisms (source: Singh *et al.*, 2004).

2.9.3 Pyraclostrobin

Pyraclostrobin is the active ingredient of a new, broad-spectrum strobilurin fungicide developed under codename BAS500F or F500 by BASF. It has a methyl *N*-methoxycarbamate toxophore (**Figure 2.15**). Regarding its properties, pyraclostrobin has translaminar activity, is transported acropetally and basipetally, and is non-systemic, but locally systemic (**Table 2.3**). Its unique physicochemical properties ensure that the agent, once applied, adheres firmly to the waxy layer of the leaf as a fat-soluble coating and reaches all areas of the leaf tissue.

Table 2.3. Redistribution properties of pyraclostrobin

Uptake into leaf	Very low
Molecular redistribution by air	No
Metabolic stability in leaf	Yes
Translaminar movement	Low
Xylem systemic	No
Systemic movement to new growth in wheat and barley	No
Phloem mobile	No

Since its launch in 2002, pyraclostrobin has made a significant contribution to keeping crops healthy against numerous fungi from the four agronomical classes. Like all strobilurins, pyraclostrobin acts as a highly selective inhibitor of fungal spore germination by blocking a specific stage of cell respiration in the mitochondria. Without this respiration process, the attacking harmful fungi are “suffocated” early in their development and die (Bartlett *et al*., 2001; 2002).

Pyraclostrobin, as well as having excellent preventative curative properties, gives a yield boost beyond that expected solely from disease control. It increases plant vitality, induces various mechanisms that help the plant to adapt better to various stress factors, and enhances the plant's performance. Field experiments have revealed that cereals treated with pyraclostrobin show significant increases in production, greater than those due only to its fungicidal effect (Köehle *et al*., 2003). Thus, the fungicide presents additional effects on plant physiology, which lead to a positive influence in the formation of production. The physiological effects of pyraclostrobin

were reviewed under several levels of complexity, from the "greening effect" frequently mentioned and the enhancement of stress factors in field and under controlled conditions, to the influence of hormonal regulation and assimilation of carbon and nitrogen by the plant (Venancio *et al*., 2003). Pyraclostrobin's mode of action in this regard, appears to be different from other strobilurins.

Indeed, pyraclostrobin appears to have a much larger effect on nitrogen reductase, so the plant can assimilate more nitrogen, which means that soil nitrogen is absorbed better by the plant and it is put to more effective use for plant protein production. This has a positive impact on plant biomass and ultimately also on plant productivity.

Moreover, the enhancement of nitrate reductase activity promotes the production of NO, a signal molecule involved in the plant's pathogen defence system. Conrath *et al*. (2004), using Restriction capillary inlet mass spectrometry (RIMS), demonstrated that pyraclostrobin triggers NO production in plants. Analytically, it was revealed that in tobacco cell cultures, F500 elicited a huge emission of NO after approximately five minutes which steadily increased over the 24-hours detection period, while respiratory O_2 uptake was dramatically inhibited. NO ensures rapid activation of defence mechanisms in plants. In this manner, treated plants are better equipped for rapid identification and effective repulsion of aggressive pathogens. It has been speculated that the enhanced pathogen resistance of tobacco induced by F500 might be because of pyraclostrobin-induced NO release (Kohle *et al*., 2003). This can be demonstrated particularly effectively for bacterial and viral pathogens, as pyraclostrobin, like all strobilurin, has no direct action against these pathogens. Indeed, suitably treated plants are better able to defend themselves against these harmful organisms than untreated controls.

Pyraclostrobin also has a much stronger hormonal effect than other strobilurins. Köehle *et al*. (2003) concluded that pyraclostrobin altered the status of phytohormones in wheat bud tissues. The most remarkable change was the inhibition of ethylene biosynthesis by the reduction of the activity of ACC synthase. Together with the increase in endogenous auxin, this change in hormonal balance would explain the retarded senescence of leaves and enhancement in the tolerance to stress. So the plant stays vital for longer, and the yield and quality are supported on a sustainable basis. Also, pyraclostrobin stimulated the levels of ABA (Köehle *et al*., 2003), and the authors believe that this might favour tolerance to cold and adaptation

to conditions of water shortage. Studies show that pyraclostrobin increases the activity of the protective enzymes naturally produced by the plant. Stimulation of the plant's own natural antioxidative processes results in a marked reduction of harmful ROS, equipping the crop to adapt effectively to adverse conditions.

In addition, increased assimilation of carbon dioxide has been observed due to pyraclostrobin treatment of plants, with increased chlorophyll activity and increased yield (Clair *et al*., 2004). Application of pyraclostrobin also reduces the incidence of symptoms caused by ultraviolet (UV) radiation and prevents the associated loss of vitality (Thorsten, 2005).

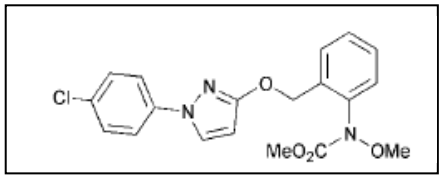

Figure 2.15. Structure of pyraclostrobin (source: Bartlett *et al*., 2001)

2.9.4 Antiviral activity of pyraclostrobin

Very little is known about the activity of pyraclostrobin against virus diseases. Pyraclostrobin enhanced the resistance of tobacco (*Nicotiana tabacum* cv Xanthi nc) against infection by TMV, and it also enhanced TMV resistance in *nahG* transgenic tobacco plants. This finding suggests that pyraclostrobin enhanced TMV resistance in tobacco either by acting downstream of SA in the SA signaling mechanism or by functioning independently of SA. The latter assumption is the more likely because in infiltrated leaves, pyraclostrobin did not cause the accumulation of SA-inducible PR-1 proteins that often are used as conventional molecular markers for SA-induced disease resistance. Though accumulation of PR-1 proteins and the associated activation of the *PR-1* genes were elicited upon TMV infection of tobacco leaves and both these responses were induced more rapidly in pyraclostrobin-pretreated plants than in the water-pretreated controls. Thus pyraclostrobin may enhance disease resistance in tobacco by accelerating the plant's ability for the induction of normal defence responses that occur once the pathogen is sensed by the plant (Herms *et al*., 2002).

The effect of pyraclostrobin in the production of potato plants cv. Agate infected by the viruses PVY or PVX was investigated by Geraldino *et al*. (2002). Potato plants treated and inoculated with PVY produced an average of 351.1 g and, the ones treated and inoculated with PVX produced 398.5 g, the plants treated and not inoculated produced 332.5 g and the plants not treated and not inoculated produced 302 g. This result was considered important, because it indicated a new alternative for the decrease of losses caused by virus in field.

However, pyraclostrobin failed to induce resistance against *Tobacco etch virus* and PVY in tobacco plants (data not shown Herms *et al*., 2002). Finally, there were indications that pyraclostrobin induced plant resistance mechanisms against CMV and delayed disease development in tomato plants, in greenhouse experiments. Lower disease incidence and slower development of CMV infection was observed when inoculation with the virus was carried out 24 hours after pyraclostrobin application. Moreover a second application of pyraclostrobin, one week after the first one, seemed to maintain the antiviral effect (Varveri *et al*., 2006).

CHAPTER THREE

Materials and methods

3.1 Plant material and cultivation

The hybrid F1 Clodin tomato without special resistance against viruses was used for all the experiments of this study with tomato seedlings. In a glasshouse equipped with double doors and insect proof net, tomato seeds were sown in 0.2 L pots containing peat moss. Seedlings in all experiments were grown at 25°C day and 19°C night, with a 16-hour photoperiod. Additional daylight illumination was provided. Plants were irrigated every second day with approximately 50 mL of water.

The Spanish hybrid Delos tomato (Fytroseeds, Greece), kindly supplied by V. Mallis, was used in three experiments aiming at fruit production. Experiments were conducted in consecutive years, from 2008 till 2010 (1st experiment: October 2008 - April 2009, 2nd: August 2009 - January 2010 and 3rd: January - June 2010). Seeds were sown in seed pans containing a blend of high grade frozen black sphagnum peat (Klasmann Potgrond P, Germany). Seedlings were transplanted 36 days after seeding in 20 L pots of 30 cm diameter in a soil mix consisting of medium decomposed white sphagnum peat moss (Klasmann TS3, Germany) and perlite (2:1 v/v respectively).

Plants were grown at temperatures ranging between 16 and 30°C in winter and between 18 and 32°C in spring with a 16-hour photoperiod. Seedlings were irrigated every second day with approximately 50 mL of water. Plants were fertilised after transplantation [$Ca(NO_3)_2$: KNO_3 : NH_4NO_3 : $NH_4H_2PO_4$: $MgSO_4$ in proportion of 0.5 : 0.55 : 0.15 : 0.3 : 0.35 g per L of water] every day initially with 0.5 L, gradually increased up to 2 L, depending on their needs. In the 2nd and the 3rd experiments plants were fertilised with potassium (KPO_4, 3 g per L of water) twice per week instead of the pre mentioned fertilization to obtain an earlier blossom.

All plants were topped at a height of 2.5 m for the first experiment and 2.3 m for the next two experiments. The side shoots were pruned as they appeared. Flowers were hand-pollinated and tagged at anthesis in order to determine the days after anthesis (DAA).

3.2 Source of inocula and challenge inoculation

3.2.1 Source of inocula

Two CMV, two PVY and one TSWV Greek isolates were used. The isolate used most in this study was an isolate of CMV named "CMV four" (CMV4). It was isolated in Korinthos, in 1995 from *L. esculentum* showing mosaic and shrinkage symptoms (Varveri and Boutsika, 1999).

The other CMV isolate, named "CMV fifty" (CMV50), was isolated from *Cucurbita pepo* in Tympaki (Crete), in 2007. The TSWV was isolated from *Lactuca sativa*, in Marathon, in 2006. The one of the two PVY isolates, "PVY one" (PVY1), a common virus isolate (PVY^{O}), was isolated from *Solanum tuberosum* in Crete, in 1995, while the other named "PVY sixty nine" (PVY69), an isolate inducing necrotic symptoms on potato tubers (PVY^{NTN}), was isolated from *S. tuberosum* in Tripoli in 2005 (Varveri, personal communication).

All used virus isolates were stored as dehydrated (by $CaCl_2$) infected plant tissues under refrigeration at Benaki Phytopathological Institute (BPI). In order to be used in the experiments the viruses were propagated in *Nicotiana tabacum* "Xanthi nc" plants showing symptoms in the glasshouse of BPI.

3.2.2 Virus inoculation

Inoculum of each virus was prepared from young symptomatic tobacco leaves by homogenizing leaf tissue in 0.03 M potassium phosphate buffer pH 7.4 in a 1:3 w/v (fresh tissue weight / volume buffer) ratio.

In experiments with seedlings virus inoculation was carried out either on cotyledons, or on cotyledons and the first true leaf, or only on the first true leaf (depending on the plant size) as stated in each experiment separately. In three experiments aiming at fruit production, the inoculation was performed on the first true leaf of each seedling, when plants had the first two fully expanded leaves. In all cases the virus was applied by gently rubbing with carborundum over the leaf surface. The leaves were then washed under a stream of tap water.

3.2.3 Virus transmission by aphids

Colonies of virus-free *Myzus persicae* aphids were propagated onto *Solanum melongena* plants at 25°C. Prior to virus acquisition aphids were left to starve into Petri-dishes for 3 h, and were then fed on infected tobacco leaves for 3-5 min, to acquire the virus. Subsequently, aphids (8-22, depending on the plant size and the used virus isolate) were transferred to tomato plants. The following day all plants were sprayed with the insecticide imidacloprid (20 % SL).

3.3 Benzothiadiazole (BTH) and pyraclostrobin application

BTH was applied once or weekly (formulation BION 50% water dispersible granule [50 WG]) at a concentration of 50 mg/L until incipient run off (15 mL per plant approximately) when the first true leaves appeared. Challenge with viruses was done four, five or seven days after application with BTH, as stated in each experiment separately.

Pyraclostrobin was applied twice with an interval of seven days (formulation F500 25% a.i. w/v) at a concentration of 0.8 mL/L until run off (15 mL per plant approximately). The first application took place when the first true leaves appeared and the next day the challenge with the virus was conducted.

3.4 Physical assessments

Plant developmental stage (stem length and leaves number) was recorded at regular time intervals for each individual plant. The stem diameter (at the bottom, middle and top of the plant) was measured. After harvesting, the fruits were individually weighed with and without calyx and their dimensions (diameter, perimeter and height) recorded. The total number of fruits per plant was also noted.

3.5 Biochemical assessments

Enzyme-linked immunosorbent assay (ELISA) was conducted to determine the presence of the virus in leaves and fruits of CMV inoculated plants. NSCs, organic acids and carotenoids of fruits were quantified by HPLC as described below. The antioxidant capacity was measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging.

3.5.1 Sample preparation for biochemical analyses

Samples were prepared from fruits immediately after harvest and all samples from inoculated plants were tested with ELISA for presence of CMV. Each fruit without calyx was divided into four sections by cutting it vertically and longitudinally. The parenchyma (tomato gel) including seeds was removed. The remained tomato pericarp was chopped to reduce its size to less than 1 cm^3 and an average fresh weight of 15 g for each sample was immediately snap-frozen in liquid nitrogen and then stored at -80°C. Frozen samples were freeze-dried in a freeze drier (HETO LyoLab 3000, **Figure 3.1**). Lyophilisation was used to dehydrate the biological material, so no enzymatic reactions in the dry state and no affect on the composition of the plant material could occur. Lyophilised samples were weighed again for determination of dry weight percentage. Subsequently the samples were ground using a hand-operated pestle into a fine powder and returned to -80°C until their transportation from Athens to United Kingdom (UK). In the Plant Science Laboratory (PSL) of Cranfield University (CU) pulverized samples were stored at -40°C until analysis.

Figure 3.1. HETO LyoLab 3000 freeze drier.

3.5.2 Enzyme-linked immunosorbent assay (ELISA)

The presence of CMV was determined regularly mainly in newly emerged leaves and selected fruits for further analysis by double antibody sandwich (DAS) ELISA (Clark and Adams, 1977).

Polystyrene 96 well microtitre plates (Corning Incorporated 3590, NY) were coated with anti-CMV immunoglobulin G (IgG) from in-house made antiserum raised against a CMV Greek isolate and diluted at 1:1000 in carbonate coating buffer, pH 9.6. The applied volume per well was 200 μL, as in all steps, and plates were incubated for 3 to 4 h at 37°C. Samples were homogenized in plastic bags, where sap of leaves or fruits was expressed using phosphate buffer saline (PBS) containing 0.05% Tween-20 PVP (PBS T PVP, pH 7.4) at a ratio of 1:10 w/v (fresh weight / volume buffer). Coated plates were washed by flooding wells with PBS T for 5 min; this being repeated three times, before adding plant samples, which were then incubated at 4°C overnight. The next day the plates were washed as above and alkaline phosphatase-conjugated IgG diluted at 1:1500 in PBS T PVP was added. After 3 to 4 h incubation at 37°C, substrate (*p*-nitrophenylphosphate at 1 mg/mL in diethanolamine pH 9.8) was added and incubated at room temperature (25°C) for 1-2 h. Absorbance values were determined using a microplate absorbance reader (BIORAD i Mark) at 415 nm (**Figure 3.2**). The blank solution to calibrate the absorbance reader consisted of extraction buffer (PBS T PVP, pH 7.4). Samples with absorbance values that exceeded three times those of the healthy controls were considered as positive (CMV infected).

In the same way the presence of TSWV or PVY was determined in newly emerged leaves of tomato seedlings using commercial reagents (LOEWE) against TSWV and an in-house made antiserum raised against a PVY Greek-derived isolate, respectively.

Figure 3.2. Microplate absorbance reader (BIORAD i Mark).

3.5.3 Immunocapture-polymerase chain reaction (IC-PCR)

To support the findings from the ELISA an analysis, IC-PCR was conducted in all BTH treated and CMV inoculated plants. Primers allowing production of a coat protein fragment of 482-501 bp were used for CMV amplification. The IC-PCR protocol of Candresse *et al*. (1995) was applied as described by Varveri and Boutsika (1999) with a few modifications.

Briefly, PCR tubes were coated with 2 μL of anti-CMV IgG from in-house made antiserum in 100 μL carbonate buffer (pH 9.6) at 37°C for 3 h. Coated tubes were emptied and washed with 150 μL PBS T. The plant extracts were prepared as described above for the ELISA test (1:10 w/v sample fresh weight / PBS T buffer, pH 7.4) and 100 μL of each sample were incubated at 4°C overnight. The next day two washes with 150 μL PBS T buffer and one wash with 150 μL bidistilled water were conducted. Subsequently, 10 μL of RT-PCR mix (0.5 μL Triton 10 %, 2 μL buffer 5x, 2 μL DTT, 0.625 μL dNTPs 10 mM, 1 μL super script II, 0.4 μL reverse primer and 3.475 μL H_2O) were added to each tube and tubes were incubated at 42°C for 45 min. Then, 40 μL of PCR mix (5 μL buffer 10x, 1.5 μL $MgCl_2$ 50 mM, 1.25 μL dNTPs 10 mM, 0.4 μL forward primer, 0.4 μL Taq DNA polymerase and 31.45 μL H_2O) were added to each tube and PCR process was carried out in a DNA thermocycler. Denaturation was accomplished by incubation for 2 min at 92°C, followed by 40 cycles of 20 s at 92°C, 20 s at 57°C and 40 s at 72°C each, with a final extension for 5 min at 72°C.

Aliquots (10 μL) of PCR product were analysed by electrophoresis on a 1% agarose gel containing ethidium bromide in Tris-borate EDTA buffer. The size of the amplicons was compared with size standards (1 kb DNA ladder).

3.5.4 High performance liquid chromatography (HPLC)

HPLC was used for all sample analyses that included separation and identification. It allows detection of several compounds simultaneously offering high selectivity and sensitivity. Sugars were detected using an evaporative light scattering detector (ELSD) and identified by the retention indices. Organic acids and carotenoids were detected using a diode array detector (DAD), which as well as tested the retention times, also monitored the spectral characteristics of the examined compounds and compared them directly with the pure standards at the ascending and

descending slopes and at the maximum. It is worth taking into consideration that reliability of data obtained by HPLC mainly depends on the accuracy of the calibration (Kimura and Rodriguez-Amaya, 2002; Periago *et al*., 2004). So once the standard solution had been prepared and the standard curves demonstrated the required characteristics, a number of samples of both treatments were analyzed in a randomized order. Standards were analyzed at the beginning and the end of each analysis in order to check for accuracy.

3.5.5 Extraction and quantification of non-structural carbohydrates (NSCs)

3.5.5.1 Extraction of NSCs

Sucrose, glucose and fructose were extracted from the tomato pericarp using a method described by Davis *et al*. (2007). Lyophilised samples (150 mg) were mixed thoroughly (using a Vortex Genie 2, G-560 E, Scientific Industries, Bohemia, NY) with 3 mL of 62.5:37.5 HPLC grade methanol : HPLC grade water (v/v) in 7 mL polystyrene bijou vials (Sterilin, Stone, UK). The vials were placed in a shaking water bath (Fisons, UK) at 55°C for 15 min and were removed and vortexed briefly every 5 min to prevent layering. Then samples were removed from the waterbath and left to cool at room temperature (25°C) for 10 min. The cooled samples were filtered using a 0.2 μm Millex - GV syringe driven filter unit (Millipore Corporation, Billerica, MA, USA). Resulting clear extracts were stored at -40°C until needed (**Figure 3.3**).

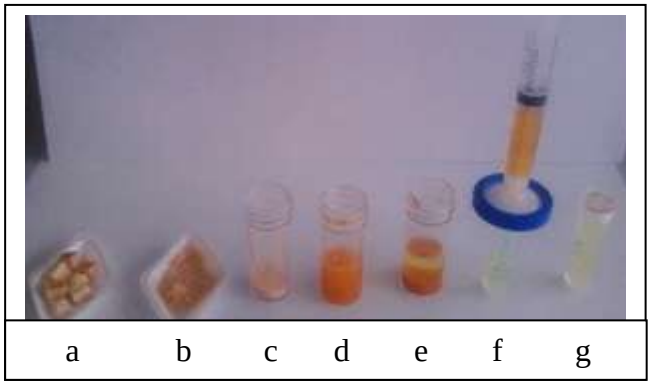

Figure 3.3. Stages of non-structural carbohydrate extraction process. Lyophilised tomato sample (a), powder of sample (b), 150 mg of sample in bijou vial (c), mixed sample with extraction solution (d), layering extract (e), filtration of extract (f), clear extract (g).

3.5.5.2 Quantification of NSCs

The NSCs content in pericarp tomato extracts was determined using a HPLC system comprising a P580 pump and GINA 50 autosampler (Dionex, Sunnyville, CA, USA) (**Figure 3.4**). The column used was a Rezex RCM monosaccharide Ca^{+} (8%) column of 300 mm × 7.8 mm diameter and 8 μm particle size (Phenomenex, Torrance, CA, USA, Part No. 00H-0130-K0, Serial No. 423216-1), fitted with a Carbo-Ca^{+} security guard cartridge of 4 mm × 3 mm diameter (Phenomenex, CA, Part No. AJO-4493). Mixed calibration standards of sucrose, glucose and fructose were prepared at concentrations of 0.05, 0.25, 0.5, 1.25 and 2.5 mg/mL. Tomato extracts were diluted 1:9 (v/v) with HPLC-grade water immediately before analysis. For the analysis of soluble sugars, 20μL of standards and of diluted extracts were injected automatically into the HPLC column. The column temperature was maintained at 75°C using a Dionex STH column thermostat. The mobile phase was HPLC grade water at a flow rate of 0.6 mL min^{-1}, which had been degassed by sparging with helium for 20 min prior to use. Eluted NSCs from extractions were detected by an evaporative light scattering detector (ELSD 2420, Waters, Milford, MA, USA) connected to the Dionex system using a UCI-50 universal chromatography interface. The total analysis time was 16 min followed by 5 min of re-equilibration. The retention times for sucrose, glucose and fructose were approximately 9.3, 11, and 13.7 min, respectively. The presence and abundance of fructose, glucose and sucrose were automatically calculated in each extract against the external standards by comparing peak area to the calibration curves using Chromeleon version 4.6 software (Dionex).

Figure 3.4. Dionex HPLC system.

3.5.6 Extraction and quantification of organic acids

3.5.6.1 Extraction of organic acids

Organic acids were extracted from tomato pericarp according to Terry *et al*. (2007) with some modifications. Lyophilised samples (150 mg) were dissolved into 3 mL of HPLC grade water in vials and left to stand for 5 min at room temperature (25°C). Then samples were agitated for 15 s using a vortex and the slurries were subsequently clarified by centrifugation at 10000 rpm (MSE Mistral 2000, Sanyo Gallenkamp, Leics., UK) for 3 min at 25°C. The supernatants were filtered using the above-mentioned 0.2 μm syringe driven filters and stored at -40°C until use.

3.5.6.2 Quantification of organic acids

Analysis of the organic acids was performed on an Agilent 1200 series HPLC binary pump system (Agilents, Berks., UK) equipped with an Agilent photodiode array with multiple wavelength detector (DAD, G1315B/G1365B) (**Figure 3.5**). L-ascorbic, citric and oxalic acid contents in extracts were detected at 210 nm. Mixed calibration standards of organic acids, including oxalic, L-tartaric, L-malic, L-ascorbic and citric acids were prepared at concentrations of 0.04, 0.08, 0.20, 0.40, 1.00 and 2.00 mg/mL. Filtered organic acid extracts (20μL) without dilution were injected automatically into an Alltech Prevail Organic Acid column 250 mm × 4.6mm diameter, 5 μm particle size (Alltech, CA; Part No. 88645, Serial No. 04061922.1), fitted with an Alltech Prevail Organic Acid guard column, 7.5 mm × 4.6 mm diameter, 5 μm (Alltech, CA, Part no. 96429). The mobile phase was 0.2% analytical grade metaphosphoric acid (HPO_3) in HPLC grade water (w/v), and before being used was filtered (Charles Austin Pump Ltd, B105 D/E, UK) using 0.4 μm filter and degassed as previously described. The flow rate of the mobile phase was 1 mL min^{-1} under isocratic conditions. The column temperature was set at 35°C and the temperature of the samples held at 4°C using an Agilent G1330B cooled autosampler. The total analysis time for the acids was 15 min, followed by 5 min of re-equilibration. All acids appearing from the tomato sample extracts were identified by comparing their absorbance spectra to the known external standards. Then the quantity of each identified acid was calculated by comparing the peak area obtained with those of standards using ChemStation Rev. B.02.01.

Figure 3.5. Agilent 1200 series HPLC system.

3.5.7 Extraction and quantification of carotenoids

3.5.7.1 Extraction of carotenoids

The carotenoids of the tomato samples were extracted using acetone as the solvent (Kimura and Rodriguez-Amaya, 2002; Porcu and Rodriguez-Amaya, 2008) with some modifications. That is, the samples were extracted in one step directly and used immediately. Freeze dried samples (100 mg) were dissolved into 5 mL of HPLC grade acetone in aluminum foil wrapped glass vials to exclude light and to allow optical view of their content (by removing the aluminum foil), at the same time. The samples were mixed thoroughly by vortexing for 20 s and then were left at room temperature (25°C) for 5 min. The supernatants were filtered in 12 mL brown glass vials using 0.2 μm syringe driven filters. The extraction and subsequent filtration were repeated once more so as the residue turned colorless. The supernatants were combined (~9 mL), mixed thoroughly and stored at -40°C in the dark for few min, because each of the extracts was immediately prepared for quantification analysis.

3.5.7.2 Preparation of carotenoid standards and determination of their concentration

Commercial carotenoid standards of lycopene (1 mg) and β-carotene (5 mg) were purchased from Sigma-Aldrich and prepared according to Scott (2001). Lycopene and β-carotene were individually dissolved in 10 mL of HPLC grade hexane (theoretical concentrations: an aliquot of 0.1 mg/mL for lycopene and 0.5 mg/mL for β-carotene were used). Each individual stock standard solution was divided into nine aliquots of 1 mL, in brown glass vials, evaporated to dryness and stored at -80°C until use for the preparation of mixed working standards. The remained volumes (~1 mL) were used to determine their actual concentrations. For the latter purpose, 0.1 mL of the solutions were diluted in hexane till 10 mL (total dilution factor DF = 100). The spectrophotometer (M501 UV/Vis Spectrophotometer, Camspec, Cambs. UK) was calibrated with hexane and the absorbance of the diluted standard solutions was measured at 470 nm for lycopene and 450 nm for β-carotene, using 1 cm pathlength vial cuvette. The actual concentrations of these standards were calculated using data given in **Table 3.1**, and the formula $(A \times V_1)/A^{1\%} \times C^{1\%}$, where A is the measured absorbance of the diluted standards, V_1 is the DF, $A^{1\%}$ is the extinction coefficient and $C^{1\%}$ is the concentration of 1% solution (Scott, 2001). The calculated concentrations of lycopene and β-carotene standards were 0.098 mg/mL and 0.2188 mg/mL respectively.

Table 3.1. λmax, extinction coefficients and measured absorbance of the carotenoid standards.

Standard	Solvent	Wavelength λmax (nm)	Extinction coefficient $A^{1\%}$ (AU)	Measured absorbance (AU)
Lycopene	Hexane	470	2592	0.567
β-carotene	Hexane	450	3450	0.338

3.5.7.3 Quantification of carotenoids

The quantification of all-trans lycopene and β-carotene in the pericarp tomato extracts was performed using a calibration curve made with a mixed working standard resulting from the above two individual standards. One mL of HPLC grade acetone was added in a vial containing lycopene dry stock and this was sonicated for 2 min to dissolve crystals. Then 900 μL of this solvent were diluted in 900 μL of acetone (DF = 2). One mL of HPLC grade dichloromethane (DCM) was added to a vial of dried β-carotene, this was sonicated to be dissolved, and then 100 μL diluted in 5 mL of DCM (DF = 50). Subsequently, a mixed calibration standard was prepared by combining 250 mL of each one of these two diluted solvents with resultant concentration of 0.0245 mg/mL lycopene (final DF = 4) and 0.002188 mg/mL β-carotene (final DF = 100). The quantification was performed using calibration curves constructed with five different injected amounts (1, 5, 10, 15 and 20 μL) of this mixed carotenoid standard (lycopene concentrations: 0.0245, 0.1225, 0.245, 0.3675, 0.49 mg/mL and β-carotene concentrations: 0.002188, 0.01094, 0.02188, 0.03282, 0.04376 mg/mL). All-trans lycopene and β-carotene contents in tomato extracts were determined using the same Agilent HPLC system as described above. HPLC apparatus coupled with DAD set to scan the wavelengths of maximum (λmax) absorption of these two carotenoids in the mobile phase. For the analysis 20 μL of extract was injected automatically into a Zorbax Eclipse XDB column 150 mm × 4.6 mm diameter, 5 μm particle size (Serial No. USKH034953), with a Zorbax XDB C18 guard column of 12.5 mm × 4.6 mm diameter. The mobile phase used consisted of ethylacetate (Fisher Scientific) stabilised by addition of 0.01% (w/v) butylated hydroxytoluene (BHT) and 0.1% (v/v) triethylamine (TEA) (A) and 90% (v/v) acetonitrile (ACN) in HPLC grade water (B), which were filtered and degassed as previously described. The gradient conditions were 0-5 min, 100% of B, 5-10 min, 100% of A and 10-15 min 100% of B, followed by 5 min re-equilibration with solvent B. The flow rate was 1 mL min^{-1}, the column temperature was set at 30°C and the temperature of the autosampler held at 4°C. Owing to the disparity in the absorption coefficient of lycopene and β-carotene two measurements were done, one at 472.4 nm and the other at 452.4 nm respectively. Eluted all-trans lycopene and β-carotene were detected at 6.5 and 6.88 min respectively. They were identified by comparing their absorbance spectra to the known external standards, and calculated by comparing peak area obtained with standards.

3.5.8 Extraction and measurement of tomato antioxidant activity

3.5.8.1 Extraction of tomato antioxidant compounds

The antioxidant capacity was studied through the evaluation of the free radical scavenging effect on the DPPH radical according to the procedure described by Xu *et al*. (2010) with slight modifications. Antioxidant compounds were extracted from tomato pericarp using MeOH/H_2O/2M HCl (70:29.5:0.5 v/v/v). Freeze-dried samples were weighed (75 mg) and transferred into 7 mL polysterene bijou vials with 1.5 mL solvent and mixed thoroughly by vortexing. The vials were placed in a shaking waterbath (HAAKE SWB 20, Germany) at 35°C for 1.5 h. They were removed briefly and shaken for 20 s every 15 min to prevent layering. Then samples were removed from the waterbath and left to cool at room temperature (25°C). The cooled samples were filtered using a 0.2 μm syringe driven filter unit. The clear extracts obtained were stored at -20°C in dark until further analysis, within 2 days.

3.5.8.2 Measurement of tomato antioxidant activity

Tomato extract in methanol (100 μL) was diluted with 400 μL PBS at pH 7.4. Then 100 μL of the diluent was added to 3.9 mL methanolic DPPH solution (0.0025 g/100 mL CH_3OH) in disposable cuvettes (1 cm × l cm × 4.5cm) used for visible absorbance measurements. The reaction mixture was mixed vigorously and incubated in darkness for 60 min at room temperature (25°C), so that the reaction could reach a plateau. Absorption of the samples was measured using a spectrophotometer (M 501 single beam scanning UV/visible, Camspec) at 515 nm. The blank solution used to calibrate the spectrophotometer consisted of 3.9 mL MeOH and 100 μL PBS. The blank was also used to check for recalibration between measurements, although no adjustments were necessary. The absorbance was recorded to determine the concentration of the remaining DPPH. The percentage of inhibition of DPPH of the test sample and known solution of Trolox (6-Hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid) were calculated by the following formula: % Inhibition = $100 \times (A_o - A)/A_o$, where A_o was the beginning absorbance at 515 nm, obtained by measuring the same volume of solvent (3.9 mL methanolic DPPH solution with 100 μL PBS), and A was the final absorbance of the test sample at 515 nm. The calibration curve between

% inhibition and known solutions of Trolox was then established. The radical scavenging activities of the test samples were expressed as micromoles of trolox equivalent antioxidant capacity per gram of dry sample (μM TE/g DW). Trolox standard solutions were prepared at a concentration ranging from 50 to 1000 μM.

3.6 Statistical analysis

All statistical analyses were carried out using Genstat for Windows version 8.1 (VSN International Ltd., Herts., UK). The differences between treatments, fruit position, the interaction of these two factors and greenhouse cabins were analysed through analysis of variance (ANOVA). Least significant difference values (LSD; $P = 0.05$) were calculated. Correlations between experimental variables were made using Spearman's rank Correlations.

CHAPTER FOUR

Investigation into possible antiviral effect of Benzothiadiazole and pyraclostrobin on tomato seedlings

4.1 Introduction

Greek horticulture is characterized by a great diversity of annual crops of high commercial value, amongst which tomato is particularly important. During the last decades epidemics of CMV have emerged as one of the most serious threats to tomato cultivation in Greece (Varveri and Boutsika, 1999; Kyriakopoulou *et al*., 2000), while TSWV and PVY epidemics on tomato have also occurred to a lesser extent. CMV causes yellow mosaic and curling of the leaves, stunting of vegetative growth and mosaic patterns on the fruits, is distributed systemically and leads to crop failure (Zitter, 1991; Conti *et al*., 1996; Zehnder *et al*., 2000).

CMV is readily transmitted by aphids, and it can be acquired by more than 80 aphid species during ingestion of food from infected plants and then can be transmitted to healthy plants during brief and superficial probing (Gallitelli, 2000). Aphid of species *Myzus persicae* (**Figure 2.8**, section 2.6.3.3) is an important pest of tomato plants, whose damage may be directly through phloem feeding or indirectly by the transmission of plant viruses such as CMV (Francki *et al*., 1979; Matthews, 1991).

Viruses, once established in a susceptible host, propagate outwards from inoculated cells to all parts of the plant and there is no curative method, in the way that fungicides protect against fungi, for suppressing viral diseases. Therefore, every effort should be made to prevent introduction of virus diseases into the plants. Among the most outstanding alternative and preventive methods is the use of plant induced resistance. Indeed, SAR is described as one of the strategies of efficient and environmentally respectful control (Zehnder *et al*., 2000).

BTH has been demonstrated to elicit SAR against a broad spectrum of plant-pathogen interactions, including solanaceous crops and viral diseases (Friedrich *et al*., 1996; Anfoka, 2000; Pappu *et al*., 2000; Csinos *et al*., 2001; Momol *et al*., 2001; 2004; Mandal *et al*., 2007; 2008; Nischwitz *et al*., 2008). All existing literature

concerns the plant-virus interactions tobacco-TSWV or tomato-TSWV (Momol *et al*., 2001) except for Anfoka (2000) who first indicated that BTH application as a drench protected tomato plants against the necrosis caused by a yellow strain of CMV. Later, Smith-Becker *et al*. (2003) established that BTH induced resistance also to cantaloupe against CMV.

Pyraclostrobin, on the other hand, has been reported to be, apart from a direct fungicide, a multi-functional crop protector by inducing a positive physiological effect in plants and making them more resistant to pathogens (Geraldino *et al*., 2008). In particular for viruses, Herms *et al*., (2002) demonstrated that pyraclostrobin enhanced the resistance of tobacco and NahG transgenic tobacco against TMV. Moreover, there were indications that pyraclostrobin induced plant resistance mechanisms against CMV and delayed disease development in tomato seedlings (Varveri *et al*., 2006).

In this study the commercially available plant defence activator Bion (a.i.: BTH) and fungicide F500 (a.i.: pyroclostrobin) were separately applied as a foliar spray on tomato seedlings and evaluated for their potential to confer resistance against different viruses such as CMV, TSWV or PVY. Below, firstly are presented the investigation and results of Bion and follow those of F500.

4.2 Examination of possible antiviral effect of Benzothiadiazole (BTH) on tomato seedlings

4.2.1 Experimental design

The possible antiviral activity of BTH (50 mg/L) was examined against CMV, TSWV and PVY. The experimental design of each case is presented separately as follows: the response against the mechanically inoculated CMV, the aphid transmitted CMV, the mechanically inoculated TSWV and finally, the mechanically inoculated PVY. Each experiment had two treatments (a) BTH treated and virus inoculated plants (either by mechanical inoculation or by aphid transmission) and (b) virus inoculated (in the same way respectively) plants as the control.

4.2.1.1 Examination of CMV mechanical inoculation in BTH treated tomato seedlings

One experiment was performed to investigate the effect of repeated applications of BTH on CMV mechanically inoculated tomato plants. Twenty-seven plants were used for each treatment (9 replications × 3 plants). Inoculation was carried out seven days after BTH treatment (50 mg/L) on the first true leaf and BTH was applied weekly. Six samplings were carried out in new expanded leaves and were followed by ELISA analysis.

4.2.1.2 Examination of CMV aphid transmission in BTH treated tomato seedlings

Two experiments were conducted to investigate the effect of BTH on aphid transmitted CMV. For each treatment of experiment 1, 16 plants (4 replications × 4 plants) were used, for experiment 2, 30 plants (6 replications × 5 plants) and for both of them 20-22 aphids per plant. In experiment 1 BTH (50 mg/L) was applied once and virus aphid transmission was carried out seven days after BTH treatment. In experiment 2 BTH was applied weekly and virus aphid transmission was carried out five days after the first BTH application. Samplings were performed in newly expanded leaves seven and five times, respectively, and ELISA analysis of all samples was conducted.

4.2.1.3 Examination of TSWV mechanical inoculation in BTH treated tomato seedlings

Three experiments were conducted in total with different incubation periods for BTH or different numbers of BTH applications. Experiment 1 was performed with the mean time of four days between BTH treatment and virus inoculation, in which 16 plants (4 replications × 4 plants) were used for each treatment and the inoculation was done on cotyledons. Six samplings were performed in newly expanded leaves and followed by ELISA analysis. The experiments 2 and 3 were performed with the meantime of seven days between BTH treatment and virus inoculation. For experiment 2, 16 plants (4 replications × 4 plants) were used for each treatment, BTH was applied once and virus inoculation was conducted on cotyledons. For experiment 3, 20 plants (4 replications × 5 plants) were used for each treatment, BTH was applied weekly and virus inoculation was conducted only on first true leaf. Samplings for ELISA analysis were performed in newly expanded leaves five and four times respectively.

4.2.1.4 Examination of PVY mechanical inoculation in BTH treated tomato seedlings

One experiment was conducted with 20 plants for each treatment (4 replications × 5 plants) and virus inoculation was carried out on the two first true leaves. BTH was applied weekly and three samplings were carried out in newly expanded leaves for ELISA test.

4.2.2 Statistical analysis

The viral disease presence in tomato plants was assessed at several times and the results were presented collectively as a disease progress curve [that is the graph of disease intensity versus days post inoculation (dpi)]. Data on the percentage of infected plants were used to calculate the area under disease progress curve (AUDPC) per treatment (Campbell and Madden, 1990) and the absolute rate of disease increase (dy/dt).

The AUDPC can be estimated as:

$$\text{AUDPC} = \sum_{i=2}^{n} [(y_i + y_{i-1})/2] \, (t_i - t_{i-1})$$

Where i: is the time dimension (in assessment)

y: is the level of infection

t: is time in days (between two successive assessments) and

n: is the number of total Elisa analysis

and areas give the rate of infected plants per day.

For the rate dy/dt:

dy denotes the difference in infection between two successive assessments and dt the lag time between two assessments.

Data of: (a) AUDPC values or (b) dy/dt were subjected to a repeated measures ANOVA, which is an analysis involving the disease presence at each time. The significance of difference was assessed by Student's unpaired *t test* which compaired the means of two independent samples (treatment versus untreated control). If the observed significance level was small enough (less than 0.05) then the null hypothesis that the treatment and the control were equal was rejected.

Statistical analysis was performed using GENSTAT for Windows version 8.1 (VSN International Ltd., Herts., UK).

4.2.3 Results

4.2.3.1 Response of BTH treated tomato seedlings to mechanically inoculated CMV

It was observed (**Figure 4.1**) that within 15 days, 100% (27/27) of CMV inoculated control plants were infected while the respective rate of CMV inoculated plants which had been treated with BTH was only 3.7% (1/27) and on the 22nd day reached the value of 14.81% (4/27) that is more than 85% difference between the two treatments. A t-test was carried out with the values of AUDPCs and it was found that the AUDPC of control [1,419 ± 73.2 (s.e.)] was significantly different ($P < 0.001$) from the respective one of BTH treatment [68.52 ± 42.5 (s.e.)] (t = 15.95 on 16 d.f.).

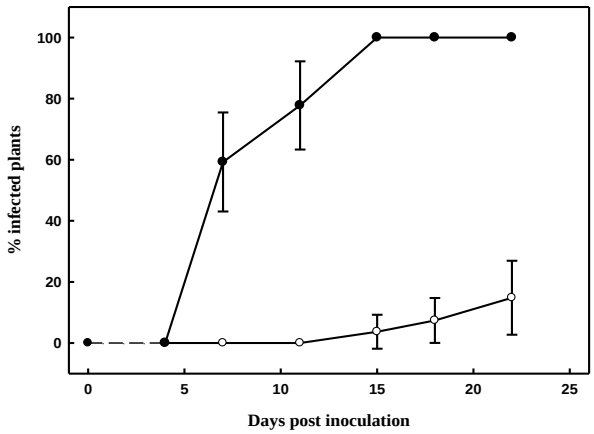

Figure 4.1. Percentage of infected tomato plants mechanically inoculated with CMV4, seven days after the first application of BTH (50 mg/L) (○) and water (●). The short dash line represents the interval between inoculation and first sampling. The bars indicate ±s.d.

4.2.3.2 Response of BTH treated tomato seedlings to aphid transmitted CMV

In the experiment 1, with the single BTH application (**Figure 4.2**, **A**), although less tomato plants of BTH treatment were CMV infected versus to control the difference was not significant, due to the high variability among different replications. The AUDPC/days of control [19.33 ± 7 (s.e.)] was not significantly different ($P = 0.365$) from the respective one of BTH treatment [10.3 ± 5.99 (s.e.)] (t = 0.98 on 6

d.f.). Moreover, a t-test was carried out with the values of the term dy/dt for the 21st day, the day with the highest difference between the two treatments, and there was not any significant difference between the two treatments (P = 0.356).

In the experiment 2, with weekly BTH applications (**Figure 4.2**, **B**), it was observed that a slower infection of BTH treated plants took place mainly for the first ten days compared with the untreated control plants. From the statistical analysis it was found that the AUDPC/days of control [29.76 ± 5.10 (s.e.)] was significantly different (P = 0.014) from the respective one of BTH treatment [11.27 ± 3.56 (s.e.)] (t = 2.97 on 10 d.f.).

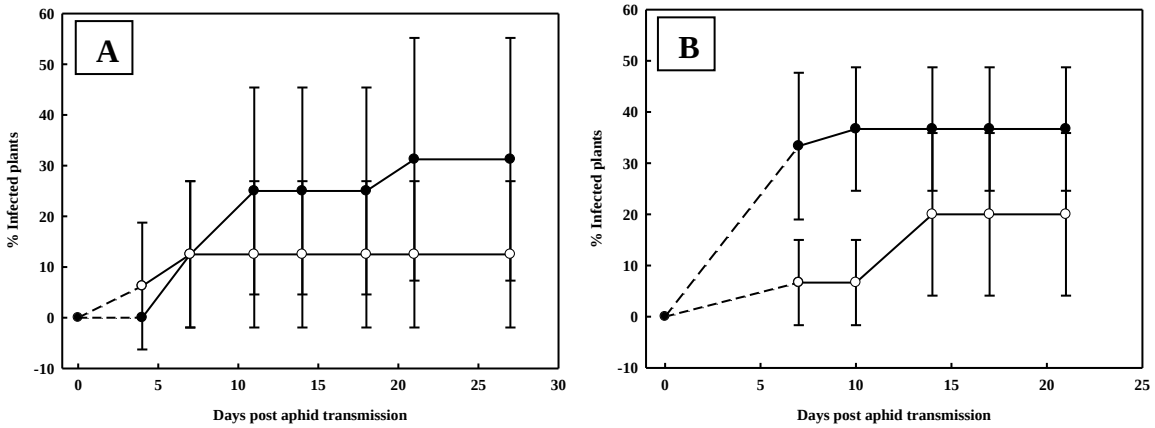

Figure 4.2. Percentage of infected tomato plants by aphid transmission of CMV4, (**A**) seven days after the single application of BTH (50 mg/L) (○) and water (●), (**B**) five days after the first application of BTH (50 mg/L) (○) and water (●). The short dash line represents the interval between aphid transmission and first sampling. The bars indicate ±s.d.

4.2.3.3 Response of BTH treated tomato seedlings to mechanically inoculated TSWV

For the experiment 1, with the mean time of four days between BTH treatment and virus inoculation, a t-test was carried out with the values of AUDPC/days. It was found that the AUDPC/days of TSWV inoculated control [45.60 ± 17.02 (s.e.)] was not significantly different (P = 0.594) from the respective one of BTH treated and TSWV inoculated plants [33.38 ± 13.45 (s.e.)] (t = 0.56 on 6 d.f.) (**Figure 4.3**, **A**).

For the experiment 2, with the meantime of seven days, at 8th day post inoculation (dpi) only 25% of the BTH treated plants were infected when all untreated control plants were infected and at 19th dpi the difference of the infected plants between the two treatments was reduced from 75% to 15% (**Figure 4.3**, **B**). The AUDPC/days of control [71.05 ± 2.15 (s.e.)] was significantly different (P = 0.009) from the respective one of BTH [41.45 ± 7.53 (s.e.)] (t = 3.78 on 6 d.f.).

In the experiment 3 (**Figure 4.3**, **C**) at 8th dpi a difference of 60% between the two treatments was observed and a t-test was carried out with this day's values of the term dy/dt and it was found that dy of control [22.5 ± 2.5 (s.e.)] was significantly different (P = 0.017) from the respective one of BTH [10 ± 2.89 (s.e.)] (t = 3.27 on 6 d.f.). Moreover, it was found that the AUDPC/days of control [62.67 ± 2.67 (s.e.)] was significantly higher (P = 0.005) than the respective one of BTH [41.33 ± 4.26 (s.e.)] (t = 4.24 on 6 d.f.).

4.2.3.4. Response of BTH treated tomato plants to mechanically inoculated PVY

Only one experiment was performed and 8 dpi all plants were infected with PVY regardless of the treatment. So, there was not statistically significant difference between the infection rate of the BTH treated plants and the control ones.

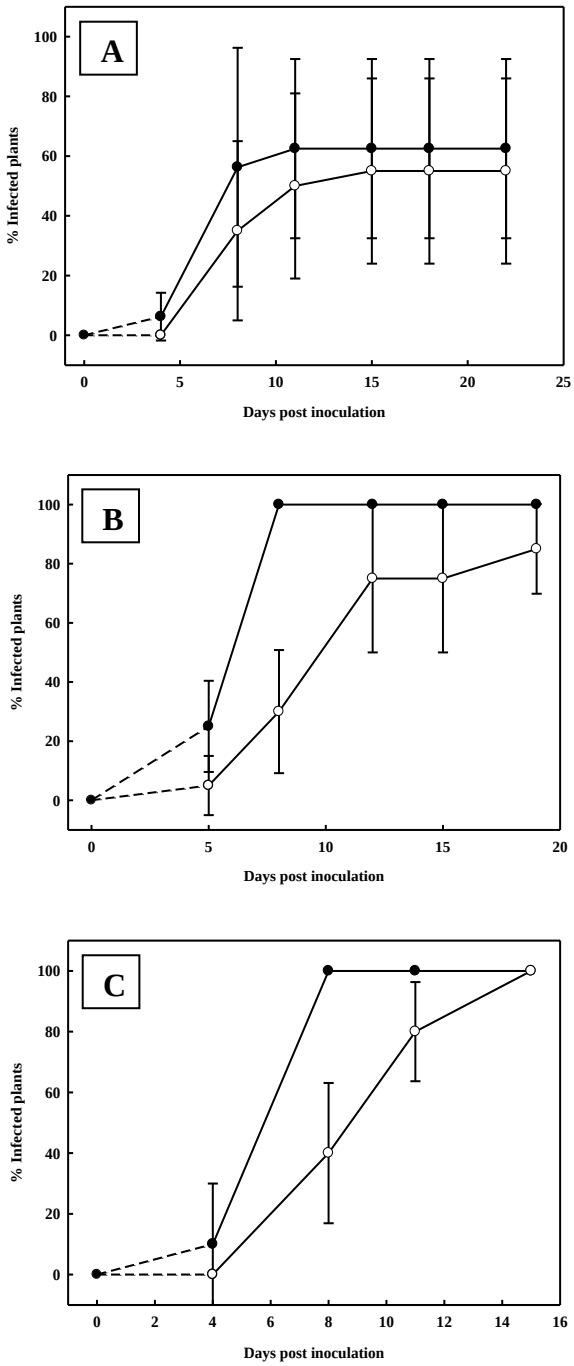

Figure 4.3. Percentage of infected tomato plants mechanically inoculated with TSWV (**A**) four days after the application of BTH (50 mg/L) (○) and water (●), (**B**) seven days after the single application of BTH (50 mg/L) (○) and water (●), (**C**) seven days after the first application of BTH (50 mg/L) (○) and water (●). The short dash line represents the interval between inoculation and first sampling. The bars indicate ±s.d.

4.2.4. Discussion of BTH antiviral effect

As BTH stimulates plants to activate their natural defence mechanisms, treated plants could be protected from a broad spectrum of pathogens. Many studies have been performed to establish the efficacy of BTH in controlling diseases caused by fungi and bacteria in diverse plants. The available literature specifically for solanaceous crops against fungi (Friedrich *et al*., 1996; Benhamou and Belanger, 1998; Inbar *et al*., 1998; Tally *et al*., 2000; Hennin *et al*., 2001; Matheron and Porchas, 2002; Perez *et al*., 2003; Achuo *et al*., 2004; Maölepsza, 2006) and bacteria (Cole, 1999; Louws *et al*., 2001; Romero *et al*., 2001; Abbasi *et al*., 2002; Buonaurio *et al*., 2002; Soylu *et al*., 2003; Obradovic *et al*., 2004; 2005; Abo-Elyousr *et al*., 2008; Herman *et al*., 2008) is abundant. However, little is known about the ability of BTH to trigger SAR against different plant species and diverse virus diseases, as the main plant-pathogen interaction studied is tobacco-TSWV (Friedrich *et al*., 1996; Pappu *et al*., 2000; Csinos *et al*., 2001; Momol *et al*., 2001; 2004; Mandal *et al*., 2007; 2008; Nischwitz *et al*., 2008).

This study provides the first evidence of the possible antiviral effect of BTH against a Greek isolate of CMV (CMV4). The experiments conducted to clarify the efficacy of BTH against CMV4 suggest that this chemical plant activator could be valuable to tomato cultivation by helping virus control.

Dicotyledonous crops have been demonstrated to require repeated applications of BTH to extend pathogen protection over time (Görlach *et al*., 1996; Cole, 1999; Morris *et al*., 1998; Louws *et al*., 2001; Romero *et al*., 2001). For instance, repeated applications of BTH were demanded for tobacco plants to acquire prolonged protection against early blight (Csinos *et al*., 2001), and for tomato plants against bacterial spot and bacterial speck (Louws *et al*., 2001). In the Laboratory of Virology at BPI, experiments were conducted in order to examine the effect of one or repeated applications on tomato plants of BTH to mechanically inoculated or aphid transmitted CMV4. Trials with only one BTH application and mechanical inoculation of CMV4 had been carried out (Varveri, unpublished results) and it was shown that a week after virus inoculation the disease incidence in the treated plants was 50-70% lower than in the control ones. In the current study where BTH was applied at weekly intervals the difference between treated and control plants was multiple (85% the 22nd dpi, **Figure 4.1**). Moreover repeated BTH application was effective the first days against aphid transmitted CMV4 in tomato (**Figure 4.2**, **B**), whereas one application was totally

ineffective (**Figure 4.2**, **A**). Therefore, repeated applications of BTH as a foliar spray are more effective than single ones against CMV4 in tomato plants, regardless of the way the virus is transmitted.

In contrast to these results, Anfoka (2000) showed that a single BTH treatment protected them against aphid transmission of CMY-Y. CMY-Y is a yellow strain of CMV, known to cause severe necrosis. It was then demonstrated that one BTH application to the roots of young tomato plants, as soil-drench, reduced the incidence and the severity of the disease. According to Friedrich *et al*. (1996), BTH is highly mobile in tobacco plants, and Anfoka (2000) concluded that BTH could translocate from the root system of tomato plants to the upper plant parts where certain SAR genes involved in the reduction of virus replication are activated. The different way of single BTH application might have affected its effectiveness. Moreover, Anfoka (2000) supposed that activation of SAR genes in BTH treated tomato plants might involve some modifications of the structure of the plasmodesmata, in such a way that virus movement from infected cells to the neighbouring cells is inhibited. The hypothesis that BTH could be effective against different strains of CMV in tomato plants either as a foliar spray or as a soil drench needs further experimentation.

Mandal *et al*. (2008) demonstrated that treatment of tobacco with BTH activates high levels of resistance against a severe isolate of TSWV after mechanical inoculation. Tobacco plants were sprayed with increased quantities (0.25, 0.5, 1.0, 2.0 and 4.0 g a.i. / 7,000 plants) of BTH and showed increased levels of SAR. The used concentration of BTH per plant in the current study was nearly the highest concentration used by Mandal *et al*. It is worth pointing out that in their experiment plants after BTH application were washed by spraying with water to move the BTH into the root zone. This is the second reference (Anfoka, 2000 was the first) for the transmission of BTH into plants through their root system, so BTH could be applied as a soil drench in agriculture, which may be easier than the foliar spray.

Resistance induced by SAR agents requires an induction period that is an interval of time between application of the agent and the challenge with the pathogen. In most cases this interval was reported to lie between three and seven days. Hence, according to Mandal *et al*. (2008) five days should be allowed after the BTH treatment for inducing a high level of resistance in tobacco plants. Godard *et al*. (1999) and Soylu *et al*. (2003) showed that the best protection of cauliflower against downy mildew and tomato against *Clavibacter michiganensis* respectively was

obtained when BTH was applied three days before inoculation. In the current study using tomato seedlings the effect of the intervals of four or seven days between BTH application and mechanical inoculation of TSWV was examined. The best protection against TSWV disease was obtained when BTH was applied seven days before virus inoculation. Indeed, BTH was ineffective when the interval of time was four days (**Figure 4.3**, **A**), less than five days and more than three days that Mandal *et al*. Godard *et al*. (1999) and Soylu *et al*. (2003) had set as limit respectivelybut it was effective when the interval was seven days irrespective of the number of BTH applications (**Figures 4.3 B** and **C**). Resende *et al*. (2002), however, found that in cocoa plants BTH needed a longer interval period (of about 15-30 days) for development of resistance against *Verticillium wilt* inoculation.

This result showed that apart from the number of BTH applications, the incubation period that BTH needs to bring about SAR, plays also an important role in tomato plants.

There were observations that tomato seedlings treated with BTH were smaller than nontreated plants in greenhouse experiments (Louws *et al*., 2001). This fact was also noted in current experiments with tomato seedlings. Furthermore, the plant growth reduction due to BTH treatment was reproduced, and assessed as well, in two other experiments (described in chapter 6) with cultivation of tomato plants leading to fruit production. Although BTH is capable of suppressing CMV and TSWV in tomato plants, stunting caused by BTH could be an issue. Mandal *et al*. (2008) used gibberellic acid in combination with BTH and reduced the stunting caused by BTH.

Moreover, Pappu *et al*. (2000) tested thrips transmission of TSWV in field trials under foliar applications of BTH singly or in combination with another plant activator, imidacloprid. The results showed that BTH significantly reduced final disease incidence in two out of four trials and when both compounds were applied significant reduction in incidence occurred at three out of four trials. So BTH may be more effective when it is used in combination with other compounds that induce plant defence mechanisms.

Taking together the above results of Pappu and Mandal it emerges that it may be possible to apply BTH with other elicitor cocktails that induce on the one hand a balance of defences (regulated by SA, jasmonic acid, ethylene, or other regulators) against specific pests or complexes of threats, and on the other hand a balance of plant growth as regards the BTH induced stunting.

Regarding the efficacy of BTH against PVY there is no previous work examining BTH and this virus. In the experiment of this study, BTH was ineffective against this virus. This could be due to the fact that PVY was able to thwart host defences or because tomato lacked the capacity to initiate defences against this pathogen, or because of lack of efficacy of BTH. This result suggests that the protective effects of BTH against pathogens in general, and viruses in particular, cannot be taken for granted and that its effect on a particular crop and pathogen should be independently evaluated.

4.3 Examination of possible antiviral effect of pyraclostrobin on tomato seedlings

4.3.1 Experimental design and statistical analysis

The possible antiviral activity of pyraclostrobin (0.8 mL/L) was examined against CMV (mechanically inoculated or aphid transmitted) and PVY (mechanically inoculated). The experimental design of each case is presented separately below. Each experiment had two treatments (a) pyraclostrobin (formulation F500) treated and virus inoculated plants (either by mechanical inoculation or by aphid transmission) and (b) virus inoculated (in the same way) plants as the control.

The statistical analysis was performed as described in section 4.2.2 by analyzing data of AUDPC values and/or dy/dt, using GENSTAT for Windows version 8.1.

4.3.1.1 Examination of CMV mechanical inoculation in pyraclostrobin treated tomato seedlings

Three experiments were carried out with CMV4 isolate under different inoculum pressures. In experiment 1, 20 plants (4 replications × 5 plants) were used per treatment and virus inoculation was applied only on cotyledons (low inoculum pressure). In experiment 2, 30 plants (6 replications × 5 plants) and in experiment 3, 27 plants (9 replications × 3 plants) were used per treatment and virus inoculation was applied on cotyledons and the first true leaf (high inoculum pressure). Samplings were performed at intervals of three or four days and ELISA analysis of all samples followed.

4.3.1.2 Examination of CMV aphid transmission in pyraclostrobin treated tomato seedlings

Three experiments were conducted. Experiments 1 and 2 were carried out using the CMV4 isolate. Fifteen plants were used for each treatment (3 replications × 5 plants) with 10 aphids per plant. Samplings were performed after aphid-virus transmission and were followed by ELISA analysis.

The experiment 3 was carried out using the CMV50 isolate, a more recent field isolate in order to achieve better aphid transmission rates, with 20 plants for each treatment (4 replications × 5 plants) and 8 aphids per plant.

4.3.1.3. Examination of PVY mechanical inoculation in pyraclostrobin treated tomato seedlings

Three experiments were performed with 20 plants for each treatment (4 replications × 5 plants) and virus inoculation was applied only on cotyledons. Two PVY isolates, namely PVY69 (for experiments 1 and 2) and PVY1 (for experiment 3), were used to evaluate the differential response of pyraclostrobin treated plants to different strains.

4.3.2 Results

4.3.2.1 Response of pyraclostrobin treated tomato seedlings to mechanically inoculated CMV

In the experiment 1 (**Figure 4.4**) under low inoculum pressure (inoculation of cotyledons) a relatively lower infection levels of pyraclostrobin treated plants compared to the control were observed. However, the t-test on the AUDPCs (up to the day 10) values showed that the AUDPC of control [150 ± 44 (s.e.)] was not significantly different (P = 0.360) to the respective one of pyraclostrobin [90 ± 41 (s.e.)] (t = 0.99 on 6 d.f.), although differences in absolute values were evident. This happened probably because the experiment showed high variation within the replications. The same analysis was carried out with the square root transformation of disease incidence values of the tenth day which verified the aforementioned findings (P = 0.364) [control: 8 ± 1.3 and pyraclostrobin: 5.6 ± 2.1 (s.d.)] (t = 0.98 on 6 d.f.).

In the experiments 2 and 3, under high inoculum pressure (inoculation of cotyledons and first true leaf) all pyraclostrobin treated plants were infected within a period of six days after inoculation and no significant slower disease development was obtained (data not shown).

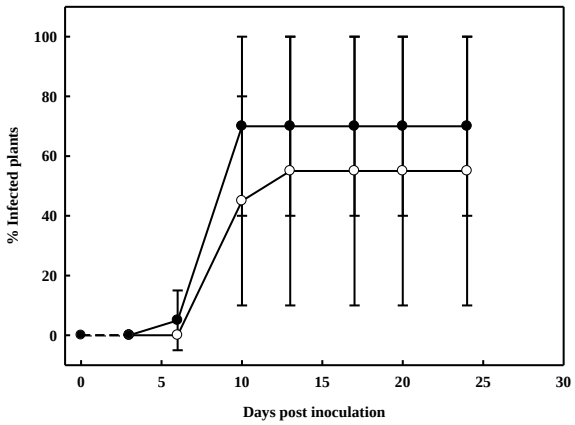

Figure 4.4. Percentage of infected tomato plants mechanically inoculated with CMV4 (under low inoculum dynamic), 24h after the first application of pyraclostrobin (0.8 mL/L) (○) and water (●). The short dash line represents the interval between inoculation and first sampling. The bars indicate ±s.d.

4.3.2.2 Response of pyraclostrobin treated tomato seedlings to aphid transmitted CMV

In the experiments where CMV4 isolate was used the following results were recorded. In the experiment 1 although slower disease development was observed in the pyraclostrobin treated plants (**Figure 4.5**, **A**), the variable response of plants within the same treatment was high resulting in no statistically significant differences between treated and untreated control plants. The statistical analysis showed that the AUDPC value of the control [50.12 ± 11.1 (s.d.)] was not significantly different (P = 0.296) to that of pyraclostrobin [29.87 ± 12.7 (s.d.)] (t = 1.20 on 4 d.f.). In the experiment 2 also there was not statistically significant difference between the two treatments (**Figure 4.5**, **B**).

Similarly, in the experiment 3 no significant differences were obtained with CMV50 isolate. In this case the t-test was carried out with the values of the apparent infection rate (dy/dt: slope of line) for the period of the first week. It was found that the rate of the control [8.57 ± 1.17 (s.d.)] was not significantly different (P = 0.620) to that of pyraclostrobin [7.14 ± 2.47 (s.d.)] (t = 0.52 on 6 d.f.).

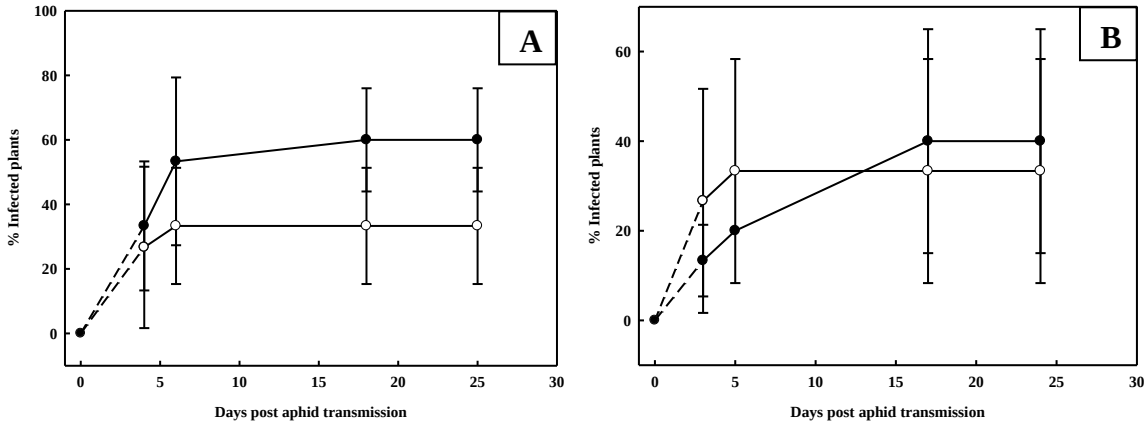

Figure 4.5 A and **B.** Percentage of infected tomato plants by aphid transmission of CMV4, 24h after the first application of pyraclostrobin (0.8 mL/L) (○) and water (●). The short dash line represents the interval between aphid transmission and first sampling. The bars indicate ±s.d.

4.3.2.3 Response of pyraclostrobin treated tomato seedlings to mechanically inoculated PVY

In the experiments where PVY69 isolate was used, pyraclostrobin treated plants showed a lower infection rate compared to the control in a period of six or seven days after inoculation. In the experiment 1 (**Figure 4.6**, **A**) the AUDPC value of the control [322.5 ± 35 (s.d.)] was found significantly higher (P = 0.017) than the respective one of F500 [235 ± 40.41 (s.d.)] (t = 3.27 on 6 d.f.).

In the experiment 2 (**Figure 4.6**, **B**) similar results were obtained with the control plants [497.5 ± 17.5 (s.e.)] showing significantly higher disease progress curve than the pyraclostrobin treated plants [427.5 ± 17.5 (s.e.)] (t = 2.83 on 6 d.f.), (P = 0.03).

On the contrary, in the experiment 3 where the PVY1 isolate was used, pyraclostrobin treated plants did not react differently to the virus compared to the untreated ones.

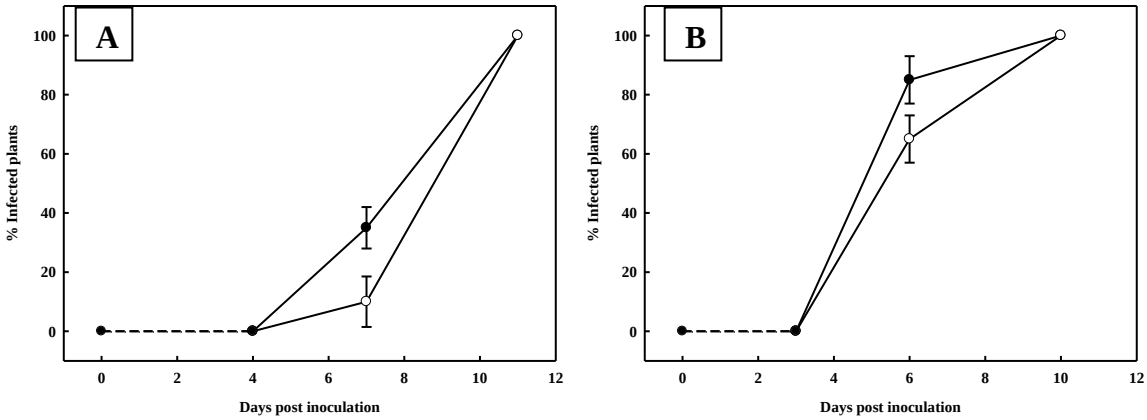

Figure 4.6 A and **B.** Percentage of infected tomato plants inoculated with PVY69, 24h after the application of pyraclostrobin (0.8 mL/L) (○) and water (●). The short dash line represents the interval between inoculation and first sampling. The bars indicate ±s.d.

4.3.3 Discussion of pyraclostrobin antiviral effect

The strobilurin class of fungicides has a broad spectrum antifungal activity as it may control plant pathogenic fungi from all of their four classes: the Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes (Bartlett *et al*., 2001). Especially for pyraclostrobin there was reference that in addition to direct antifungal activity, it may also prime plants for activation of subsequently pathogen induced defence responses. Indeed, Herms *et al*. (2002) demonstrated that pyraclostrobin enhanced the resistance of tobacco against TMV. Varveri *et al*. (2006) examined the effect of pyraclostrobin in tomato plants against mechanically inoculated CMV and TSWV. They observed lower disease incidence and slower development of CMV infection in pyraclostrobin treated plants, when inoculation with the virus was carried out 24 hours after pyraclostrobin application. However, there were no significant differences between the treated and untreated plants when TSWV was the pathogen.

In the current study, as a continuation of the above work, new experiments were carried out in order to estimate the effect of pyraclostrobin against aphid transmitted CMV. Experiments were conducted using two CMV isolates, the CMV4, a well established isolate propagated in the greenhouse of BPI since 1995 , and the CMV50, a more recent field isolate (2007) in order to achieve better aphid transmission rates. It was shown that no significant differences in virus incidence were obtained between pyraclostrobin treated and untreated control plants for both isolates (**Figures 4.5 A** and **B** for CMV4). Moreover, repetition of the experiments conducted by Varveri *et al*. (2006) with mechanical CMV inoculation did not result in confirmation of the pre-mentioned results (**Figure 4.4**).

The fact that the results obtained in 2006, leading to the conclusion that CMV development was delayed in tomato plants mechanically inoculated, were not reproduced in 2007 could be explained by the different seasons that the experiments were conducted (May-July 2006; July-November 2007) as all the other parameters were approximately the same. Although all the experiments were conducted in the same greenhouse of controlled environmental conditions, light quality and to some extent temperature were different at the different seasons. Moreover, season influences the physiological condition of the plants which react differently towards chemical stimulants of their defence system resulting in variability of disease resistance induction (Terry and Joyce, 2003; 2004). According to Koricheva *et al.*

(1998) and Terry and Joyce (2004) in resource rich environments growth process demands carbon for protein synthesis and reduces the availability of carbon skeletons for carbon-based secondary defence related compounds. So, although the necessary data to come to a conclusion are not available, it could be presumed that plants reacted better to pyraclostrobin during spring 2006 than summer 2007 due to the season difference. The influence of different cultivation seasons on tomato plant growth, CMV severity and a plant's activator (BTH) effectiveness is discussed in chapter 6, based on two experiments which were conducted during winter and spring of two consecutive years.

Herms *et al*. reported that pyraclostrobin failed to induce resistance against PVY in tobacco cv. Xanthi (2002). In the current study, experiments were conducted in order to investigate the effect of pyraclostrobin against two isolates of PVY mechanically transmitted in tomato plants. Significant difference was obtained with one of them only for ten to eleven days after inoculation (**Figures 4.6**, **A** and **B**). This result denotes that pyraclostrobin seems to have an effect of delaying PVY disease development on tomato depending on the virus isolate.

Furthermore, in this study, the spectrum of pyraclostrobin antiviral activity and the influence on virus multiplication and movement at very early stages of infection was studied using *Tobacco rattle virus* (TRV) in tobacco plants (data not shown). An infectious clone of an English isolate of TRV of reduced fitness (TRV-PpK20) genetically modified to express the green fluorescent protein (GFP), emitting green fluorescence under UV light, was used to visualize the replication and the movement of the virus in the pyraclostrobin treated and untreated tobacco plants. During the first days after mechanical virus transmission, treated plants showed delay in virus local lesion expression, movement and replication. Finally, systemic infection occurred in 21% fewer plants of pyraclostrobin treatment than those of the untreated control. Additional experiments with a highly virulent Greek isolate (TRV-GR) and the English wild type (TRV-PpK20 not modified) strain were carried out, but no differences were observed between pyraclostrobin treated and untreated plants.

According to all the above mentioned results, regarding the effect of pyraclostrobin against viruses, the overriding conclusion is that pyraclostrobin induced resistance may act against some host-virus isolate combinations under certain conditions.

Synoptically all the results of the experiments conducted to examine the antiviral effect of BTH and pyraclostrobin are presented in **Table 4.1**.

Table 4.1. Effectiveness of BTH and pyraclostrobin against tomato viruses.

Virus	**CMV**		**TSWV**	**PVY**
Compound	Aphid transmission	Mechanical inoculation	Mechanical inoculation	Mechanical inoculation
BTH	++	+++*	+++	-**
pyraclostrobin	-	-	-***	+

*+++, ++, +: difference between treatment and control >50%, 25-50%, 10-20% respectively, one week after the inoculation of viruses

**-: no statistical significant differences were observed

***-: Varveri, personal communication

4.4 General discussion of Benzothiadiazole and pyraclostrobin antiviral effect on tomato seedlings

It is generally recognised that there is no viable control of plant viruses, except the preventive measures, yet these often are inadequate. Therefore, control of CMV remains a major problem, because it is very widespread, can infect many species of plants, tomato included, and is very efficiently and quickly spread by many species of aphids. Inducing NDR may be one long-term effective virus control measure, although it is not a stand-alone method for pest control, but another tool that will need to be further integrated into pest management systems.

One form of NDR is SAR, the process of a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens, mediated by accumulation of endogenous SA. The classic form of SAR can be triggered artificially with chemical compounds called "plant activators", such as BTH. Benhamou and Bélanger (1998) were the first who demonstrated that BTH induces SAR in tomato plants.

A plant defence strategy aimed at viruses could conceivably target any one or all of these processes: replication, trans-plasmodesmata movement or transport through the vasculature. Thus, according to Anfoka (2000), BTH may target the structure of plasmodesmata and reduce the rate of virus movement. Evidence is provided in this study that BTH treatment confers increased protection of tomato plants against infection by CMV or TSWV, though whether the induced resistance is directed to act against virus replication or movement has not been determined.

Exogenous application of BTH to diverse plants has been shown to activate a number of SAR-associated genes, leading to enhanced plant protection against various pathogens (Friedrich *et al* 1996; Görlach *et al*., 1996; Lawton *et al* 1998). There are many references about the positive results of BTH in suppressing a wide range of diseases but there are also some references that demonstrate the ineffectiveness of BTH to induce resistance. Indeed, there are some studies stating that BTH derivatives are relatively ineffective in some plant-pathogen systems and/or environments. In the current study BTH was ineffective against PVY. This may be due to biological limitations hindering the practical use of BTH, such as the pathogen that may not respond to elicitors, the lapse of time between treatment and expression of resistance that occurs, the part of the plant that will be treated, or the growing

environment. Moreover, there may be side effects on growth or yield characteristics when BTH is used.

In contrast to BTH, the strobilurin class of fungicides, with broad-spectrum activity against all four classes of plant pathogenetic fungi, has positive physiological effects on the plants, the so-called "greening effect". This physiological effect makes plants treated with strobilurins look healthier than untreated plants, and improves crop production and quality, even in the absence of challenge by fungal pathogen attack.

Herms *et al*. (2002) investigated whether strobilurins might enhance the capability of plants to ward off other pathogens other than fungi. They used pyraclostrobin, a relative new strobilurin fungicide, and reported an enhancement of TMV resistance not only in tobacco plants (*N*. *tabacum* cv. Xanthi nc) but also in *nahG* transgenic tobacco plants. In the same study in infiltrated leaves, pyraclostrobin did not cause the accumulation of SA-inducible PR-1 proteins that are often used as conventional molecular markers for SA-induced disease resistance, so Herms *et al*. concluded that pyraclostrobin enhanced TMV resistance in tobacco by functioning independently of SA.

The fungicidal activity of the strobilurins lies in their ability to inhibit mitochondrial respiration by affecting the mitochodrial bc1 complex, thereby blocking electron transfer. Since the bc1 complex persists in all eucaryotae, at least one partial inhibition in the transportation of electrons must also be expected in plant cells after absorbing the strobilurin fungicides (Venancio *et al*., 2003). So strobilurin type fungicides may cause an increase in ROS with subsequent effect in movement and viral replication.

The effect of pyraclostrobin in the production of potato plants cv. Agate infected by the viruses PVY or PVX was investigated by Geraldino *et al*. (2002) and it was shown that potato plants pyraclostrobin-treated and inoculated with PVY or PVX increased their production compared to the plants treated and not inoculated, and those not treated and not inoculated. This extraording result indicated a new alternative for the decrease of losses caused by viruses in the field. So, pyraclostrobin, in addition to exerting direct antifungal activity, might protect plants by priming them for potentiated activation of subsequently pathogen-induced cellular defence responses and more interestingly may improve crop production and quality.

The results obtained from this study have given useful information about the antiviral effect of BTH and pyraclostrobin against Greek virus isolates in tomato

plants. In particular BTH showed to have an antiviral activity against CMV and TSWV, whereas pyraclostrobin was ineffective against these viruses, but effective against one PVY isolate during the immediate period after inoculation. So, from the examined compounds only BTH, the plant activator of SAR, seems to play an important role in the ability of tomato plants to defend themselves against different viruses.

Thus far, the phenomenon of SAR has attracted much attention as a new strategy for controlling plant diseases, but there are many unresolved questions about the use of these kinds of chemical compounds, such as optimal timing and method of application on different crops, integration with other types of pesticide, and interactions with the physiology of the plant. Effective disease control strategies will come from a better understanding of disease and resistance. Newer and more effective elicitors of SAR will be developed, perhaps in part as the result of a growing understanding of the underlying mechanisms of these pathways within the plant. In the future, it may be possible to apply a combination of different elicitors, for instance BTH and pyraclostrobin, in order to succeed in solving both the problems of controlling various plant pathogens and having the best plant growth respectively. However, this future will require a shift in conventional agriculture away from the total reliance on pesticides to solve pest problems, and a concerted effort to manage pests as opposed to eliminating them. Hence, the control of crop pathogens should be based on IPM on an annual basis.

CHAPTER FIVE

Impact of *Cucumber mosaic virus* (CMV) infection on quantity and quality traits of marketable tomato fruits

5.1 Introduction

On a worldwide scale, tomato (*Solanum lycopersicum* L.) continues to increase in importance for both fresh market and processed foods. CMV disease of greenhouse and field tomato frequently causes serious damage and remarkable economic losses. Especially for Greece, CMV epidemics, of various symptomatology on tomato, have occurred during the last decades (Varveri and Boutsika, 1999; Kyriakopoulou *et al*., 2000). It has been observed that severely diseased plants present typical filiformity of leaf blades, or shrinkage, or lethal necrosis, or tomato fruit necrosis, or small misshaped bumpy fruits (Bem 1989; Katis and Avgelis 1991; Kyriakopoulou *et al*., 1991; Conti *et al*., 1996; Varveri and Boutsika, 1999; Kyriakopoulou *et al*., 2000). However, there have been CMV infected tomato plants with mild symptoms, such as slight yellowing mosaic and mottling on leaves and few fruits with marketable external appearance, but internal browning or immature areas (Conti *et al*., 1996). This implies that fruits of CMV infected plants are frequently consumed.

Apart from symptomatology of CMV on tomato fruits, there is no information on the morphological and biochemical changes in ripened tomato fruits of CMV infected plants and especially in marketable fruit. A study was conducted by Georgieva *et al*. (2000) to examine the metabolic changes in tomato fruits infected with CMV. It was found that the peroxidase and glucose-6-phoshate dehydrogenase activities were enhanced by CMV, which was presumed to be because the former enzyme is an important component of plant defence responses and the latter enzyme is related to *de novo* synthesis of the virus' RNA.

The aim of the present study was to elucidate the impact of infection with a Greek isolate of CMV, "CMV4", on plant development, fruit size, quality and biochemical traits of visually attractive tomatoes of hybrid Delos. Specific emphasis was given to quantifying the concentration of some taste-related or health-related

compounds such as NSCs (fructose, glucose and sucrose), organic acids (ascorbic, citric and oxalic) and carotenoids (β-carotene and lycopene). It was also of interest to measure the total antioxidant capacity of tomatoes.

5.2 Experimental design

One experiment was performed (October 2008 – April 2009) to investigate the impact of a Greek isolate CMV4 on quantity and quality traits of tomato fruits, followed by two more, incorporating more treatments, described in chapter 6. Tomatoes of Delos hybrid were cultivated (October 2008 - April 2009) in two cabins of an insect-proof glasshouse at BPI (**Figure 5.1**). There were two treatments (CMV infected and healthy control plants) of 20 plants each. Plants of each treatment were grouped together and were divided equally between the two cabins. In the glasshouse of the Laboratory of Virology at BPI basically experiments with seedlings take place and there is lack of space due to the workbenches (**Figure 5.2**). So complexly randomised design could not be applied because adjacent plants touched each other (**Figure 5.3**) and CMV is mechanically transmitted. Other greenhouses at BPI, free from workbenches, could not be used because either they are not equipped with double doors and insect proof net, or they have not controlled conditions.

Inoculation with CMV was carried out on the first true leaf of tomato seedlings. Fruits with CMV symptomatology were harvested but were not used for further analysis, as the purpose was to examine marketable fruits (**Figure 5.4**). The first three marketable ripened fruits which developed on different trusses (at the bottom, middle and top of the plant) were selected from each plant, therefore, sixty fruits were harvested from each treatment (n = 3 fruits × 20 replications of plants). All fruits were picked almost at the same red stage based on a colour classification map (**Figure 5.5**). According to this map the different maturity stages of each fruit were characterised by progressive numbers (from 1 to 12). Samples were harvested at the stage 9. ELISA test was conducted to determine the presence of the virus in fruits of CMV inoculated plants and then fruit samples of both treatments were prepared as mentioned in materials and methods in chapter 3 (described in section 3.5) in order to quantify their NSCs, organic acids and carotenoids contents by HPLC, and their antioxidant capacity using DPPH radical scavenging.

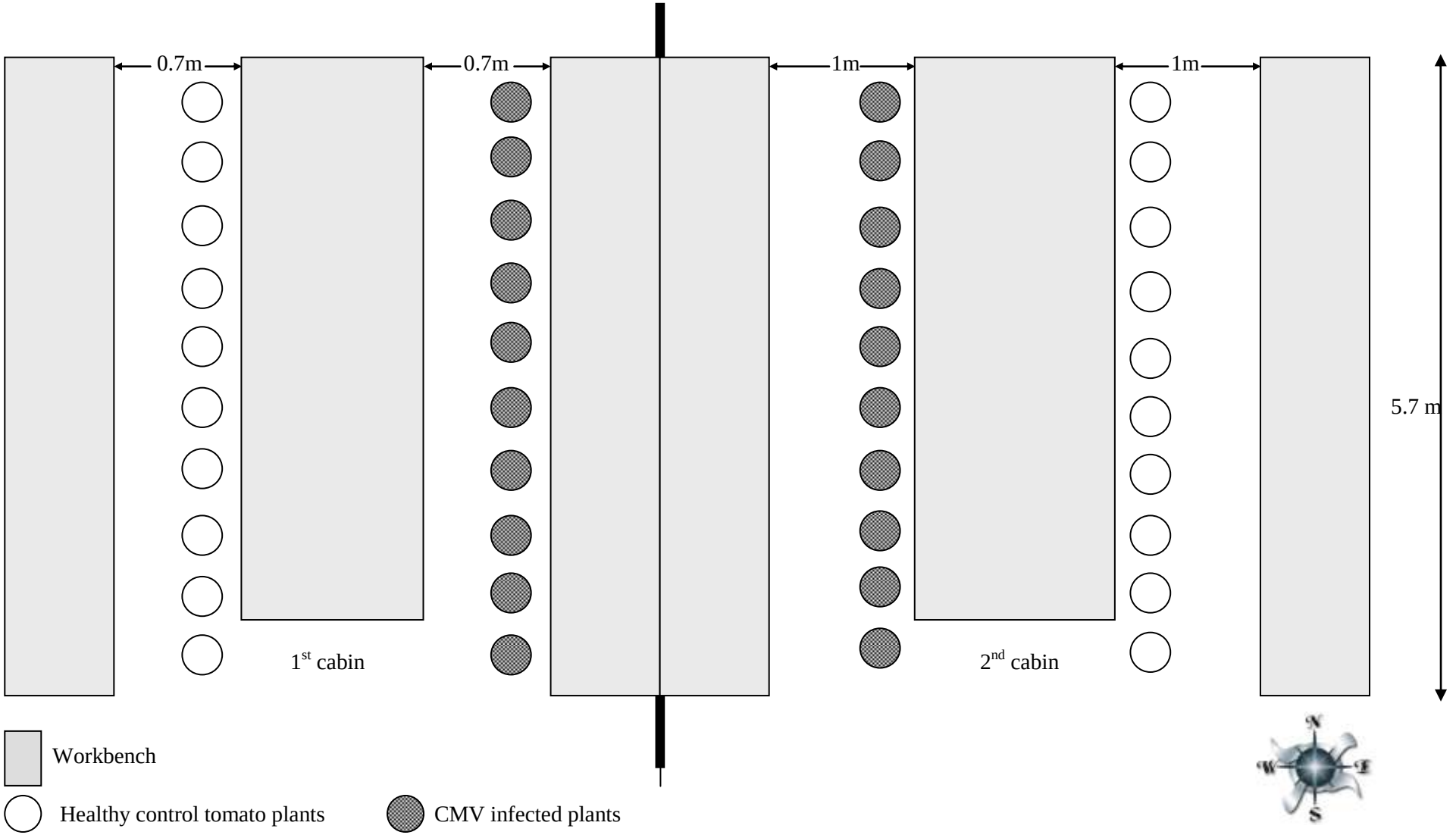

Figure 5.1. Ground plan of the glasshouse showing the tomato plants of the examined treatments arrangement (October 2008 - April 2009).

Figure 5.2. View of the 2nd cabin of the glasshouse showing an early stage of tomato plants placed in the corridors among the workbenches (left corridor: CMV inoculated and infected plants, right corridor: healthy control plants).

Figure 5.3. View of the 1st cabin of the glasshouse showing adjacent tomato plants touched each other due to lack of space (left corridor: healthy control plants, right corridor: CMV inoculated and infected plants).

Figure 5.4. Hybrid Delos tomato fruits of CMV4 infected plant, showing unmarketable fruit that was not used for further analysis (left) and fruit with attractive appearance that was selected (right).

Figure 5.5. Samples were harvested at stage 9 based on this colour classification map.

5.3 Results

5.3.1 Physical assessments

5.3.1.1 Plant morphology

After the CMV4 mechanical inoculation was carried out, the plant height and the leaf number were recorded about twice a week for the 40 individual plants, until topping. Plant development was significantly different between the two treatments as is shown on **Figures 5.6** and **5.7**. Plants of both treatments were at or near the same height until 39 dpi. Subsequently, the growth rate of CMV infected plants progressively declined over an 11 day period to reach a statistically significant difference at 50th dpi compared to the healthy controls. The significant difference was maintained till the end of the experiment. CMV4 caused leaf yellow mosaic symptoms (**Figure 5.8**), leaflet size reduction and, in a small percentage, downward curling of expanding leaves was observed. But the emergence rate of leaves was almost the same for both treatments (**Figure 5.9**). Therefore, plants of CMV treatment were stunted with shorter internodes than healthy controls (**Figure 5.7**). The stem diameter was measured at three different points, at the bottom (50 cm), middle (130 cm) and top (210 cm), of each plant and results are presented in **Table 5.1**. Stem diameter of healthy control plants was significantly thicker than that of CMV infected plants in all measurements.

Table 5.1. Effect of CMV4 infection on stem diameter of hybrid Delos tomato plants measured at three different points, at bottom (50 cm), middle (130 cm) and top (210 cm).

Stem length (cm)	Stem diameter (mm)	
	Healthy control	CMV4
50	11.5 (±0.6)* a**	10.0 (±0.5) b
130	12.8 (±0.5) a	10.9 (±0.5) b
210	10.7 (±0.5) a	9.2 (±0.5) b

*Numbers in brackets indicate the ±s.d. of 20 plants

**Different letters within lines indicate significantly different values ($P < 0.05$)

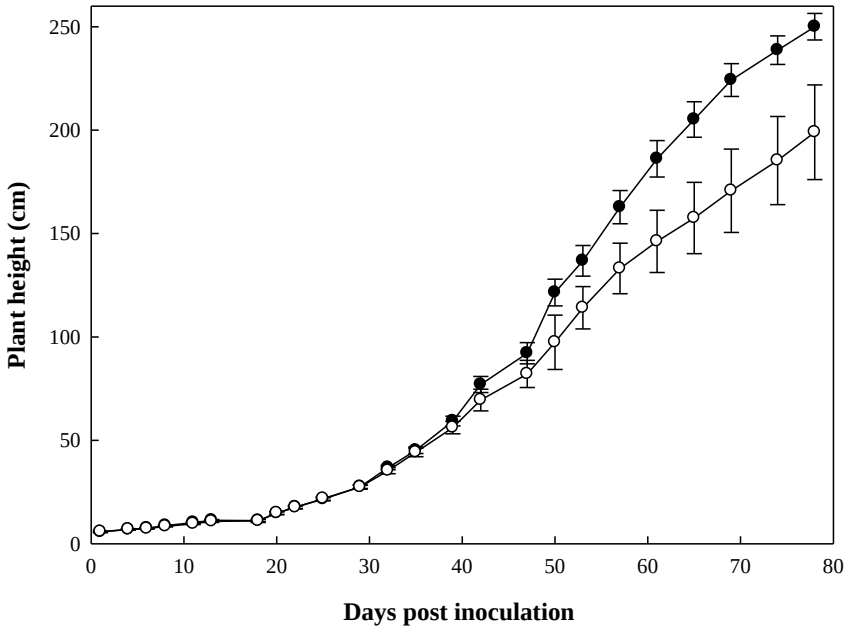

Figure 5.6. The plant height (cm) of CMV4 infected (○) and healthy control (●) tomato plants after the inoculation. The bars represent the ±s.d. of 20 plants.

Figure 5.7. Hybrid Delos tomato plants of healthy control treatment (left) and CMV4 treatment (right). CMV infected plants were stunted with shorter internodes compared to controls.

Figure 5.8. Yellow mosaic symptoms on CMV4 infected Delos hybrid tomato leaf.

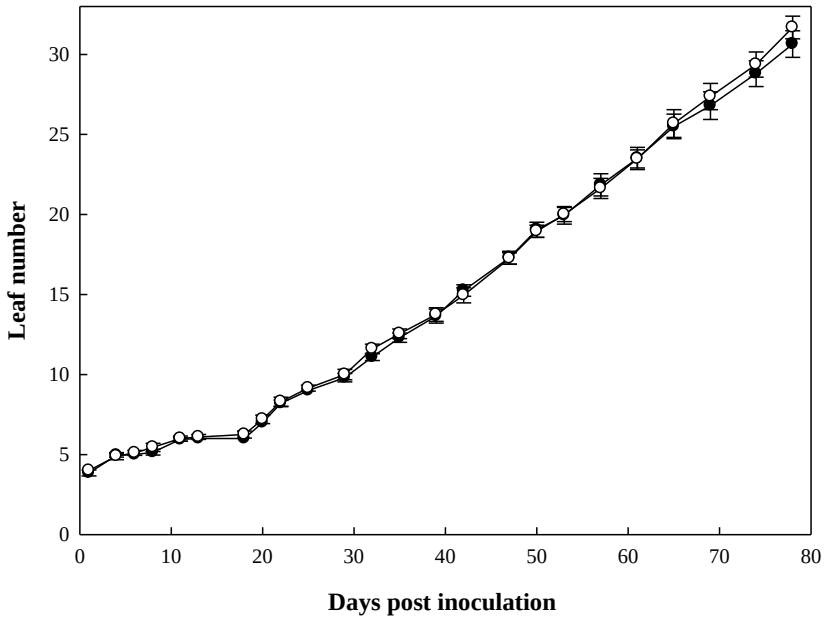

Figure 5.9. The leaf number of CMV4 infected (○) and healthy control (●) tomato plants after the inoculation. The bars represent the ±s.d. of 20 plants.

5.3.1.2 Fruit morphology and production

Fruits of CMV4 infected plants were smaller compared to healthy control and some of them (nearly 27%) had symptoms. The characteristic symptom of the Greek CMV4 isolate on hybrid Delos tomatoes was yellow bumps on their surface (**Figure 5.4**) which made them unmarketable.

In order to estimate the effect of CMV4 infection on tomato production of hybrid Delos all ripened detached fruits (marketable and not) were recorded and weighed with calyx. Fruits were harvested at about the same maturity stage based on their external colour and according to the colour classification map (**Figure 5.5**). The overall (including sample fruits) fruit number, crop weight and mean fruit weight are presented in **Table 5.2**. Although the 20 plants of both treatments produced in total almost the same fruit number (~300, the CMV treatment production being slightly increased) the CMV treatment presented a reduction by 24% in total crop weight and 25.6% in the mean fruit weight. Taking into account only the marketable fruits of CMV treatment the reduction in fruit number came up to 25.6%, in total crop weight to 40.7%, and in mean fruit weight to 20.4% compared to healthy control.

Table 5.2. Effect of CMV4 infection on tomato production of hybrid Delos, expressed as number, total weight and mean weight of ripen fruits.

Treatment (20 plants)	Number of ripe fruits	Total weight of yield (kg)	Mean weight of ripe fruits (g)
Control	305	55.0	180.2 (± 25)*
CMV (all fruits)	312	41.8	134.0 (± 25)
CMV (marketable fruits)	227	32.6	143.5 (± 25)

*Numbers in brackets indicate the ±s.d. of 305, 312 and 227 fruits for healthy control, all CMV fruits and marketable CMV fruits respectively

DAA was monitored and showed that fruit maturation was significantly slower under CMV infection. Fruits from infected plants needed more than 59 DAA in order to ripe and get the same red colour as healthy control fruits, which needed less than 55 DAA (**Table 5.3**). The fruit position on the plant independently of treatment, also, affected the DAA, as the primary fruit needed nearly one more day to become ripe compared to secondary and tertiary fruits (**Table 5.4**). The interaction between

treatment and fruit position showed significant differences in DAA as presented in **Table 5.5**. From the data it is obvious that the difference in DAA regarding fruit position was only observed on healthy control plants.

In both treatments the dry matter of pericarp was 5.9% (**Table 5.3**). In fruit samples of CMV infected plants the fresh weight, either with or without calyx, was reduced by 24% and the fresh weight of calyx was reduced by 18.5% versus healthy control samples. Mean weight of tomato fruit samples (201.7 g healthy control, 153.3 g fruit of infected plant) was also increased compared to mean weight of all fruits (180.2 g healthy control, 143.5 g marketable fruit of infected plant). This happened because samples consisted of the first fruits developed on different trusses, and these fruits as usual, were bigger compared to the rest. To obtain information on the variation in fruit size of the treatments the diameter, the height and the perimeter of each sample fruit were recorded and significant differences in all measurements were observed (**Table 5.7**). Generally, fruits of CMV infected plants were considerably smaller than healthy control fruits as depicted in **Figure 5.10**. In particular, they had 11.2% smaller diameter, 10.6% height and 9.3% perimeter compared to healthy control. The fruit density of samples was calculated and it was found that the fruit density of infected plants was significantly increased by 7.6% (**Table 5.7**). Independent of treatment, fruit position resulted in differences in the calyx fresh weight, fruit diameter and density as presented in **Table 5.4**. According to Spearman's rank correlations there was a high correlation coefficient ($r^2 > 0.9$, $P < 0.05$) between perimeter-fruit weight and perimeter-diameter as is shown in **Table 5.6**.

Table 5.3. Effect of CMV4 infection on days after anthesis (DAA) and DW as a proportion of FW (DW/FW) of hybrid Delos tomato fruits.

Treatment	DAA (days)		DW/FW of pericarp	
Control	54.9	(a)*	0.059	(a)
CMV infected plant	59.2	(b)	0.059	(a)
LSD ($P < 0.05$)	0.3		0.001	

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 5.4. Effect of the fruit position on DAA, calyx weight and some morphological characteristics in tomato fruit of hybrid Delos independent of treatment.

Fruit	DAA (days)	Calyx fresh weight (g)	Diameter (cm)	Density (g cm^{-3})
1 (bottom)	57.7 (a)*	0.9 (a)	7.4 (a)	1.1 (a)
2 (middle)	56.9 (b)	0.8 (b)	7.1 (ab)	1.2 (b)
3 (top)	56.6 (b)	0.8 (b)	6.9 (b)	1.2 (b)
LSD (P=0.05)	0.3	0.06	0.2	0.04

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 5.5. Effect of the interaction of treatment and fruit position on DAA of hybrid Delos tomato fruits.

Fruit position	1	2	3
Treatment	DAA (days)		
Control	56.0 (b)*	54.8 (a)	53.9 (a)
CMV	59.4 (c)	59.1 (c)	59.2 (c)
LSD (P=0.05)		0.5	

*Different letters in brackets indicate significantly different values ($P < 0.05$)

Table 5.6. Spearman's rank correlation coefficient on fruit weight and morphological characteristics ($P < 0.05$).

	Diameter	Perimeter
Weight with calyx	0.87*	0.96
Weight without calyx	0.87	0.96
Height	0.75	< 0.6
Perimeter	0.92	< 0.6

*Value > 0.6 and close to 1 means good correlation between the two elements

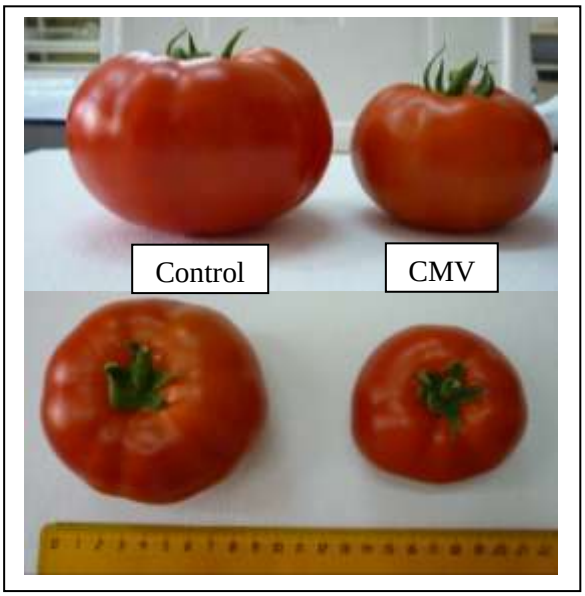

Figure 5.10. Tomato samples of hybrid Delos harvested from healthy control and CMV4 infected plant. Fruits of CMV infected plants were considerably smaller than healthy control fruits.

Table 5.7. Effect of CMV4 infection on weight, morphological characteristics and density of tomato fruit of hybrid Delos.

Treatment	Fresh weight with calyx (g)	Fresh weight without calyx (g)	Calyx fresh weight (g)	Diameter (cm)	Height (cm)	Perimeter (cm)	Fruit density (g cm^{-3})
Control	201.7 (a)*	200.8 (a)	0.89 (a)	7.54 (a)	5.91 (a)	24.43 (a)	1.14 (a)
CMV infected plant	153.3 (b)	152.5 (b)	0.73 (b)	6.69 (b)	5.28 (b)	22.1 (b)	1.22 (b)
LSD ($P < 0.05$)	8.8	8.8	0.05	0.15	0.12	0.44	0.03

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

5.3.2 Biochemical assessments

5.3.2.1 ELISA

Results of ELISA tests referring to CMV inoculated plants are presented in **Table 5.8**. It was observed that 21 dpi only 10% (2/20) of plants were infected in new, fully expanded leaves and within 25 dpi all plants were infected. This result was verified for 46 dpi. An ELISA test was carried out 61 dpi on samples from three different parts of each plant (top, middle and bottom) and it was found that 20% (4/20) of plants showed recovery, as the virus was not detected in their uppermost leaves, although it was detected in the other two plant parts. The last ELISA test (100 dpi) indicated that 45% (9/20) of plants showed recovery in their newly emerged leaves.

Table 5.8. Percentage of CMV infected tomato plants according to ELISA tests.

	% infected plants		
	Examined part of plant		
dpi	top	middle	bottom
21	10% (2/20)*		
25	100% (20/20)		
34	100%		
46	100%		
61	80% (16/20)	100%	100%
100	55% (11/20)	100%	

*Numbers in brackets indicate the number of infected plants divided to inoculated plants

Regarding the fruit samples ELISA tests showed that CMV could not be detected in 25% (15/60) of them. In particular, all three selected fruits were found ELISA positive in 45% (9/20) of plants, two out of three fruits were infected in 35% (7/20) of plants and only one fruit was positive in 20% (4/20) of plants. For this reason fruits of CMV treatment are not denominated CMV infected fruits, but fruits of CMV infected plants.

5.3.2.2 Non-structural carbohydrates

Three different columns were tried in order to aquire the chromatogram with the best separation (**Figure 5.11**). The **Figure 5.12** shows HPLC chromatograms of a representative tomato sample of each treatment. As depicted in the chromatograms the retention times for sucrose, glucose and fructose were approximately 9.3, 11 and 13.7 min, respectively.

Sucrose in almost all fruits from CMV infected plants was at low but detectable quantities (irrespective of the actual virus presence in the fruit) while in healthy control fruits sucrose was below the limit of detection. Thus, the findings of sucrose are underpinned with the counts of values below quantification limit per 60 fruit measured (healthy control n = 32; CMV infected plants n = 1). With this assumption, sucrose was 2.3-fold higher on a DW basis and 2.1-fold on a FW basis in fruits from infected plants as compared to tomatoes from healthy control plants (**Table 5.9**).

Fructose was also significantly higher on a DW (8.06%) and FW (8.17%) basis in fruits of infected plants than for healthy control fruit. Glucose was slightly increased in fruits from CMV infected plants, but there was no significant difference between the two treatments. Although total sugars were significantly increased on DW basis in fruits from infected plants compared with healthy control, on FW basis there was no difference (**Table 5.9**). Significant Spearman's rank correlations were observed between fructose and glucose with a high coefficient of correlation (**Table 5.10**).

Table 5.9. Effect of CMV4 infection on the concentration of sucrose, fructose, glucose, ascorbic, citric, oxalic acid, lycopene and β-carotene and the antioxidant capacity of marketable tomato fruit of hybrid Delos, expressed per dry weight (DW) and per fresh weight (FW).

	Treatment	Sucrose (mg g^{-1})	Fructose (mg g^{-1})	Glucose (mg g^{-1})	Total sugars (mg g^{-1})	Ascorbic acid (mg g^{-1})	Citric acid (mg g^{-1})	Oxalic acid (mg g^{-1})	Lycopene (μg g^{-1})	β-carotene (μg g^{-1})	Antioxidant capacity (μM TE/g)***
DW	Control	3.21 (a)*	177.5 (a)	165.9 (a)	346.6 (a)	4.78 (a)	80.7 (a)	10.13 (a)	1054 (a)	28.40 (a)	34.25 (a)
	CMV infected plant	7.39 (b)	191.8 (b)	169.4 (a)	368.0 (b)	5.33 (b)	87.7 (b)	10.33 (a)	1148 (a)	32.27 (b)	39.88 (b)
	LSD ($P < 0.05$)	0.57	4.2	3.9	7.7	0.16	2.4	0.32	50.8	1.56	1.19
FW	Control	0.19 (a)**	10.52 (a)	9.85 (a)	20.57 (a)	0.285 (a)	4.77 (a)	0.60 (a)	62.2 (a)	1.68 (a)	2.03 (a)
	CMV infected plant	0.41 (b)	11.38 (b)	9.94 (a)	21.73 (a)	0.314 (b)	5.16 (b)	0.61 (a)	67.6 (a)	1.87 (b)	2.36 (b)
	LSD ($P < 0.05$)	0.04	0.36	0.36	0.71	0.012	0.16	0.03	3.1	0.08	0.08

*Different letters in brackets within columns referred to DW indicate significantly different values ($P < 0.05$)

**Different letters in brackets within columns referred to FW indicate significantly different values ($P < 0.05$)

***Results expressed as μM Trolox equivalents (μM TE/g DW or FW)

Table 5.10. Spearman's rank correlation coefficient on fructose, glucose and DW/FW ($P < 0.05$).

	Fructose DW	Fructose FW	DW/FW
Glucose DW	0.68*	0.67	< 0.6
Glucose FW	< 0.6	0.89	0.77
Fructose FW	< 0.6	< 0.6	0.66

*Value > 0.6 and close to 1 means good correlation between the two elements

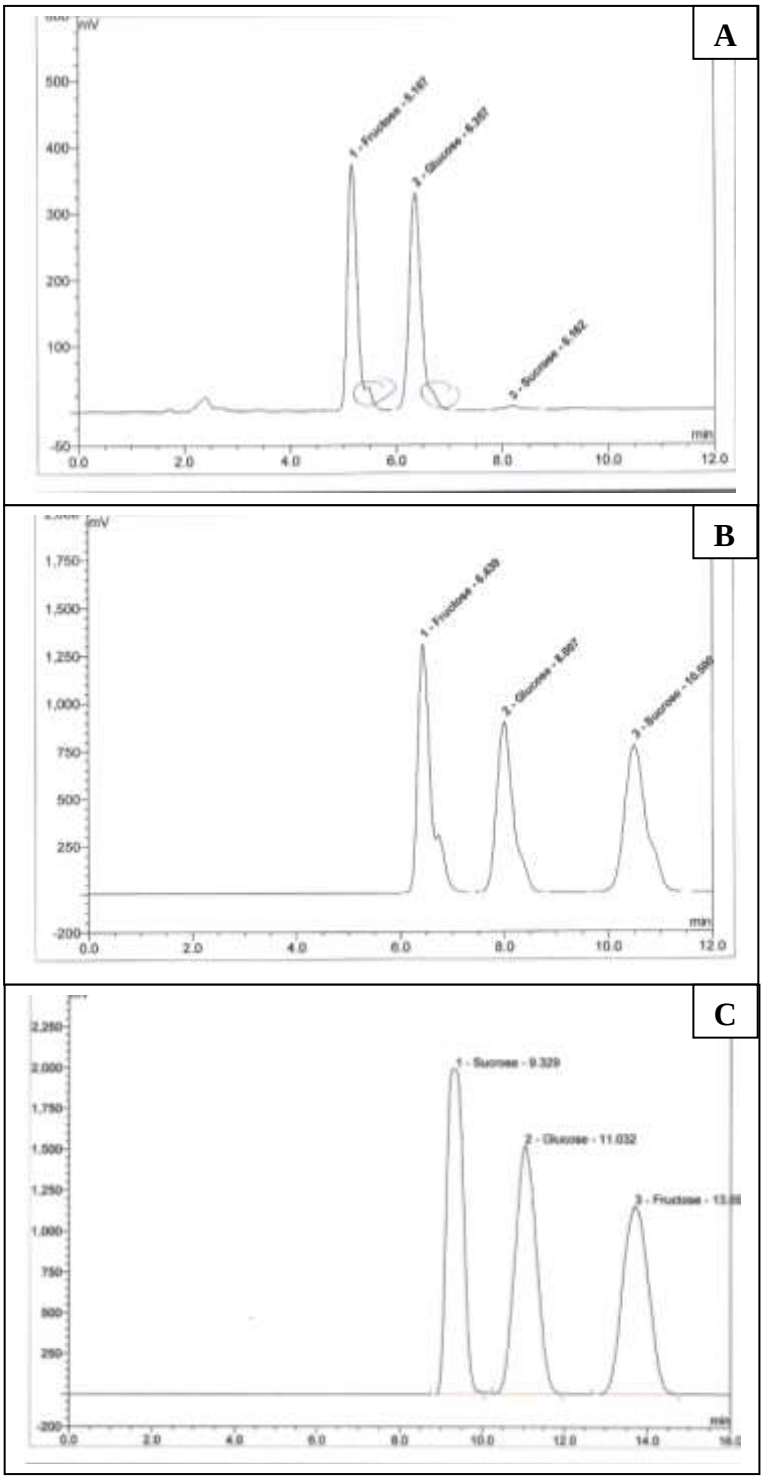

Figure 5.11. Comparison of HPLC chromatograms of a nonstructural carbohydrates (NSCs) standard mixture solution injected onto different columns, which separate the NSCs in different directions. **A**: Fructose and glucose peaks have uneven curves (Waters carbohydrate Alltech column of 250 × 4.6 mm diameter, Part No. 35101), **B**: Fructose and sucrose peaks have uneven curves (Waters carbohydrate analysis column of 300 × 3.9 mm diameter and 10 μm particle size, Part No. WAT084038), **C**: Chromatogram with the best separation and curves of sucrose, glucose and fructose [Rezex RCM monosaccharide Ca^{+} (8%) column of 300 × 7.8 mm diameter and 8 μm particle size, Phenomenex, Part No. 00H-0130-K0].

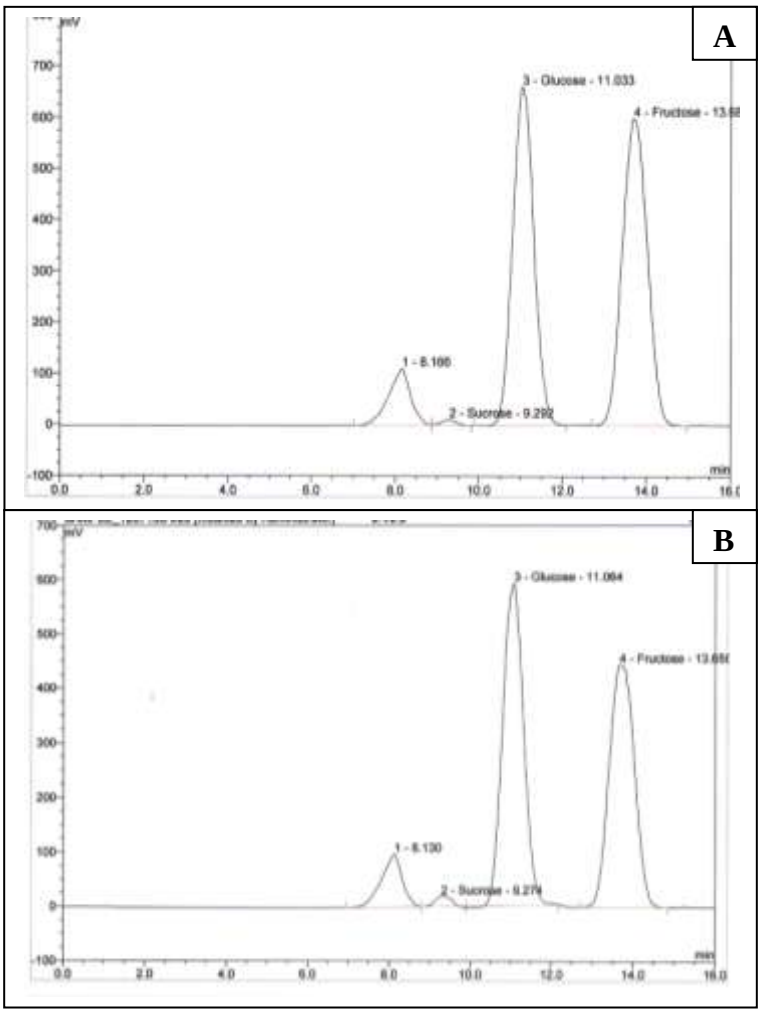

Figure 5.12. HPLC chromatograms of the nonstructural carbohydrates (NSCs) profile of tomato representative sample fruit solution derived from (**A**) healthy control plant and from (**B**) CMV inoculated and infected plant. The first peak in chromatograms of both treatments was not identified.

5.3.2.3 Organic acids

Many compounds were detected in tomato sample extracts as illustrated in **Figure 5.13** (**B** and **C**), but only three organic acids (oxalic, ascorbic and citric) could accurately be identified and quantified using retention times and spectral data (**Figure 5.14**) obtained from different tested standards (oxalic, tartaric, malic, ascorbic, citric, maleic, succinic, fumaric, pyruvic). **Figure 5.13** (**A**) presents the HPLC chromatogram of the organic acids standard mixture solution, consisted of oxalic, tartaric, malic ascorbic and citric acids that were used for calibration. According to chromatograms of the standard and those of tomato samples of both treatments (**Figure 5.13 A**, **B** and **C**) the retention times for oxalic, ascorbic and citric acids were approximately 2.7, 4.4 and 6.5 min, respectively.

Oxalic acid concentration on both DW and FW basis was not significantly affected by CMV infection. Ascorbic and citric acids were both significantly higher in fruits of plants infected with CMV than healthy controls. Ascorbic acid was 11.4% on DW basis and 10.4% on FW basis higher compared to healthy control fruit and citric was 8.7% and 8.1% higher, respectively (**Table 5.9**). Moreover, the concentration of ascorbic acid presented significant difference regarding the fruit position on the plant, independent of CMV treatment. In primary fruits ascorbic acid was significantly lower than that for secondary (by 10.4% FW, 12.5% DW) and tertiary (by 12.5% FW, 13.3% DW) fruits. Concentration of ascorbic acid in fruits from middle and top of plant did not differ (**Table 5.11**).

Table 5.11. Effect of the fruit position on the concentration of ascorbic acid and β-carotene in tomato fruit of hybrid Delos, independent of treatment.

Fruit (40 replicates)	Ascorbic acid (mg g^{-1}) DW		Ascorbic acid (mg g^{-1}) FW		β-carotene (μg g^{-1}) FW	
1 (bottom)	4.70	(a)*	0.28	(a)	1.63	(a)
2 (middle)	5.18	(b)	0.31	(b)	1.78	(ab)
3 (top)	5.28	(b)	0.31	(b)	1.91	(b)
LSD (P=0.05)	0.20		0.01		0.10	

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

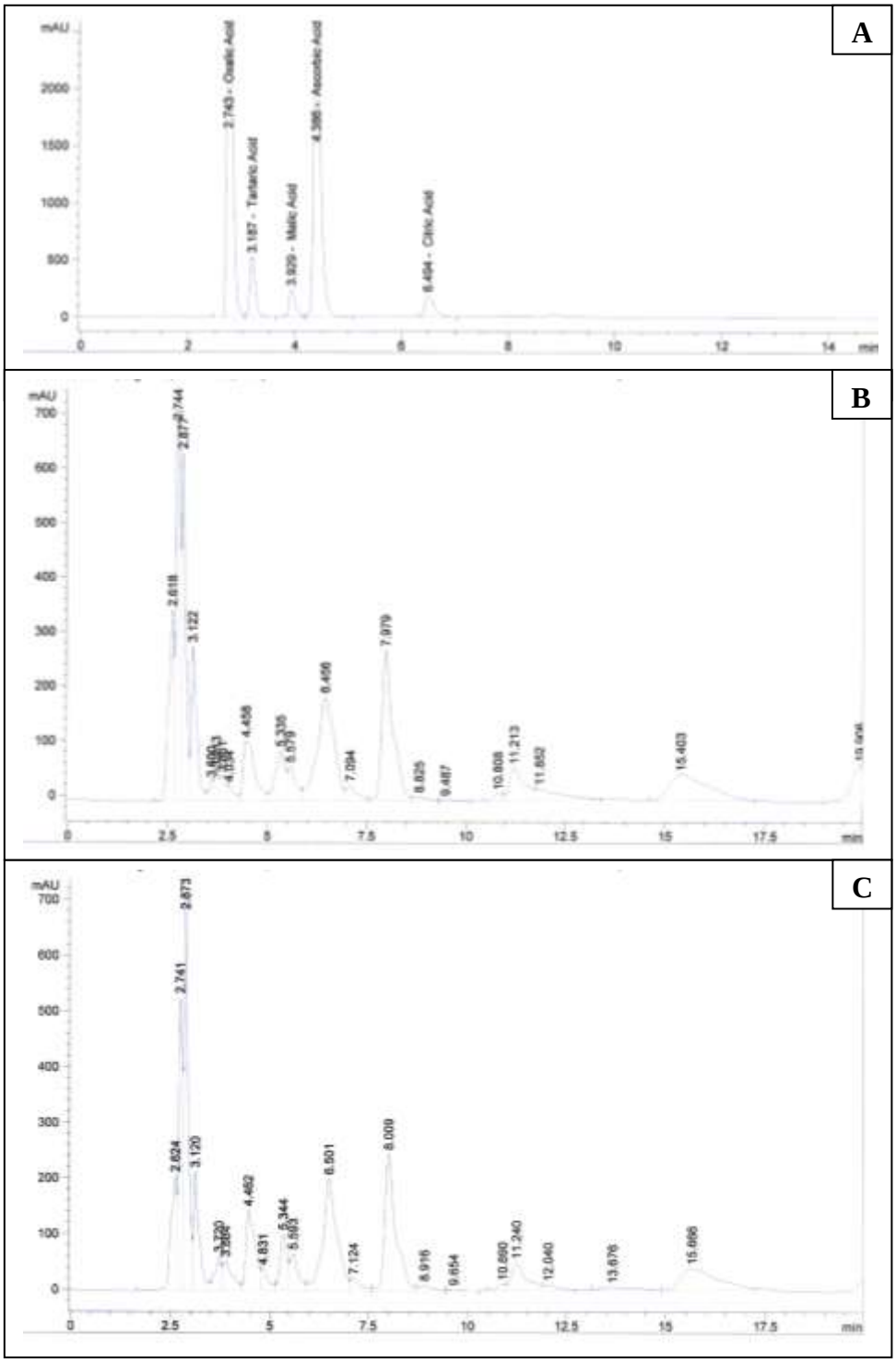

Figure 5.13. HPLC chromatograms of (**A**) organic acids standard mixture solution and the organic acids profile of tomato representative sample fruit solution derived from (**B**) healthy control plant and from (**C**) CMV inoculated and infected plant. Only oxalic, ascorbic and citric acids of tomato sample solutions were accurately identified using retention times and spectral data.

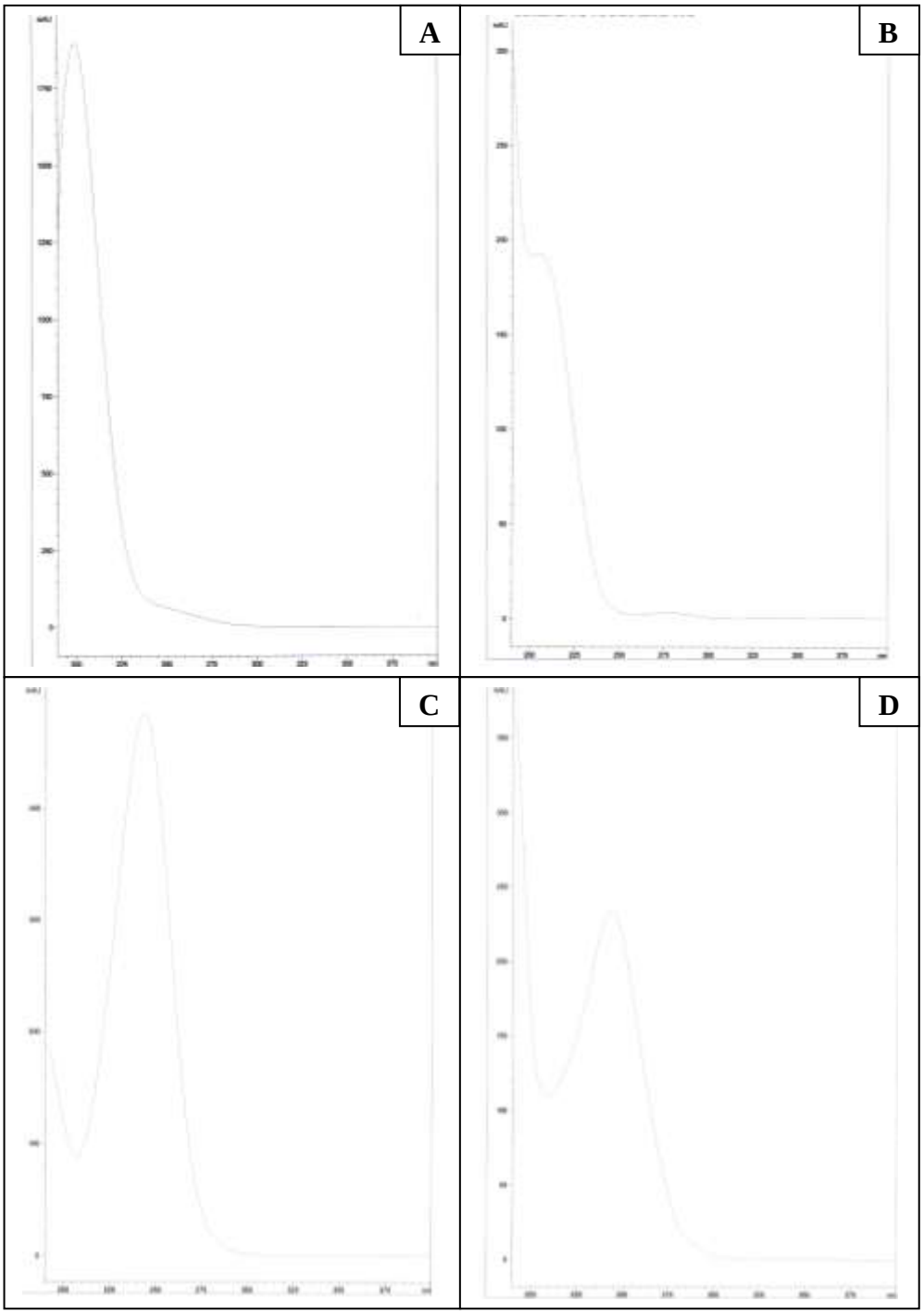

Figure 5.14. The spectra of peaks correspond to (**A**) oxalic, (**B**) citric and (**C**) ascorbic standards. (**D**) The spectrum of dehydroascorbic acid (DHA), the oxidized form of ascorbic, which on purpose was avoided to be co-eluted with it resulting in false measurement of ascorbic acid content.

5.3.2.4 Carotenoids

The modified method used for carotenoids quantification was tested for reproducibility and repeatability by running the standard and the same tomato sample solution six times on two consecutive days, respectively. **Figure 5.15** illustrates the result of the overlaid chromatograms of the tomato sample and shows the reproducible retention times and the almost identical peak areas of measurements. As shown in **Figure 5.16**, (**A**) for the standard, (**B** and **C**) for the tomato samples, the lycopene content was detected at 472.4 nm, while the β-carotene at 452.4 nm, because they present disparity in their absorption coefficient. Tomato samples of both treatments presented three peaks, two of which were identified based on comparison of their absorbance spectra (**Figure 5.17**) to the known lycopene and β-carotene standards (**Figure 5.16**, **A**). Moreover, the retention time of examined carotenoids was considered, which was at about 6.5 min for trans-lycopene and 6.8 min for β-carotene.

The trans-lycopene content of tomato pericarp was not found to be different between the two treatments on both DW (1054 μg g^{-1} for healthy control and 1148 μg g^{-1} for fruits of CMV infected plants) and FW (62.2 μg g^{-1}, 67.6 μg g^{-1} respectively) basis. Although β-carotene was detected in trace amounts, it was significantly higher by as much as 13.6% in DW and 11.5% in FW in fruits of CMV treated plants versus to healthy control fruits (**Table 5.9**). Regardless of CMV infection there was a spatial difference for β-carotene on FW basis since β-carotene content tended to be higher on fruits derived from the upper parts of plant (**Table 5.11**).

5.3.2.5 Antioxidant capacity

Tomato antioxidant capacity measured using the DPPH radical scavenging was enhanced under CMV infection. Fruits of CMV infected plants presented on FW basis 16.4% and on DW basis 16.3% more antioxidant capacity than healthy control (**Table 5.9**).

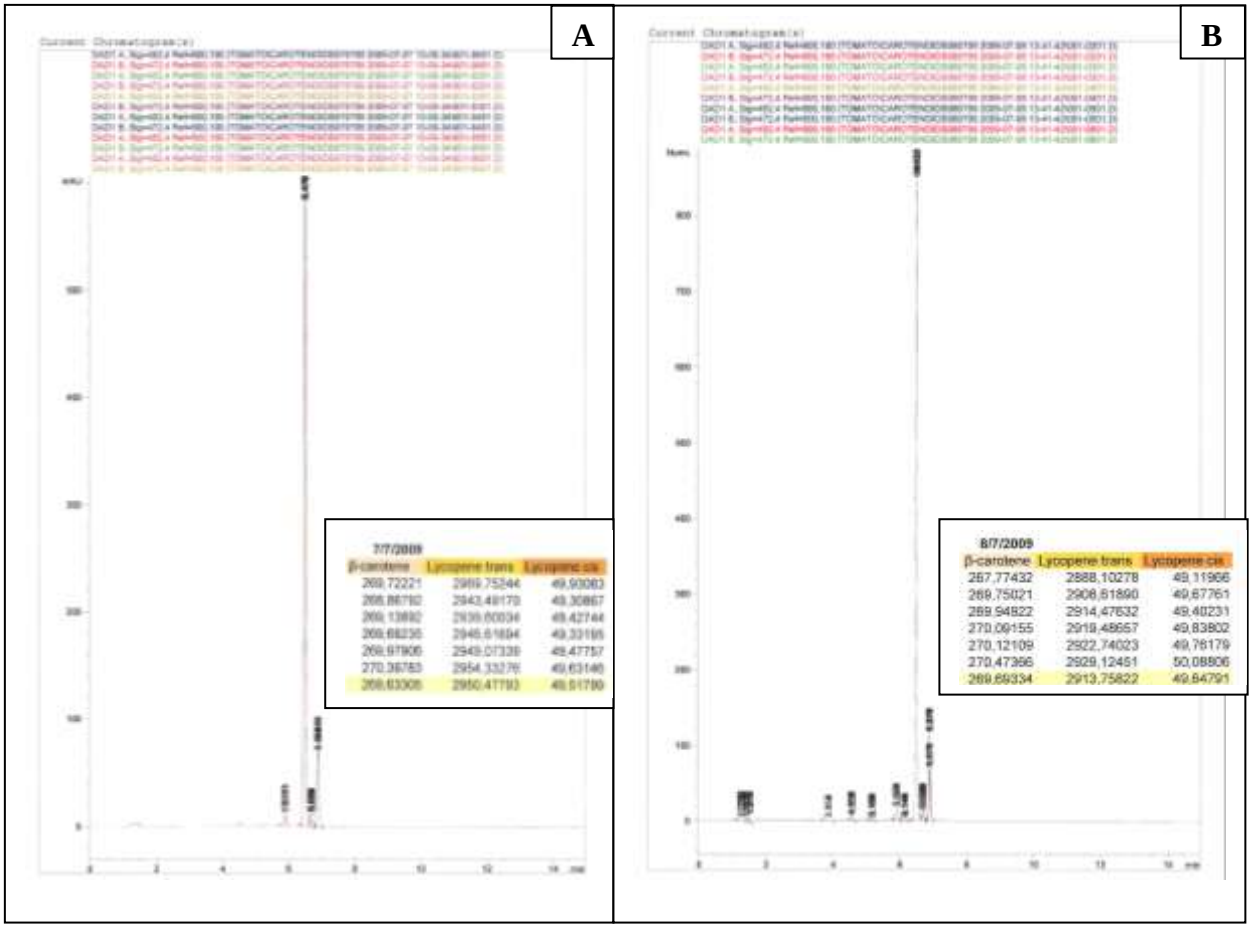

Figure 5.15. The overlaid chromatograms of the carotenoids profile of the same tomato sample measured six times repeatable, two consecutive days (**A**: 7/7/2009 and **B**: 8/7/2009). The insert tables show the peak areas as concerns β-carotene, trans and cis-lycopene, which were almost identical. The mean values of the six measurements are recorded in the last yellow marked line.

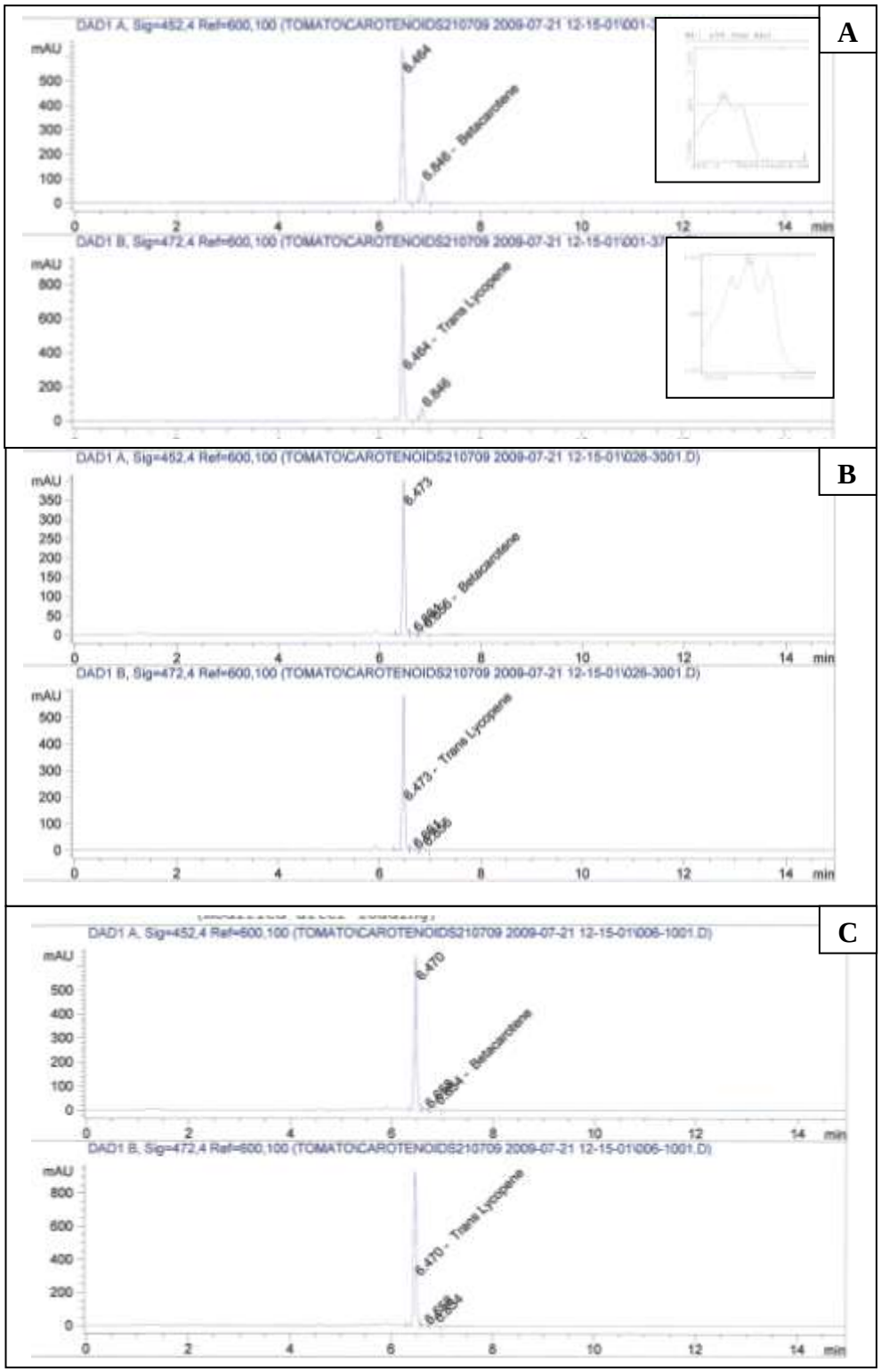

Figure 5.16. HPLC chromatograms of (**A**) trans-lycopene (measured at 472.4 nm) and β-carotene (measured at 452.4 nm) standard mixture solution and the carotenoids profile of tomato representative sample fruit solution derived from (**B**) healthy control plant and from (**C**) CMV inoculated and infected plant. The inserts in panel (**A**) represent the spectra peaks of β-carotene and lycopene standards.

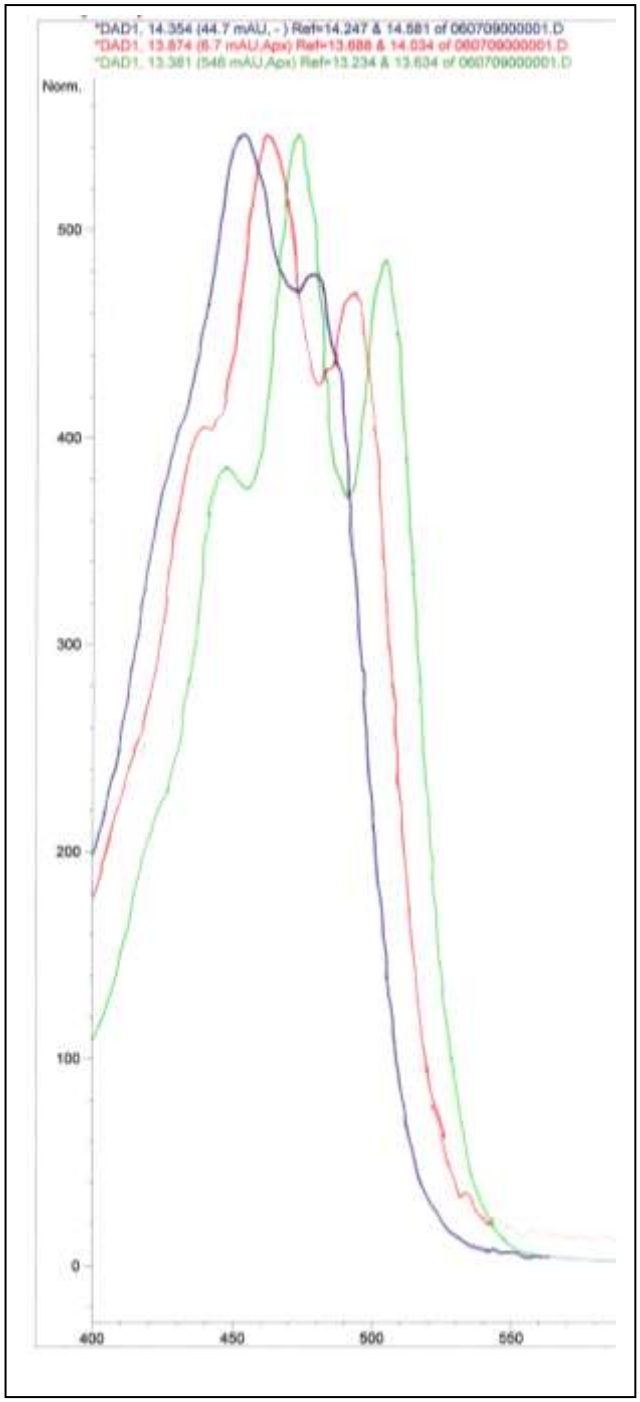

Figure 5.17. The spectral characteristics of β-carotene (blue line), trans-lycopene (red line) and cis-lycopene (green line) of tomato sample extracted with 100% acetone.

5.4 Discussion

5.4.1 Impact of CMV4 infection on tomato plants and fruit production of hybrid Delos

Tomatoes are a significant source of supplementary diet and also present high economic value as they are the second most consumed fresh produce type worldwide. On the other hand, CMV is a pathogen of special importance which causes severe economic losses in the tomato crop. Although, there are many references about CMV symptomatology on tomato plant and fruits (MacNab *et al*., 1983; Bem 1989, Katis and Avgelis 1991; Kyriakopoulou *et al*., 1991; Conti *et al*., 1996; Šutić *et al*., 1999; Varveri and Boutsika, 1999; Cerkauskas, 2004; Zitter and Murphy, 2009) there is no published information on the impact of CMV infection on the quantity and quality traits of tomato fruits. This is the first piece of research exhibiting the effect of CMV and especially of a Greek CMV isolate, "CMV4", on plant development and on physical and chemical characteristics of visually attractive tomato fruits of hybrid Delos.

This specific CMV isolate caused symptoms both on fruits and on the vegetative tomato plant parts of hybrid Delos, including fruit yellow bumps areas (**Figure 5.4**, left fruit), leaf size reduction, curling downward and yellow and green mosaic symptoms (**Figure 5.8**). Some plants showed severe symptoms and were stunted with shorter internodes and weak stems, while other were less affected (**Figure 5.7**). Miteva *et al*. (2005) also demonstrated that CMV infection had a negative effect on tomato plants by limiting the growth of their stems. At about 60 dpi the new leaves of some plants appeared normal and symptomless. ELISA was conducted in three different parts of each plant (top, middle and bottom) and was found that some plants showed recovery in their uppermost leaves (**Table 5.8**). The present results are in line with references which have reported that CMV symptoms can be transitory, that is, often the leaves on one portion of the plant show severe symptoms (extremely distorted and malformed leaves), while other leaves are less affected or appear normal (Zitter and Murphy, 2009; Kyriakopoulou personal communication).

The effect of CMV infection on stem diameter followed the same trend in the three measurements, with infected plants having significantly thinner stems than

healthy control plants (**Table 5.1**). This fact was in agreement with tomato plants singly and mixed infected with PVX and TMV, where in all cases healthy plants had the thickest stem diameter (Balogun, 2009).

The number of ripened fruits produced by the two treatments was almost the same (slightly increased in CMV4 infected plants) (**Table 5.2**). In contrast to statements that CMV infected plants produce fewer fruits (Conti *et al*., 1996; Šutić *et al*., 1999; Cerkauskas, 2004; Agrios, 2005) this result was reproduced in two more experiments following this one using the same CMV isolate and tomato hybrid. Although, the leaf-to-fruit ratio was almost the same, the fruit mean weight was decreased by 26% in CMV infected plants, hence the total yield per treatment (expressed as kg/treatment) was 24% less under CMV pressure. Considering that 27% of the fruit had symptoms, the reduction in marketable fruit yield production increased to 40.7%. This is in agreement with many reports which have stated that CMV infected plants produce smaller fruits (Conti *et al*., 1996; Cerkauskas, 2004; Agrios, 2005; Malathrakis, 2007).

Biotic stress, which could be caused by viral infection, may reduce crop yield because it affects the photosynthesis process (Nogués *et al*. 2002; Zhou *et al*. 2004; Funayama-Noguchi and Terashima 2006; Song *et al*., 2009). It is known that photosynthetic and respiratory electron transport chains are the main energy-transducing processes in eukaryotic organisms (Song *et al*., 2009). Several studies have evidenced that plant virus infection decreased their photosynthetic rate (van Kooten *et al*. 1990; Balachandran *et al*., 1997; Rahoutei *et al*. 2000). Moreover, Song *et al.* (2009) studied the photosynthetic electron transport in CMV infected tomato plants, and they found that electron transport chains in chloroplasts and mitochondria were disrupted and resulted in decreased photosynthesis (approximately by 60%) rate. In the current study, CMV infection detrimentally affected tomato fruit size (**Figure 5.10**). There was no change in the leaf-to-fruit ratio between the two treatments as plants produced the same number of leaves (**Figure 5.9**) and fruits (**Table 5.2**). Notwithstanding this fact, CMV infection had repercussions on fruit yield. Therefore, the photosynthesis of CMV infected plants should be limited due to the lower leaf area, the downwards leaf curling and the prementioned alteration of the photosynthesis process.

Many significant ($P < 0.05$) correlations were found with reference to fruit weight and morphology characteristics and this relationship was positive and

predictable (nearly all $r^2 > 0.87$) (**Table 5.6**). No significant differences among and within used glasshouse cabins for quantity and quality measurements were found, whereas there were few spatial differences (**Tables 5.4** and **5.11**).

5.4.2 Impact of CMV4 infection on the quality of tomato fruits of hybrid Delos

The maturity stage of tomato fruits is harmonised according to their external colour. Fruits were selected on the basis of colour because tomato colour serves as a measure of total quality (Shi and Le Maguer, 2000), and also has a strong influence on the buying behaviour of consumers. In accordance with Zitter and Murphy (2009), CMV presence delayed fruit maturity. Fruits of CMV infection needed more than four days extra to get the same red colour as the healthy control (**Table 5.3**). After collecting representative fruit samples of each treatment, pericarp tissues were snap frozen in two hours to ensure that changes in compounds composition did not take place between collection and analysis, as tomato is a perishable product.

No significant difference was found between the ratio DW/FW in fruits of CMV inoculated and non-inoculated plants. These data imply that any difference in measured compounds between the two treatments are not due to a dilution effect, as happened in water stress treatments of strawberry (Terry *et al*., 2007), but to a metabolism effect. According to Afeal *et al*., (1996) tomato plants infected with CMV did not show any change in their moisture content. Hence, CMV does not cause any difference in the percentage of dry weight for both fruit and tomato plant.

As well as colour, the quality of tomato fruit is determined by a wide range of desirable characteristics such as nutritional value (total antioxidants, carotenoids and ascorbic acid), development of aroma and flavour (sugars and organic acids), texture and shelf-life. Many studies and reviews have dealt with organoleptic quality and compositional parameters in a lot of different tomato varieties (Hart and Scott, 1995; Wang *et al*., 1996; Ben-Amotz and Fishler, 1998; Arias *et al*., 2000; Baysal *et al*., 2000; Raffo *et al*., 2002; Rotino *et al*., 2005; Olives Barba *et al*., 2006; Rodrígues-Bernaldo de Quirós and Costa, 2006; Topal *et al*., 2006; Chang and Liu, 2007; Kortstee *et al*., 2007; Vermeir *et al*., 2007; Porcu and Rondriguez-Amaya, 2008; Hernández Suárez *et al*., 2008a,b; Toma *et al*., 2008; Choudhary *et al*., 2009; Odriozola-Serrano *et al*., 2009; Cámara *et al*., 2010; Luengwilai *et al*., 2010; Yin *et*

al., 2010; Georgé *et al*., 2011). It is usual for the content of some tomato compounds to vary because their concentration may be affected by many factors such as variety, maturity, growing conditions, soil, light intensity, and season of the year (Hernández Suárez *et al*., 2008b; Georgé *et al*., 2011). Though studying all these references sometimes showed such wide differences that either the examined tomato varieties are totally different in nutrient content or, as according to Kimura and Rodriguez-Amaya (2002), analytical inaccuracies appear to be involved.

5.4.2.1 Quantification of NSCs

To examine the effect of CMV4 infection on sugar profile of hybrid Delos, sucrose, glucose and fructose were measured. Sucrose concentration showed a general trend to be below quantification limits in fruits of healthy control treatment and was underestimated, as zero, for 32 out of 60 samples. On the contrary, sucrose concentration of fruits from CMV infected plants was in low but detectable quantity, with only one sample non-detectable. Taking into account this fact, sucrose was 2.3-fold higher on a DW basis and 2.1-fold on a FW basis in fruits of infected plants versus healthy control (**Table 5.9**). According to Landahl *et al*. (2009) these results should not be considered since most of the values were below quantification limit. Other authors, who studied the nutritional value of some tomato varieties, also noticed that sucrose was non-detectable (Raffo *et al*., 2002; Galiana-Balaguer *et al*., 2006; Vermeir *et al*., 2007).

Fructose was significantly increased under CMV infection, while glucose did not differ between the two treatments. Total sugars concentration was higher on DW basis in fruits of infected plants than healthy controls, whereas on FW basis no difference occurred. Many reports have indicated that carbohydrate metabolism basically in source leaves is altered by viral infection. Afeal *et al*. (1996) demonstrated that reducing sugars were significantly increased in CMV infected tomato plants versus healthy controls. Herbers *et al*. (2000) showed that infection of tobacco plants with PVY led to accumulation of soluble sugars in leaves, probably due to the increased activity of cell wall invertase which inhibited sugar export. Moreover, a sharp increase in fructose and glucose concentrations was observed in CMV infected melon leaves, due to the increased demand for soluble sugars to maintain the high respiration rate, which then was accompanied by reduced soluble sugars accumulation (Shalitin and Wolf, 2000). Gil *et al*. (2011) indicated that the

changes in the sugar content in CMV infected plants were due to alteration in the metabolism and translocation of the assimilated carbohydrates. Indeed, the viruses are obligate intracellular parasites and use the plant host's cells to replicate over a prolonged period of time. In addition, tissues of infected plants have increased nutrients demands due to the activation of defence responses (Herbers *et al*., 2000).

Fructose, glucose and sucrose in sugar-beet, oats and carrot leaves were greatly increased, by infection with *Beet yellows virus* (BYV), *Cereal yellow dwarf virus* (CYDV) and *Carrot motley dwarf virus* (CMDV) respectively, but in petioles and roots of carrots fructose and glucose were decreased (Goodman *et al*., 1965). Gonçalves *et al*. (2005) proved an increase in sugar content in sugarcane leaves infected by *Sugarcane yellow leaf virus* (ScYLV), but a reduction in sucrose content in stalks. Furthermore, Islam *et al.* (2003) showed that overall sugar content was significantly reduced in tomato fruits infected with *Tomato yellow leaf curl virus* (TYLCV). So, the sugar content change in host metabolism is equivocal because different virus-host systems have been studied with conflicting results. Besides, it is difficult to come to a conclusion about the effect of viruses on physiology of fruits, since literature has a lack of information about carbohydrate content in fruits.

Independent of CMV infection, fructose was the dominant sugar, but with similar mean content to glucose, which agrees with other references (Loiudice *et al*., 1995; Islam *et al*., 1996; Hernández Suárez *et al*., 2008b, c). Furthermore, Spearman's rank correlations analysis showed that glucose and fructose concentrations were strongly and possitively correlated (**Table 5.10**), which suggests their common origin probably from sucrose (Hernández Suárez *et al*., 2008b). In tomato, the ratio glucose: fructose tends to be about 0.8-1 according to a code of practice for evaluation of fruit and vegetable (AIJN, 1999) and the ratio obtained from both treatments was within this range (healthy control: 0.94, CMV treatment: 0.88).

5.4.2.2 Quantification of organic acids

The organic acids chromatogram analysis in tomato sample solutions presented many peaks (**Figure 5.13 B** and **C**). Only three organic acids were identified in all the tomato samples: oxalic, ascorbic and citric. The unknown peaks did not match identically against any of succinic, fumaric, pyruvic, tartaric, malic or maleic acids standards that have been reported by others to be in tomato fruits (Hernández Suárez *et al*., 2008). Analytically, the times that maleic and fumaric acids came up did

not match with time of any unknown peak of the samples. Besides, in the peaks corresponding to the retention times of succinic, pyruvic, tartaric and malic there were changes in the absorption spectrum along the peaks. This suggests that either there was a mixture of more than one compound in each peak, or these acids had been unstable.

Stability is a major problem for organic acids analysis because these compounds are known to be very unstable in aqueous solution. Ascorbic acid may be oxidized to DHA (**Figure 5.14**, **D**), so care was required during sample preparation. For example, Nováková *et al*. (2008) demonstrated that the initial concentration of ascorbic acid under the influence of natural light decreased to 84.2% in a transparent flask. In current study stability problems of ascorbic acid in sample solutions were resolved by avoiding storing samples and analysing them as soon as they were prepared, by decreasing temperature in the auto-sampler and keeping samples cool at 4°C during analysis, and finally by protecting samples from light.

In the tomato samples tested, citric acid was the major organic acid followed by oxalic which agrees with reports in other papers (Islam *et al*., 1996; Hernández Suárez *et al*., 2008). Runs of freshly prepared tomato samples showed two adjacent peaks at the retention time of oxalic, which were identified as oxalic. In order to positively identify these, a tomato sample extract was spiked with known amount of this organic acid. The oxalic acid was the only organic acid without significant difference in its content ($P < 0.05$) between the two treatments.

Little data is available on the concentration of organic acids in virus infected plants, although these compounds play an important role in plant metabolism and fruit taste and pH as well. These data report an increase in the tested organic acids of virus infected tissues. Thus more malic and citric acids were found in CMV infected tobacco leaf compared to healthy control (Porte and Weinstein, 1957). The ascorbic acid content of pepper fruit infected with *Pepper mild mottle virus* (PMMoV) was increased versus healthy control (Tsuda, 2007). These are in accordance with the results of current experiment in which citric and ascorbic acids were significantly increased in fruits of CMV infected plants.

It has been noted that there is a correlation between high ascorbic acid levels and relatively poor tomato yields (Stevens 1986; Wang, 2009), and also that the larger the tomato fruit, the lower the ascorbic acid content tends to be (Dumas *et al*., 2003). These statements agree with the increased ascorbic acid concentration in small tomato

fruits accompanied by reduced yield of CMV infected plants. Regardless of virus infection a spatial difference was found in ascorbic acid content with fruits derived from the lower parts of the plant having less ascorbic acid (**Table 5.11**). This could be due to the lower light intensity or shade that usually occurs at the bottom of a tomato plant, because under shady conditions a decrease of 15-20% has been noticed in vitamin C (ascorbic acid) levels of tomato fruits compared to fruits exposed to the sunlight (Venter, 1977; Dumas *et al*., 2003).

5.4.2.3 Quantification of carotenoids

Several published procedures (Alba *et al*., 2000; Baysal *et al*., 2000; Arias *et al*., 2000, Kimura and Rodriguez-Amaya, 2002; Datta *et al*., 2003; Olives Barba *et al*., 2006; Porcu and Rodriguez-Amaya, 2008) were tested and modified for carotenoid extraction and analysis. The purpose was to achieve the best pigment separation and the ideal chromatogram conditions, at low cost, with a simple sample preparation and short run times. Usual solvent extraction of carotenoids consumes large amounts of organic solvents (hexane, acetone, ethanol, methanol, tetrahydrofuran, petroleum ether or many mixes of them, some of which are hazardous) is time consuming and requires multiple steps.

In this study acetone was used and the samples were measured as soon as they were prepared. The extraction with acetone was sufficient as it decreased the extraction efficiency only by 3.5% for β-carotene and 1.4% for lycopene compared with other mix solvents (data not shown). Saponification was also tried and considered unnecessary because there is no fat and not enough chlorophyll in ripe tomatoes. Both isocratic and gradient elution systems were tested and the gradient solvent method was chosen for better resolution compared to isocratic. Finally, the precision intra-day and between days repeatability of the method used was obtained by running the standard and one sample many times on two consecutive days. The chromatograms were overlaid showing reproducible retention times and almost identical peak areas (**Figure 5.15**).

Fruits of virus infected plants were found to have high β-carotene content with respect to healthly fruits. On the other hand, the lycopene content was not significantly increased by CMV presence. However, fruits of infected plants reached this colour four days later (**Table 5.3**). These perhaps imply that lycopene production

in CMV infected plants was delayed, while β-cyclase, (the enzyme that converts lycopene to β-carotene) continued to be active.

There are few data referring to the impact of virus infections on carotenoids. Petrova *et al*. (2009) showed that carotenoid content was increased in upper and lower leaves of CMV infected pepper plants depending on the degree of plant susceptibility to the virus. Kapinga *et al*. (2009) proved that *Sweetpotato chlorotic stunt virus* (SPCSV) and *Sweetpotato feathery mottle virus* (SPFMV) on single and mixed infections had negative effect (reduction of 43%, 16% and 37% respectively) on total carotenoid accumulation (represented mainly by β-carotene) in orange-fleshed sweetpotato tubers. At first sight, the results of this study on sweetpotato seem to be contradictory to the ones obtained on tomato. The fact that sweepotatoes from virus infected and healthy control plants were harvested at the same time, could lead to a delay in carotenoid production. Hanssen *et al*. (2011) analyzed mature tomatoes infected by *Pepino mosaic virus* (PepMV) with typical fruit marbling and revealed a decrease in carotenoids content. This result was predictable because the reduced carotenoids concentration is responsible for the marbled phenotype of PepMV infected tomatoes. In current experiment only marketable fruits with homoemorphous colour were analyzed.

Akanda *et al*. (1998) found that leaves of CMV infected tomato plants had reduced concentration of chrorophyll and β-carotene versus healthy leaves. Similarly, Haider and Hossain (1994) showed that *Yellow vein mosaic virus* (YVMV) infected okra had less chlorophyll and β-carotene compared to healthy plants. Hemida (2005) also demonstrated that the concentrations of chlorophyll a, b and carotenoids in leaves of *Bean yellow mosaic virus* (BYMV) infected *Phaseolus vulgaris* were decreased compared with healthy plants. Recently, Muqit *et al*. (2007) examined the biochemical changes of some components in ash gourd (*Benincasa hispida*) due to three different viruses [*Bottlegourd mosaic virus* (BgMV), *Watermelon mosaic virus* 2 (WMV2) and *Papaya ringspot virus* (PRSV)]. They found in all cases that chlorophylls a and b, total chlorophyll and β-carotene contents of the infected plants were decreased compared to healthy control plants. As mentioned above, the photosynthetic process is decreased due to virus infection (Rahoutei *et al*. 2000). This fact normally leads to less chlorophyll concentration and consequently less β-carotene in leaves. All these articles deal with β-carotene in leaves and there are no reports of what happens with carotenoids content in fruits. Even in case that analogous β-

carotene reduction would happen in virus infected fruits, then the increase in β-carotene found in the current experiment could be explained by allowing the prolongation of fruit ripening. An alternative or additional explanation might be that carotenoids have some antioxidant capacity and this capacity is reported to be increased under virus stress (Clarke *et al*., 2002; Mittler, 2002; Shigeoka *et al*., 2002; Hafez *et al*., 2004; Radwan *et al*., 2010).

Independent of virus infection, the lycopene content, 62.2 μg/g and 67.6 μg/g on FW basis for fruits from healthy control and infected plants respectively, lies in the range of values given in literature [30-200 μg/g FW, the widest range mentioned by Topal *et al*. (2006) and 55-80 μg/g FW, the most frequent range mentioned by Dumas *et al*., (2003)]. The β-carotene content 1.68 μg/g and 1.87 μg/g on FW for fruits from healthy control and infected plants respectively, lies slightly above the minimum published values [1-7 μg/g FW (USDA national nutrient database mentioned by Georgé *et al*. (2011)]. The highest concentration of β-carotene in tomato is 12 μg/g on FW basis, found on a cherry tomato (Olives Barba *et al*., 2006).

The variability in the data above could be explained by the high dependence of various factors on the carotenoid content in tomatoes. Some of these factors are maturity, variety, cultivar, environmental and agronomic conditions (Dumas *et al*., 2003), technical processing, extraction method (Georgé *et al*., 2011), preparation and calibration of stock and working carotenoid standard (Hart and Scott, 1995), assay method (Scott *et al*., 1996) and the examined part of tomato fruit. In a recent study the impact of lyophilisation on carotenoids and on two other major antioxidant micronutrients (total polyphenols and vitamin C) was evaluated. It was found that lyophilisation decreased by 47% and 14% the lycopene and β-carotene contents, respectively, in ripe tomatoes compared to the same fresh analysed tomatoes (Georgé *et al*., 2011). So absolute comparison of data related to carotenoids content is difficult due to all the prementioned factors of variability among laboratories.

5.4.2.4 Antioxidant capacity

Several methods have been used to evaluate the antioxidant profile of food products and according to Cao *et al*., (1993) results may greatly vary depending on the experimental conditions and the specificity of the used free radical. Herein the antioxidant capacity of tomatoes was determined using the free, stable radical DPPH, which is not specific to any particular antioxidant (Gil *et al*., 2000). DPPH has an

absorption band at 515 nm that disappears after reduction by an antioxidant compound (Ordiozola-Serrano *et al*., 2009). To evaluate the antioxidative activity of tomato extracts, the latter reacted with DPPH in a methanol solution and the reduction of DPPH was followed by the decrease in its absorbance. Trolox was used as an antioxidant standard.

To assess the nutritional quality of fresh tomatoes, it is important to study all the main compounds having antioxidant activity. Tomatoes apart from lycopene, contain many different antioxidant micronutrients, such as A, C and E vitamins, carotenoids (β-carotene, γ-carotene), lutein, phytoene, phytofluene and phenolics (flavonoids, hydroxycinnamic acids) (Wang *et al*., 1996; Martinez-Valverde *et al*., 2002; Dumas *et al*., 2003; Chang and Liu, 2007). DPPH provides an easy and rapid way to measure the overall antioxidant capacity of tomato samples (Brand-Williams *et al*., 1995).

As was demonstrated above, β-carotene of tomato fruits from CMV infected plants was significantly increased compare to healthy control, the same effect occurred with the antioxidant capacity which was significantly higher by more than 16% both on the FW and DW basis versus healthy control plants.

Information on the fate of antioxidant activity in fruits during virus infection is lacking, but there are reports referring to the effects on plant antioxidant systems. So, there is ample evidence indicating that plants under pathogen attack, viruses included, may rapidly cause oxidative burst. The oxidative burst produces high levels of ROS and plants activate the antioxidants enzymes to protect cellular damages by regulating ROS's levels (Shigeoka *et al*., 2002; Hafez *et al*., 2004). The main antioxidant enzymes are CAT that dismutates H_2O_2 to oxygen and water, superoxide dismutase (SOD) catalyzing the dismutation of superoxide radical (O_2^-) to H_2O_2 and O_2, and ascorbate peroxidase (APX) that reduces H_2O_2 to water by using ascorbic acid as specific electron donor (Mittler, 2002; Radwan *et al*., 2010).

The changes in antioxidant content and the accumulation of some antioxidant metabolites indicate a kind of plant defence response against pathogen invasion. Thus, Radwan *et al*. (2010) demonstrated that BYMV infection caused pronounced increase in the antioxidant activity of faba bean leaf extracts detected by DPPH assay, indicating an increase in the amounts of phenolics and flavonoids. According to Huang *et al*. (2006) phenolic and flavonoids compounds have strong free radicals scavenging capacity. Moreover, *Cucurbita pepo* plants infected with CMV (Técsi *et*

al., 1996) or *Zucchini yellow mosaic virus* (ZYMV) (Radwan *et al*., 2006) presented increased peroxidase activity. Riedle-Bauer (1997) inoculated two varieties of cucumber plants with CMV and infected plants showed a significant rise in activity of peroxidase compared to healthy control plants, whereas no variation in SOD and CAT was observed.

Specifically for CMV infected tomato plants Song *et al*. (2009) demonstrated that CMV infection disturbed the photosynthetic and respiratory electron transport in tomato plants, affected at the same time the antioxidative systems, and led to oxidative stress in leaves. They also demonstrated that CMV infection increased the activities of antioxidant enzymes in chloroplasts and mitochondria of tomato leaves. Hence, a hypothesis could be made that the same processes could take place in tomato fruits leading to an increase of the total antioxidant compounds.

Generally, the values of total antioxidant content in tomatoes found in this study range around or diverge little from the values stated in literature, but again direct comparison cannot be conducted as there are many differences in the extraction methods, assays used and free radicals (Wang *et al*., 1996; Rotino *et al*., 2005; Ordiozola-Serrano *et al*., 2009).

5.5 Conclusions

This study was conducted to evaluate the impact of CMV and especially of a Greek CMV isolate on the quantity and quality traits of a Spanish tomato hybrid Delos. CMV infection had profound effects mainly on plant and fruit morphology and secondarily on fruit biochemistry.

Virus infection stressed the growth of plants as it reduced their height, stem diameter and leaf area. Although, the leaf-to-fruit ratio was almost the same between the two treatments, the fruit size of CMV infected plants was significantly decreased and as a result the production expressed as weight of total yield was reduced by 24%. Colour uniformity of some fruits (27%) was affected due to CMV symptoms, but the rest had high colour quality and could be easily consumed. Consequently, fruit production as measured by marketable fruit yield, was reduced by 40.7% under CMV infection.

Fruit maturity of CMV infected plants was delayed, for more than four days, demonstrating that viral infection affected the fruit metabolism/ development. For the first time, the nutritional composition of tomatoes derived from CMV infected plants was compared by analysing three different families of phytonutrients (sugars, organic acids and carotenoids) and total antioxidant capacity on tomato pericarp. Carotenoids and antioxidants are health-related molecules, while sugars and organic acids are important determinants of taste in fruits.

The present study quantified development decrease, small fruits production and reduced yield of a Spanish tomato hybrid infected by a Greek CMV isolate. Regarding the quality traits measured the conclusion that arises is that fruits produced from CMV4 infected tomato plants which have attractive appearance are of equivalent or even higher value from a dietary perspective compared to healthy control.

CHAPTER SIX

Impact of Benzothiadiazole (BTH) application and *Cucumber mosaic virus* (CMV) infection on quantity and quality traits of marketable tomato fruits in different cultivation seasons

6.1 Introduction

It has been shown that inducers of SAR, like BTH, influence plant metabolism by increasing plant resistance to several pathogens, including viruses. In the first year of this study it was demonstrated that when tomato seedlings were sprayed with BTH at weekly intervals and had been mechanically inoculated by CMV after the first BTH application, then significant differences in virus incidence were obtained. The incidence was lower by 85% in BTH treated plants compared to untreated.

In the second year, one experiment was performed to investigate the impact of a Greek CMV isolate on quantity and quality traits of tomato fruits of hybrid Delos. The size and fresh weight of marketable fruits of infected plants were significantly lower compared to control plants. It was also found that the levels of sucrose, fructose, ascorbic acid, citric acid, β-carotene and the total antioxidant activity were significantly higher in fruits of infected plants compared to control.

As a continuation of the above work, two experiments were performed in order to assess the influence of weekly BTH applications (12 in total), CMV infection and their combination on tomato plants and fruits. The experiments were carried out at different times of the year to estimate possible environmental effects.

6.2 Experimental design

Two tomato cultivations of hybrid Delos were conducted, one during winter (August 2009 - January 2010) and the other during spring (January - June 2010). There were four treatments: BTH treated (BTH), CMV inoculated and infected (CMV), BTH treated and CMV inoculated (BTH+CMV) and healthy (Control) plants, of 10 plants each. As mentioned in the previous experiment in chapter 5, adjacent plants touched each other and a complexly randomised design could not be applied,

because on the one hand, CMV is mechanically transmitted and on the other hand, it would be difficult for BTH application. Hence, plants of each treatment were grouped together, were divided equally between the two cabins of an insect-proof glasshouse and were differently arranged in each experiment (**Figures 6.1** and **6.2**). BTH was firstly applied when the first true leaf appeared and then was sprayed weekly until run off. Inoculation with CMV was carried out a week after the first BTH application on the first two true leaves of tomato seedlings and ELISA tests were conducted to determine virus presence. PCR analyses were also carried out to cross-check the CMV presence or absence on BTH treated and CMV inoculated tomato plants.

All ripe fruits were weighed and the first three marketable ripe fruits, developed on different trusses of each plant, were selected as samples for further analysis. The total number of samples used in each experiment was 120 (30 fruits per treatment).

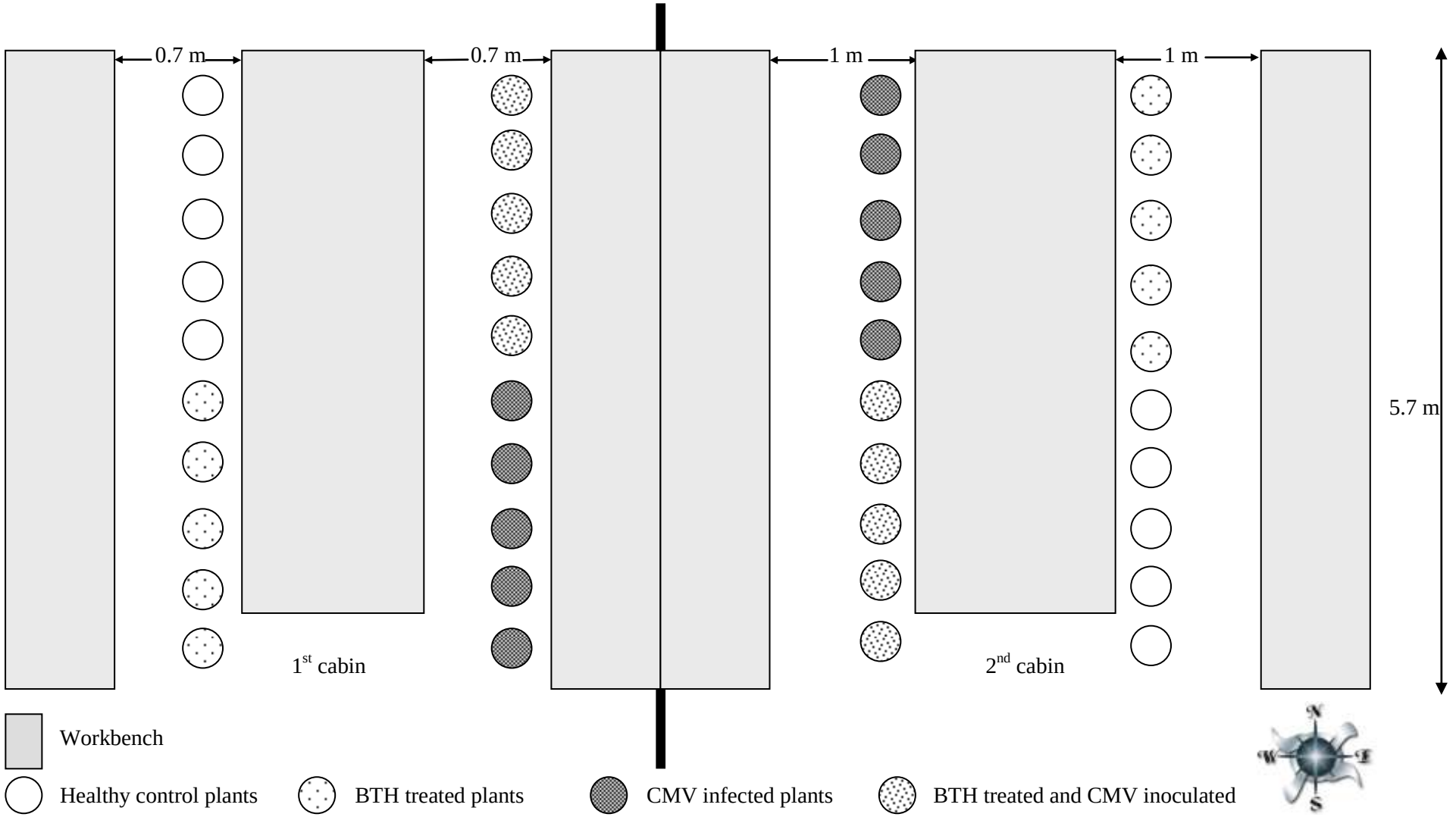

Figure 6.1. Ground plan of the glasshouse showing the tomato plants of the examined treatments arrangement of winter cultivation (August 2009 - January 2010).

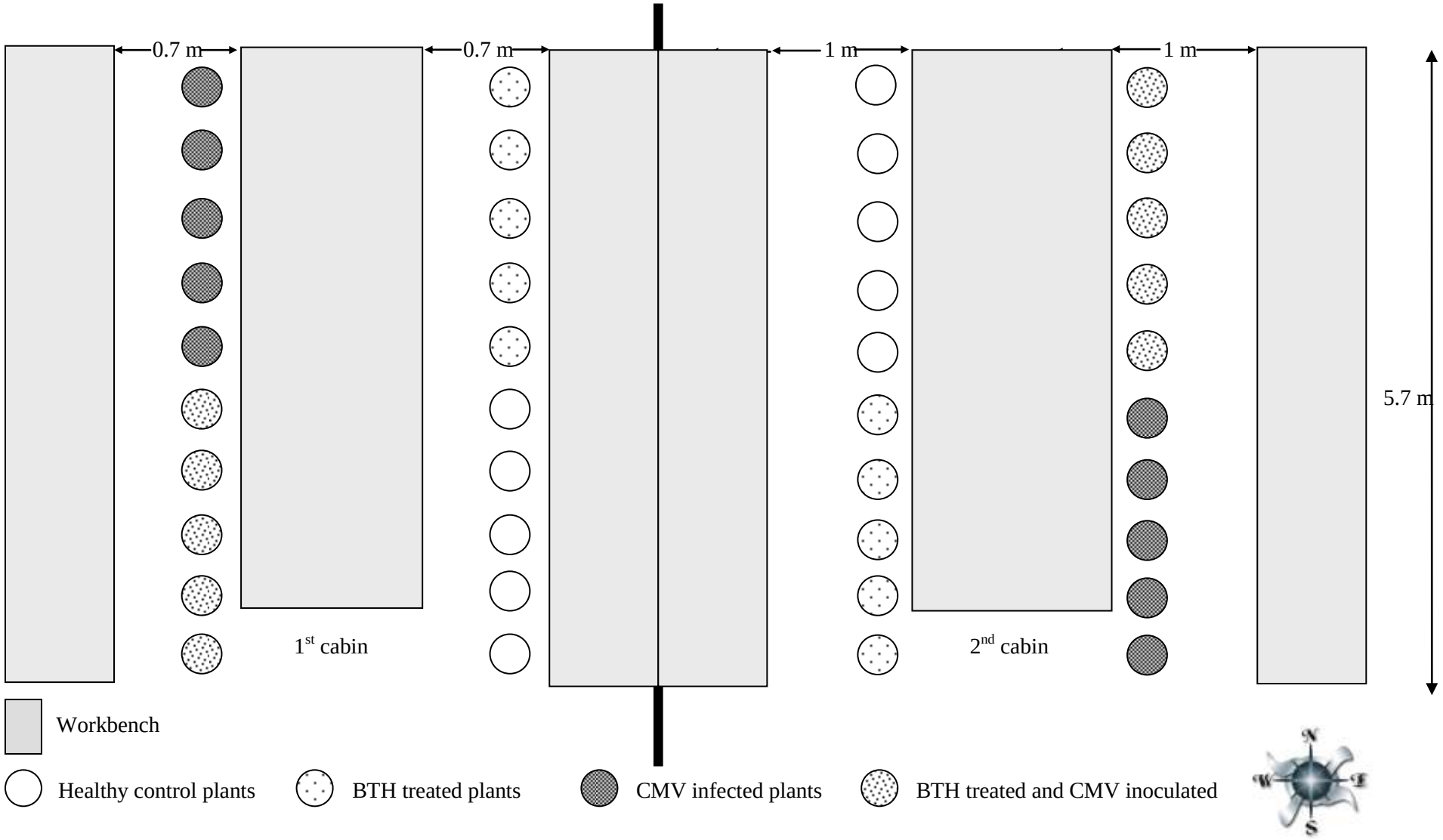

Figure 6.2. Ground plan of the glasshouse showing the tomato plants of the examined treatments arrangement of spring cultivation (January – June 2010).

6.3 Results

6.3.1 Physical assessments

6.3.1.1 Plant morphology

Differences in plant development were observed among the four treatments regardless of the growth season. The emergence rate of leaves was the same for all treatments, but the leaf area of BTH and/or CMV treated plants was reduced compared to healthy control plants. The reduction in leaf area was more pronounced for plants of both BTH (BTH and BTH+CMV) treatments than plants of CMV treatment as illustrated in **Figure 6.3**. Moreover, this reduction was more pronounced in plants of winter cultivation for both BTH treatments than plants of spring cultivation.

The height of all plants had been recorded from the 25th dpi, in intervals of about 10 days, until topping of healthy control plants (**Figure 6.2 A** and **B**). In both experiments control plants were significantly higher than BTH treated plants, followed by BTH+CMV treated plants and CMV infected plants, last in the rank. The biggest difference occured in plant mean height between control and CMV infected plants and it was 26.4% during the winter and 28.5% during the spring, between control and BTH+CMV treated plants it was 16.6% and 22.2%, between control and BTH treated plants it was 10.9% and 12.9% respectively. In the experiment conducted during the spring the plant height of each treatment was significantly different from the others at the last measurement (**Figure 6.4 B**). In particular BTH treated plants were significantly higher than BTH+CMV treated plants by 10.7% and than CMV infected plants by 19.4%, BTH+CMV treated plants were significantly higher than CMV infected plants by 11%. In the experiment conducted during the winter the height of BTH+CMV treated plants did not significantly differ from either BTH treated plants or CMV infected plants (**Figure 6.4 A**). But BTH treated plants were significantly higher than CMV infected plants, and the difference between them amounted to 18.2%.

The differences given below refer to the last measurement of mean height made when the healthy control plants reached 2.3 m. In both experiments, the height of plants of CMV treatment was significantly lower than that of healthy control plants

(by 19.6% in winter and 28.5% in spring), as well as that of BTH treated plants (by 14.5% and 19.4% respectively), and lower but without statistically significant difference than plants of BTH+CMV treatment. Only the last measurement of plant height of CMV treatment was significantly lower (by 11%) compared to that of BTH+CMV treated plants in spring experiment (**Figure 6.4 A**, **B**). Plants that remained infected were severely top stunted compared to plants that showed recovery in their new vegetation. Thus, plants of CMV treatment, especially of winter experiment, when 60% of plants showed recovery, had mean heights with greater s.d. numbers (ranged from ± 3 to ± 11 cm) than plants of all the other treatments.

The stem diameter at three points (bottom: 50 cm, middle: 130 cm and top: 210 cm) of each plant differed among the treatments as it is shown on **Table 6.1**. For both cultivations control plants had significantly thicker stem than that of the other treatments, followed by BTH treated plants.

Figure 6.3. Optical view of leaf area of hybrid Delos tomato plants from different treatments, healthy (Control), CMV4 inoculated and infected (CMV), BTH treated (BTH), BTH treated and CMV4 inoculated (BTH+CMV) plants.

Table 6.1. Effect of BTH application and/or CMV4 infection on stem diameter of hybrid Delos tomato plants measured at three different points, at bottom (50 cm), middle (130 cm) and top (210 cm).

Stem lenght (cm)	Stem diameter (mm)							
	Healthy control		BTH		CMV		BTH+CMV	
	winter	spring	winter	spring	winter	spring	winter	spring
50	11.4 (±0.4)*a**	11.6 (± 0.5) d	9.9 (±0.3) b	9.9 (±0.5) e	10.4 (±0.4) b	10.0 (±0.4) e	9.9 (±0.2) b	8.9 (±0.5) f
130	13.2 (±0.2) a	12.7 (±0.4) d	12.1 (±0.3) b	11.2 (±0.4)e	11.5 (±0.4) b	8.6 (±0.4) f	10.4 (±0.1) c	8.8 (±0.4) f
210	10.5 (±0.3) a	10.7 (±0.6) d	10.1 (±0.2) a	9.7 (±0.4) d	8.5 (±0.5) b	8.0 (±0.5) e	9.6 (±0.3) c	8.7 (±0.4) e

*Numbers in brackets indicate the ±s.d. of 10 plants

**Different letters within lines indicate significantly different values among treatments of the same cultivation (a, b, c for winter and d, e, f for spring) ($P < 0.05$)

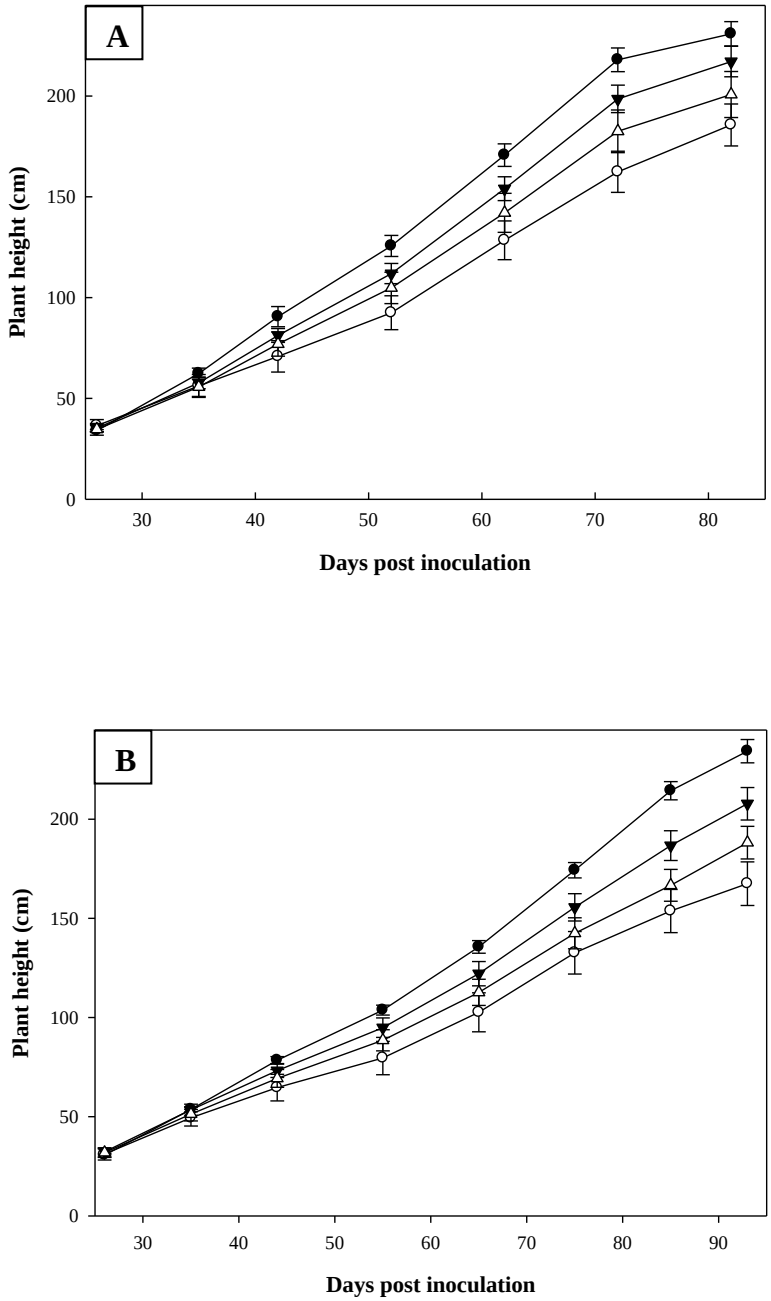

Figure 6.4. The plant height (cm) of BTH treated (▼), CMV4 inoculated and infected (○), BTH treated and CMV inoculated (∆) and healthy control (●) tomato plants after the inoculation (**A:** winter cultivation, **B:** spring cultivation). The bars represent the ±s.d. of 10 plants.

6.3.1.2 Fruit morphology and production

Fruit morphology and production was quite different between the two experiments and among the four treatments. The total number of fruits, the weight of yield and the mean fruit weight per treatment were reduced in the winter cultivation compared to the spring one (**Table 6.2**). For both experiments more ripe fruits (5.7% for the winter cultivation and 9.1% for the spring cultivation) were produced from CMV infected plants compared to healthy control plants. The BTH treatment presented reduction in number of ripe fruits by 32.5% in winter and 20% in spring versus healthy control, and the BTH+CMV treatment by 30% and 24% respectively. In both experiments CMV treatment produced symptomatic and symptomless fruits. Taking into account only the marketable fruits (integers in brackets in second column of **Table 6.2**) the number of CMV treatment fruits was less by 19.5% in winter and by 41% in spring compared to healthy control. BTH+CMV treatment produced only two symptomatic fruits in spring experiment, which additionally reduced the number of marketable fruits by 25.3% versus healthy control treatment.

The total yield weight and the mean fruit weight were reduced in all treatments for both experiments compared to healthy control. During winter BTH treated plants produced crop with 48.6% less weight and fruits with 23.9% less mean weight versus healthy control, and during spring 24.5% and 6.7% respectively. CMV infected plants presented 24% less crop weight and fruits with 28% less mean weight than healthy control in winter, and 31.9% and 38.4% in spring respectively. The BTH+CMV treatment produced reduced yield weight and fruit mean weight by 53.4% and 33.4% contrary to healthy control in winter, and 39% and 20.1% in spring respectively.

The characteristics of selected tomato samples for each treatment and experiment are listed in **Tables 6.3** and **6.4**. **Figure 6.5 (A** and **B)** shows the differences in size and overall appearance among representative fruit samples of all treatments and both cultivations. Fruits of healthy control plants were larger-sized compared to the other treatments, with a mean weight of 229.3 g for the winter cultivation and 256.3 g for the spring one. The mean weight of fruits with and without calyx from BTH treatment was reduced by 26.5% for winter cultivation and 9.6% for spring cultivation, of those from CMV treatment by 35.6% and 36.4% and from BTH+CMV treatment by 40.1% and 21% respectively. The greatest differences in

calyx fresh weight between control and all other treatments were noticed during spring cultivation. In particular, the calyx weight of fruits from BTH treated plants was reduced by 12.9% in winter cultivation and 15.5% in spring cultivation compared to fruits from control plants, of CMV infected plants by 19.3% and 38.2% and of BTH+CMV treated plants by 21.3% and 22.3% respectively (**Table 6.3**). In winter cultivation the fruit weight and all dimensions of control treatment were significantly increased as compared to the other three treatments. But in spring cultivation all these measurements of control fruits were not significantly different from fruits of BTH treatment. Fruits of BTH treatment had 9.8% and 4.6% smaller diameter versus control fruits in winter and spring cultivation respectively, 8.6% and 1.5% height, 10% and 4.7% perimeter respectively. Fruits of CMV infected plants had 14.25% and 14.65% smaller diameter compared to control fruits in winter and spring cultivation respectively, 10.5% and 12.5% height, 15% and 15.5% perimeter respectively. Fruits of BTH+CMV treated plants had 15.36% and 9.9% smaller diameter versus control fruits in winter and spring cultivation respectively, 12.7% and 5.6% height, 16.3% and 9.6% perimeter respectively (**Table 6.4**).

There was no significant difference in the dry weight as a propotion of the same fresh weight of sample pericarp among all treatments for both experiments, and this proportion was higher in spring (**Table 6.5**). The fruit density presented few differences among the four treatments and between the two experiments. For both experiments fruits of CMV infected plants needed significantly more days in order to get the same maturity stage compared to the other treatments. During winter cultivation fruits of CMV treatment needed 2.5 more days in order to ripen compared to healthy control fruits and during spring they needed nearly 7 more days. No significant difference was found in spring in the DAA among fruits of control, BTH and BTH+CMV treatments, and in winter between control and BTH treated fruits, and between BTH and BTH+CMV treated fruits (**Table 6.5**). Independent of treatment, the fruit position presented differences in fruit height, density and in DAA as shown in **Table 6.6**. The interaction between treatment and fruit position showed significant differences only in calyx weight and fruit density in spring cultivation (**Table 6.7**). For both experiments there was a high correlation coefficient (Spearman's rank correlation) between fruit weight and the diverse dimensions (**Table 6.8**).

Table 6.2. Effect of BTH application and/or CMV4 infection on tomato production of hybrid Delos, expressed as number, total weight and mean weight of ripe fruits during the winter or spring cultivation.

Treatment	Number of ripe fruits*		Total weight of yield (kg)		Mean weight of ripe fruits (g)	
(10 plants)	winter	spring	winter	spring	winter	spring
Control	123	154	24.1	35.0	195.8 (±27.4)**	230.4 (±32.0)
BTH	83	123	12.4	26.4	149.1 (±23.1)	214.9 (±27.7)
CMV	130 (99)	168 (91)	18.3	23.9	141.0 (±30.9)	142.0 (±16.4)
BTH+CMV	86	117 (115)	11.2	21.4	130.5 (±25.2)	184.1 (±25.5)

*integers in brackets indicate the marketable fruits, in cases without number in brackets means that all produced fruits were symptomless and marketable.

**numbers in brackets indicate the ±s.d. of the respective number of ripen fruits for each treatment.

Table 6.3. Effect of BTH application and/or CMV4 infection on tomato weight with and without calyx and on calyx weight of hybrid Delos.

Treatment	Fresh weight with calyx (g)		Fresh weight without calyx (g)		Calyx fresh weight (g)	
(30 fruits)	winter	spring	winter	spring	winter	spring
Control	229.3 (a)*	256.3 (a)	228.5 (a)	255.3 (a)	0.85 (a)	1.07 (a)
BTH	168.6 (b)	231.6 (ac)	167.9 (b)	230.7 (ac)	0.74 (b)	0.91 (b)
CMV	147.6 (bc)	163.0 (b)	146.9 (bc)	162.3 (b)	0.69 (b)	0.66 (c)
BTH+CMV	137.3 (c)	202.3 (c)	136.6 (c)	201.5 (c)	0.67 (b)	0.83 (b)
LSD (P=0.05)	13.8	15.2	13.8	15.2	0.05	0.06

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.4. Effect of BTH application and/or CMV4 infection on tomato dimensions and volume of hybrid Delos.

Treatment	Diameter (cm)		Height (cm)		Perimeter (cm)	
(30 fruits)	winter	spring	winter	spring	winter	spring
Control	7.58 (a)*	7.81 (a)	5.35 (a)	5.71 (a)	26.12 (a)	26.86 (a)
BTH	6.84 (b)	7.45 (a)	4.89 (b)	5.62 (a)	23.50 (b)	25.61 (ac)
CMV	6.50 (bc)	6.66 (b)	4.79 (b)	4.99 (b)	22.21 (bc)	22.70 (b)
BTH+CMV	6.41 (c)	7.03 (b)	4.67 (b)	5.39 (c)	21.86 (c)	24.28 (c)
LSD (P=0.05)	0.19	0.20	0.14	0.11	0.66	0.68

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.5. Effect of BTH application and/or CMV4 infection on DW as a proportion of FW (DW/FW), fruit density and days after anthesis (DAA) of hybrid Delos tomato fruits.

Treatment	DW/FW of pericarp		Fruit density (g cm^{-3})		DAA (days)	
(30 fruits)	winter	spring	winter	spring	winter	spring
Control	0.051 (a)*	0.060 (a)	1.39 (a)	1.37 (a)	55.5 (a)	53.1 (a)
BTH	0.049 (a)	0.059 (a)	1.39 (a)	1.38 (ab)	54.2 (ac)	53.5 (a)
CMV	0.048 (a)	0.059 (a)	1.32 (b)	1.36 (a)	57.9 (b)	59.8 (b)
BTH+CMV	0.050 (a)	0.062 (a)	1.34 (ab)	1.41 (b)	53.5 (c)	54.8 (a)
LSD (*P*=0.05)	0.001	0.002	0.026	0.02	1.0	1.2

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.6. Effect of the fruit position on fruit height, density and on days after anthesis (DAA) of hybrid Delos independently of treatment during winter or spring cultivation.

Fruit position	Height (cm)		Fruit density (g cm^{-3})	DAA (days)
	winter	spring	winter	spring
1 (bottom)	5.15 (a)*	5.69 (a)	1.33 (a)	56.7 (a)
2 (middle)	4.87 (b)	5.41 (b)	1.35 (ab)	55.2 (ab)
3 (top)	4.77 (b)	5.19 (c)	1.39 (b)	54.0 (b)
LSD (*P*=0.05)	0.12	0.10	0.02	1.1

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.7. Effect of the treatment and fruit position on the weight of calyx and on the fruit density of hybrid Delos during spring cultivation.

Fruit position	1 (bottom)	2 (middle)	3 (top)	1 (bottom)	2 (middle)	3 (top)
Treatment	Weight of calyx (g)			Fruit density (g cm^{-3})		
Control	0.86 (cde)*	1.30 (f)	1.06 (e)	1.33 (a)	1.39 (abcd)	1.40 (bcd)
BTH	0.99 (de)	0.84 (bcd)	0.89 (de)	1.37 (ab)	1.45 (d)	1.33 (a)
CMV	0.72 (abc)	0.65 (ab)	0.62 (a)	1.36 (ab)	1.36 (ab)	1.36 (ab)
BTH+CMV	0.84 (bcd)	0.82 (bcd)	0.84 (bcd)	1.41 (bcd)	1.39 (abc)	1.44 (cd)
LSD (*P=0.05*)		0.10			0.03	

*Different letters in brackets indicate significant difference values ($P < 0.05$).

Table 6.8. Spearman's rank correlation coefficients between fruit weight and morphological characteristics in winter or spring cultivation ($P < 0.05$).

	Diameter		Height		Perimeter	
	winter	spring	winter	spring	winter	spring
Height	0.68*	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
Perimeter	0.98	0.99	0.68	0.61	< 0.6	< 0.6
Weight with calyx	0.98	0.95	0.73	0.73	0.99	0.97
Weight without calyx	0.98	0.95	0.73	0.73	0.99	0.97
Weight of calyx	0.80	< 0.6	< 0.6	< 0.6	0.82	0.63

*Value > 0.6 and close to 1 means good correlation between the two elements

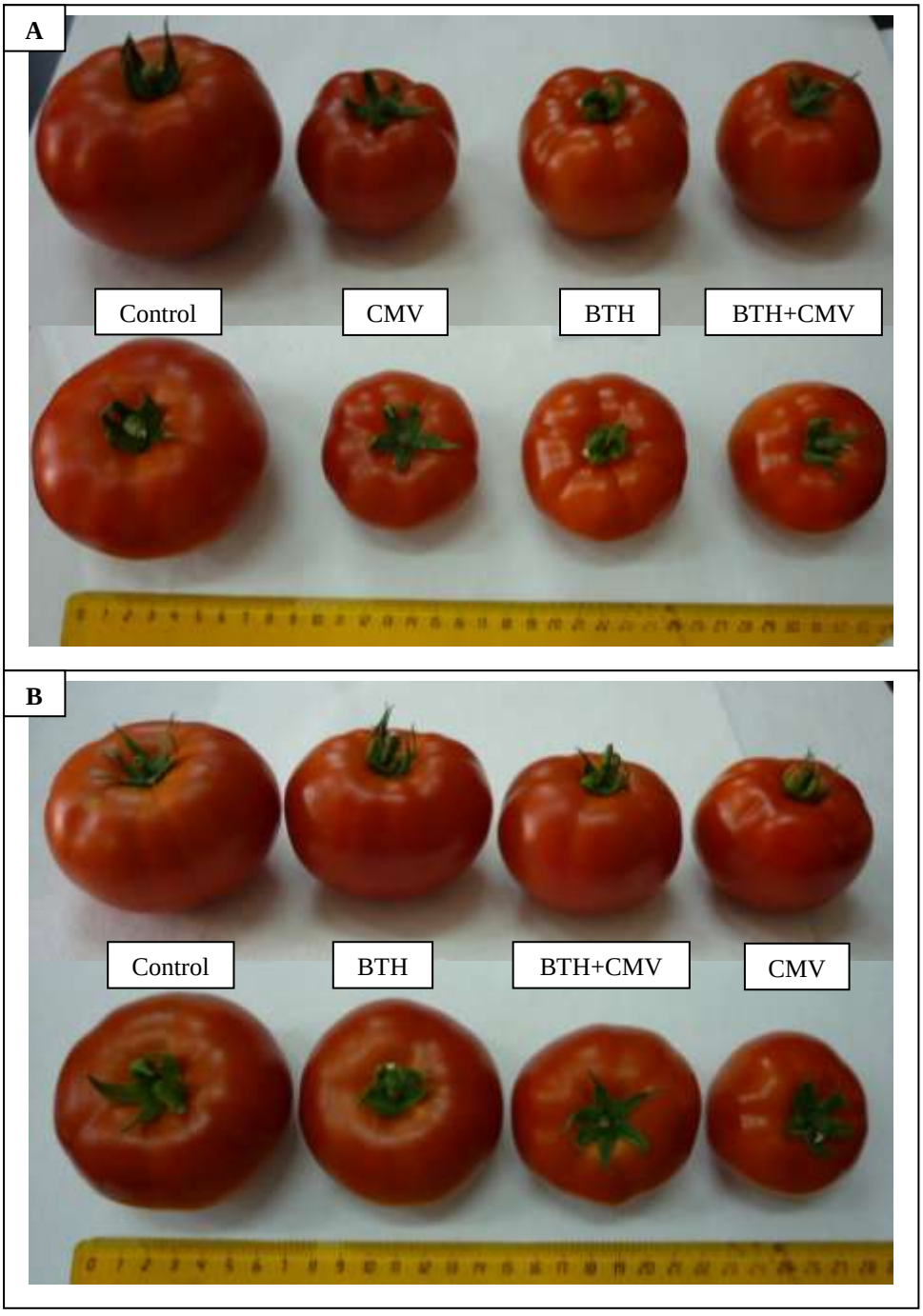

Figure 6.5. Tomato samples of hybrid Delos harvested from plants of different treatments, healthy (Control), CMV4 inoculated and infected (CMV), BTH treated (BTH), BTH treated and CMV4 inoculated (BTH+CMV) during winter (**A**) and spring cultivation (**B**).

6.3.2 Biochemical assessments

6.3.2.1 ELISA

New fully emerged leaves were selected at different intervals of time post inoculation for the two experiments and ELISA tests were conducted to determine the presence of CMV. As indicated in **Figure 6.6** within 38 dpi all plants of CMV treatment (n = 10) were infected during winter cultivation (**A**) while the respective rate of BTH+CMV treatment was 20% (two infected plants out of ten). Then plants of both treatments showed recovery in their uppermost leaves and the result of the last ELISA was that 40% (4/10) of plants of CMV treatment and none of BTH+CMV treatment remained infected (**Figure 6.6**, **A**). During spring cultivation (**Figure 6.6**, **B**) all plants of CMV treatment were infected at 28 dpi and remained infected in their newly emerged leaves till the end of the experiment. On the other hand, for BTH+CMV treatment 20% (2/10) of plants were infected at 28 dpi and last ELISA at 63 dpi showed that the percentage of infected plants was increased to 40% (4/10).

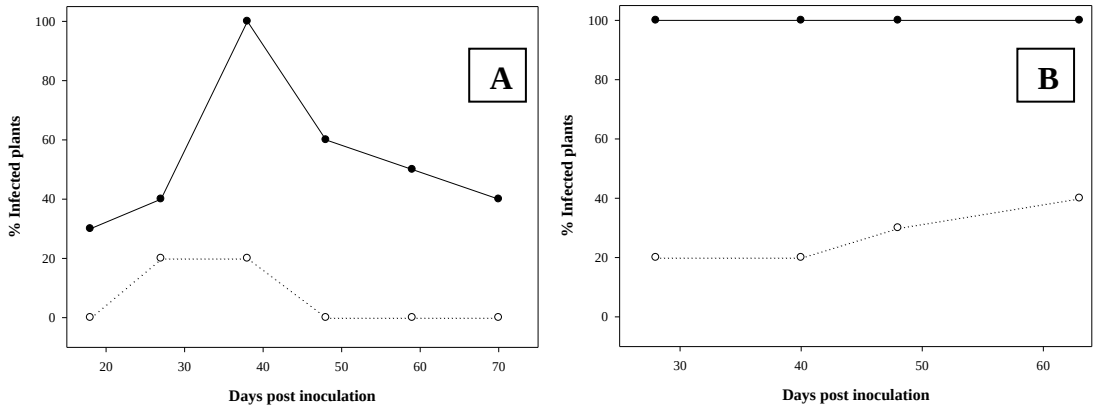

Figure 6.6. Percentage of infected tomato plants of hybrid Delos inoculated with CMV4 (●) or inoculated with CMV4 and weekly BTH treated (○) (**A**: winter cultivation, **B**: spring cultivation). ELISA tests were conducted using new emerged leaves on the top of each plant.

ELISA test was also carried out on fruit samples. In the winter cultivation 63.3% (19/30) of fruits from CMV inoculated plants were infected. In particular, all three tomato samples were infected in 30% (3/10) of CMV inoculated plants, two out of three fruits were infected in 30% (3/10) of plants and only one fruit was infected in 40% (4/10) of plants. For the spring cultivation all selected samples (30/30) of CMV treatment were infected. Referring to BTH+CMV treatment no fruit was infected for the winter cultivation, while 13.3% (4/30) of fruits were infected in spring cultivation, especially the first harvested fruit out of three was infected in each one of the BTH treated and CMV infected plant.

6.3.2.2 IC-PCR

IC-PCR analyses were carried out to cross-check the presence or absence of CMV on tomato plants of BTH+CMV treatment. The results of the IC-PCR analyses were consistent with that of the ELISA. **Figure 6.7** shows the result of the last sampling of spring cultivation (63 dpi), where four plants out of ten were infected.

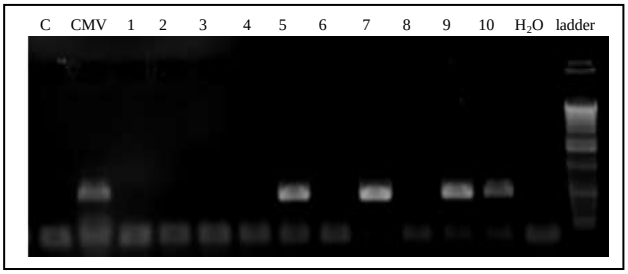

Figure 6.7. IC-PCR products demonstrating the presence of CMV4 particles in new emerged leaves of CMV inoculated and weekly BTH treated tomato plants, 63 days post inoculation in spring cultivation. Lane 1 (C), negative control healthy plant; lane 2 (CMV), positive control CMV4 infected plant; lanes 3-12 (1-10), examined samples of BTH+CMV treated plants; lane 13 (H_2O), negative control H_2O used in the reaction; lane 14 (ladder), molecular weight standard (1 kb DNA ladder).

6.3.2.3 Non structural carbohydrates (NSCs)

Sucrose was below the detection limit in most of samples for all treatments in winter cultivation and in some samples in spring cultivation. Results below the quantification limit were set at zero, which causes an unavoidable underestimation of

the mean value of sucrose content presented in **Table 6.9**. For clarity, the number of values below sucrose quantification were 66.6% (20/30) of winter samples and 30% (9/30) of spring samples of control treatment, 83.3% (25/30) and 16.7% (5/30) of samples from CMV treatment, 86.7% (26/30) and 23.3% (7/30) of samples from BTH treatment and 70% (21/30) and 40% (12/30) of samples from BTH+CMV treatment respectively. According to this statement sucrose concentration for both DW and FW basis was not affected by CMV either BTH or BTH+CMV treatment for spring cultivation, while was reduced by 78% on DW and FW basis for BTH treatment and by 75.5% on DW and 78.8% on FW basis for BTH+CMV treatment compared to control in winter cultivation.

Fructose was significantly higher on DW (9% in winter and 15.3% in spring) and FW (13.8% and 16.5% respectively) basis in fruits of healthy control plants than CMV infected plants. In winter experiment fructose of control fruits was also significantly increased by 11.4% on DW basis and 15.3% on FW basis compared to fruits of BTH treatment and by 22.1% on DW basis and 23.9% on FW basis compared to fruits of BTH+CMV treatment. In the spring experiment fructose of fruits from control, BTH and BTH+CMV treatments did not differ statistically.

In winter cultivation glucose was significantly decreased in fruits of all treatments compared with healthy control. Fruits of BTH treatment presented 16.3% less glucose concentration on DW basis and 19.8% on FW basis versus control fruits. Fruits of CMV treatment presented a reduction by 10.4% on DW and 15.2% on FW and fruits of BTH+CMV treatment by 28.9% on DW and 30.4% on FW compared to healthy control fruits. In spring cultivation, BTH or CMV treatment had no main effect on the amount of glucose in fruit compared to healthy control, but BTH+CMV treatment increased glucose concentration by 6.3% on DW and 9.9% on FW.

Total sugars for both experiments were significantly increased in fruits of healthy control compared to fruits of CMV treatment by 9.8% on DW and 14.6% on FW in winter, and by 9.3% on DW and 10.6% on FW in spring. In winter experiment total sugars of control were significantly increased by 13.9% on DW and 17.7% on FW compared to BTH treatment and by 25.4% on DW and 27.1% on FW compared to BTH+CMV treatment. Moreover, in winter cultivation each NSC measured was quite reduced compared to the respective one of spring cultivation as it is shown in **Table 6.9**, and the total NSCs were also reduced in winter versus spring by more than

17% for healthy control and CMV treatments, 29.3% for BTH treatment and 39.8% for BTH+CMV treatment.

In both experiments fructose and glucose presented Spearman's rank correlations with a high coefficient. In winter glucose FW had coefficients 0.73 with fructose DW and 0.93 with fructose FW. In spring glucose DW showed coefficients 0.71 with fructose DW and 0.67 with fructose FW.

Table 6.9. Effect of BTH application and/or CMV4 infection on the concentration of sucrose, fructose and glucose of tomato fruit of hybrid Delos, expressed per dry weight (DW) and per fresh weight (FW), for winter and spring cultivation.

	Treatment	Sucrose (mg g^{-1})		Fructose (mg g^{-1})		Glucose (mg g^{-1})		Total sugars (mg g^{-1})	
	(30 fruits)	winter	spring	winter	spring	winter	spring	winter	spring
DW	Control	1.55 (a)*	3.30 (a)	235.5 (a)	280.1 (a)	203.8 (a)	248.4 (ab)	440.9 (a)	531.8 (a)
	BTH	0.34 (b)	3.87 (a)	208.7 (b)	279.3 (a)	170.6 (b)	253.4 (a)	379.6 (b)	536.6 (a)
	CMV	0.76 (ab)	4.58 (a)	214.2 (b)	237.3 (b)	182.6 (b)	240.2 (b)	397.6 (b)	482.1 (b)
	BTH+CMV	0.38 (b)	3.26 (a)	183.4 (c)	279.0 (a)	145.0 (c)	264.1 (c)	328.7 (c)	546.4 (a)
	LSD (P=0.05)	0.47	0.69	7.3	4.5	7.8	4.4	14.8	8.4
FW	Control	0.086 (a)	0.20 (a)	11.97 (a)	16.85 (a)	10.38 (a)	14.95 (a)	22.44 (a)	32.00 (ac)
	BTH	0.019 (b)	0.24 (a)	10.14 (b)	16.52 (a)	8.32 (b)	15.01 (a)	18.47 (b)	31.78 (a)
	CMV	0.039 (ab)	0.28 (a)	10.32 (b)	14.07 (b)	8.80 (b)	14.26 (a)	19.16 (b)	28.62 (b)
	BTH+CMV	0.018 (b)	0.22 (a)	9.11 (c)	17.34 (a)	7.22 (c)	16.43 (b)	16.35 (c)	33.99 (c)
	LSD (P=0.05)	0.025	0.05	0.44	0.49	0.46	0.51	0.89	1.00

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

6.3.2.4 Organic acids

The results of organic acids assessments are presented in **Table 6.10**. Ascorbic acid concentration in fruits was not significantly affected by BTH treatment for both cultivations and only by BTH+CMV treatment for spring cultivation. In winter experiment BTH+CMV treatment had increased concentration of ascorbic acid compared to control by 26.7% on DW basis and 24.8% on FW basis. CMV treatment significantly increased ascorbic acid content by 13% on DW basis in winter cultivation and by 39.7% on DW and 36.8% on FW basis in spring.

In winter experiment citric acid was significantly increased in BTH and BTH+CMV treatments versus healthy control and was also increased in CMV treatment but with no significant difference compared to healthy control. In particular BTH treated plants produced fruits with 9.3% more citric acid on DW, and BTH+CMV treated plants produced fruits with 18% on DW and 16% on FW more citric acid than control fruits. In spring experiment citric acid was reduced in all treatments compared to control with statistical significant differences in CMV and BTH+CMV treatments. CMV treated plants produced fruits with 7.4% on DW and 9.1% on FW less citric acid than control fruits, and BTH+CMV treated plants produced fruits with 6% on DW less citric acid than control fruits.

The oxalic acid content of tomato samples was not found to be different among the four treatments for both DW and FW basis in spring cultivation. In winter cultivation oxalic acid also was not affected by the CMV infection, but it was increased by 16.45% in DW in fruits of BTH treated plants and by 21.3% in DW and 19% in FW in fruits of BTH+CMV treated plants versus to control.

The fruit position on the plant, independently of treatment affected the ascorbic acid concentration in spring as shown in **Table 6.11**. The primary fruits had lower concentration of ascorbic acid than secondary and significantly lower than tertiary (by 9.6% on DW). Moreover, from the three examined organic acids only ascorbic presented significant Spearman's rank correlations with some of the other biochemical measurements referring to lycopene, β-carotene and antioxidant capacity (**Table 6.12**).

Table 6.10. Effect of BTH application and/or CMV4 infection on the concentration of ascorbic, citric and oxalic acid of tomato fruit of hybrid Delos, expressed per dry weight (DW) and per fresh weight (FW).

	Treatment (30 fruits)	Ascorbic acid (mg g^{-1}) winter	Ascorbic acid (mg g^{-1}) spring	Citric acid (mg g^{-1}) winter	Citric acid (mg g^{-1}) spring	Oxalic acid (mg g^{-1}) winter	Oxalic acid (mg g^{-1}) spring
DW	Control	4.09 (a)*	4.64 (a)	83.5 (a)	65.18 (a)	7.78 (a)	3.48 (a)
	BTH	4.38 (ab)	4.74 (a)	91.3 (b)	63.87 (ab)	9.06 (b)	4.03 (a)
	CMV	4.62 (b)	6.48 (b)	89.0 (ab)	60.34 (b)	7.24 (a)	3.99 (a)
	BTH+CMV	5.18 (c)	4.56 (a)	98.5 (c)	61.24 (b)	9.44 (b)	3.92 (a)
	LSD (P=0.05)	0.24	0.22	3.2	1.93	0.49	0.3065
FW	Control	0.207 (a)	0.279 (a)	4.22 (a)	3.92 (a)	0.395 (ab)	0.209 (a)
	BTH	0.219 (a)	0.279 (a)	4.39 (a)	3.77 (ab)	0.440 (ac)	0.242 (a)
	CMV	0.223 (a)	0.381 (b)	4.30 (a)	3.56 (b)	0.356 (b)	0.235 (a)
	BTH+CMV	0.258 (b)	0.282 (a)	4.90 (b)	3.80 (ab)	0.470 (c)	0.242 (a)
	LSD (P=0.05)	0.010	0.014	0.16	0.14	0.028	0.020

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.11. Effect of the fruit position on the concentration of ascorbic acid, lycopene and β-carotene in tomato fruit of hybrid Delos, independently of treatment, during winter or spring cultivation.

Fruit position	Ascorbic acid DW (mg g^{-1}) spring	Lycopene DW (μg g^{-1}) winter	Lycopene DW (μg g^{-1}) spring	Lycopene FW (μg g^{-1}) winter	β-carotene FW (μg g^{-1}) winter
1 (bottom)	4.83 (a)*	994 (a)	760 (a)	49.4 (a)	2.21 (a)
2 (middle)	5.20 (ab)	918 (ab)	690 (b)	45.7 (ab)	2.30 (ab)
3 (top)	5.29 (b)	858 (b)	679 (b)	42.0 (b)	2.49 (b)
LSD (P=0.05)	0.19	46.7	30.4	2.7	0.11

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.12. Spearman's rank correlation coefficient between ascorbic acid and lycopene or β-carotene in winter cultivation and with antioxidant capacity in spring cultivation ($P < 0.05$).

	Lycopene DW winter	Lycopene FW winter	β-carotene FW winter	Antioxidant capacity DW spring	Antioxidant capacity FW spring
Ascorbic acid DW	< 0.6*	< 0.6	< 0.6	0.76	< 0.6
Ascorbic acid FW	0.65	0.67	0.66	0.71	0.69

*Value > 0.6 and close to 1 means good correlation between the two elements

6.3.2.5 Carotenoids

The carotenoids content of tomato fruits was quite different between the two experiments, with the samples of spring cultivation having lower values of trans-lycopene and β-carotene versus winter. For lycopene the reduction was 12% for BTH treatment, 23% for both CMV treatment and healthy control, and 34% for BTH+CMV treatment, while for β-carotene the reduction was 21% for BTH treatment, 20% for CMV treatment, 36% for healthy control, and 53% for BTH+CMV treatment (**Table 6.13**).

In both experiments fruits of control treatment presented the lowest carotenoid quantity compared to the other three treatments. Thus, trans-lycopene in fruits of BTH treated plants was significantly increased by 26.3% on DW basis and 20.3% on FW in winter cultivation and by 44.8% on DW and 42.2% on FW in spring versus fruits of healthy control plants. In tomatoes of CMV treatment a 24% increase on trans-lycopene was observed on DW basis and 18.3% on FW in winter experiment and 24.6% on DW and 22.6% on FW in spring compared to control. Lycopene was also raised in fruits derived from BTH+CMV treatment by 33.9% on DW and 31.6% on FW in winter and by 15.7% on DW and 18.6% on FW in spring against control.

Similarly, β-carotene concentration was increased in samples of BTH treatment by 35.4% on DW basis and 31.1% on FW in winter experiment and by 67.8% on DW and 63.7% on FW in spring. CMV treatment raised β-carotene content in tomato samples by 17% on DW in winter and by 45.9% on DW and 42.8% on FW in spring compared to healthy control fruits. Tomato samples from BTH+CMV treatment also contained 55.9% on FW and 56% on DW more β-carotene in winter and 15.1% on DW and 17.4% on FW in spring than control.

A positive Spearman's rank correlation between lycopene and β-carotene was revealed for both experiments as shown in **Table 6.14**. Moreover, lycopene and β-carotene of winter cultivation presented significant Spearman's rank correlations with ascorbic acid (**Table 6.12**). The concentrations of lycopene and β-carotene in spring or winter experiment were affected by the fruit position on the plant, regardless of the treatment, as shown in **Table 6.11**. The primary fruits had higher concentration of lycopene than secondary and significantly higher (by 13.7% on DW and 15% on FW) than tertiary in winter experiment. In spring experiment, lycopene concentration of primary fruits was also significantly higher than secondary and tertiary (by 9.2% and 10.7% on DW respectively). As regards β-carotene, the primary fruits had lower concentration of this compound than secondary and significantly lower than tertiary (by 12.3% on FW) in winter cultivation.

6.3.2.6 Antioxidant capacity

The antioxidant capacity measured in tomato pericarp was affected by the season of cultivation, as in all treatments its values were higher in spring compared to those in winter by 25.2% on DW basis and 53% on FW for BTH treatment, 30.9% and 59.7% for CMV, 31.6% and 56.3% for healthy control and 45.2% and 79.8% for BTH+CMV respectively.

Referring to the different treatments, the antioxidant capacity was significantly increased in fruits of CMV treatment for both experiments and of BTH+CMV treatment in spring experiment (**Table 6.13**, **Figure 6.8**). In winter, CMV treatment enhanced the antioxidant capacity of fruits by 21.6% on DW and 15.9% on FW, while in spring by 21% on DW and 18.4% on FW compared to healthy control fruits. Furthermore, in spring cultivation BTH+CMV treatment increased the antioxidant capacity of fruits by 13.4% on DW and 16.7% on FW versus control. Significant Spearman's rank correlation was observed between antioxidant capacity and ascorbic acid (**Table 6.14**).

Table 6.13. Effect of BTH application and/or CMV4 infection on the concentration of lycopene and β-carotene and the antioxidant capacity of tomato fruit of hybrid Delos, expressed per dry weight (DW) and per fresh weight (FW).

	Treatment	Lycopene (μg g^{-1})		β-carotene (μg g^{-1})		Antioxidant capacity (μM TE g^{-1})	
	(30 fruits)	winter	spring	winter	spring	winter	spring
DW	Control	763 (a)*	585 (a)	37.5 (a)	23.9 (a)	32.2 (a)	42.4 (a)
	BTH	964 (b)	847 (b)	50.7 (b)	40.2 (b)	33.8 (a)	42.3 (a)
	CMV	946 (b)	729 (c)	43.8 (c)	34.9 (c)	39.2 (b)	51.3 (b)
	BTH+CMV	1022 (b)	677 (c)	58.4 (d)	27.6 (d)	33.1 (a)	48.1 (c)
	LSD (P=0.05)	53.9	35.1	2.8	1.5	1.1	1.5
FW	Control	38.9 (a)	35.1 (a)	1.9 (a)	1.4 (a)	1.6 (a)	2.6 (a)
	BTH	46.8 (b)	50.0 (b)	2.5 (b)	2.4 (b)	1.6 (a)	2.5 (a)
	CMV	46.0 (b)	43.1 (c)	2.1 (a)	2.1 (c)	1. 9 (b)	3.0 (b)
	BTH+CMV	51.2 (b)	41.7 (c)	2.9 (c)	1.7 (d)	1.7 (a)	3.0 (b)
	LSD (P=0.05)	3.065	2.2	0.1	0.08	0.06	0.1

*Different letters in brackets within columns indicate significantly different values ($P < 0.05$)

Table 6.14. Spearman's rank correlation coefficient between lycopene and β-carotene in winter and spring cultivation ($P < 0.05$).

	β-carotene DW		β-carotene FW	
	winter	spring	winter	spring
Lycopene DW	0.66*	0.64	0.83	0.60
Lycopene FW	< 0.6	0.55	0.66	0.64

*Value > 0.6 and close to 1 means good correlation between the two elements

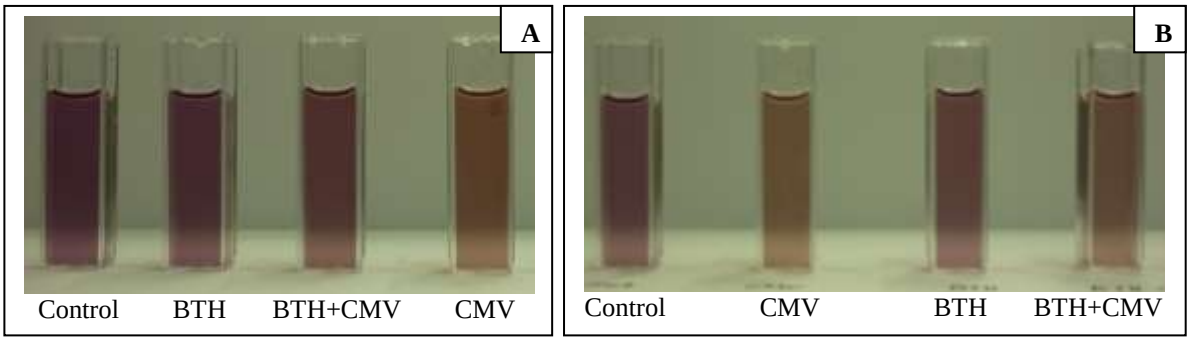

Figure 6.8. A representative sample of each treatment [healthy (Control), CMV4 inoculated and infected (CMV), BTH treated (BTH), BTH treated and CMV inoculated (BTH+CMV)] of both experiments (**A**: samples of winter cultivation, **B**: samples of spring cultivation) for the measurement of the antioxidant capacity by the use of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging. Each reaction mixture was incubated in darkness for 60 min at room temperature (25°C) and then its absorption was measured using a spectrophotometer (M501 UV/Vis Spectrophotometer, Camspec, Cambs. UK) at 515 nm. The different total antioxidant content among samples is evident from the changed colour. CMV samples of both cultivations and BTH+CMV sample in spring had significantly increased antioxidant capacity compared with the others treatments.

6.4 Discussion

6.4.1 CMV infection of tomato test plants

All plants of CMV treatment were infected for both experiments, however there were differences in the onset of the disease, the symptom appearance after inoculation and the recovery tendency regarding the growth season. Indeed, environmental conditions such as season of the year with temperature or light variations are mentioned to affect the efficiency of virus infection and the symptom development (Matthews, 1991; Balogun, 2009).

Thus, in the winter experiment (August 2009 - January 2010), some plants of CMV treatment did not manifest symptoms and 60% showed recovery in the longer term (70 dpi). The remaining 40%, which remained infected, were equally divided between the two cabins in the glasshouse (two plants out of five in each cabin, **Figure 6.1**). On the other hand, in spring (January - June 2010), all CMV inoculated plants remained infected until the end of the experiment, presented severe yellow and green mosaic symptoms in the short run and became stunted. Moreover, their leaf area was slightly reduced and curled downward compared to healthy control plants.

Balachandran *et al*. (1997) suggested that virus infections may have greater effect and be more strongly expressed symptoms in high light environments than in shaded conditions. Moreover, Handford and Carr (2007) demonstrated that virus infected wild-type *Arabidopsis thaliana* plants with CMV, *Turnip vein-clearing* (TVCV) or *Cauliflower mosaic virus* (CaMV) grown under continuous light, exhibited more severe leaf symptoms compared with plants grown under diurnal illumination. During the spring experiment light intensity was higher compared to winter, due to the sun light, independently of the additional given illumination. So, probably due to different light quality between the two seasons, in spring all plants of CMV treatment showed severe symptoms, while in winter some of them were symptomless.

ELISA tests revealed that all plants of CMV treatment were infected in newly emerged leaves as late as 38 dpi in winter experiment, when plants were inoculated during hot period (28 August 2009, with minimum temperature 23.6°C and maximum 30.4°C), and in less than 28 dpi in spring experiment, when plants were inoculated in colder period (27 January 2010, minimum temperature 16.6°C and maximum 20.2°C)

(**Figure 6.4**). These results are in accordance with Balogun (2009) who showed that the appearance of PVX symptoms in tomato plants (cv. Fukuju No.2) was earlier in plants inoculated with the virus in winter (5 dpi) compared to that inoculated in summer (20 dpi). In the first experiment described in chapter 5, aiming at fruit production of CMV infected tomato plants (October 2008 - April 2009) all plants were infected within 25 dpi, as was determined by ELISA (**Table 5.8**). In that case the inoculation took place also at a rather cool period (23 October 2008, with minimum temperature 18.6°C and maximum 23.3°C).

These data indicate that CMV4, which was used in all these experiments, was favoured in medium ranged temperatures (at about 20°C) in order to be established, propagated and become systemic in tomato plants of hybrid Delos. There are studies demonstrating that high temperature inactivates CMV. Walkey (1976) indicated that CMV could not be detected in Nicotiana cultures at 32°C, was detected at 28-30°C but resulted in a partial recovery, and remained highly infective at 22°C. Nono-Womdim *et al*. (1991) showed that the multiplication and migration of CMV from the inoculated leaf lamina to the petiole of three pepper varieties was affected by plant genotype and temperature. One variety (Yolo wonder) was systemically CMV infected regardless the season, the other (Milord) was systemically infected during the winter, while it was not infected during the summer, whereas in the third variety (Vania), CMV was not detected beyond the inoculated leaf during both seasons. In addition, Kaper *et al*. (1995) studied the effect of temperature on the response of tomato to infection with four strains of necrogenic CMV and proved that at 24°C lethal necrosis was observed, while at 32°C the response varied for the different strains from total absence or reduction of necrosis to an accelerated necrotic response. Celebi-Toprak *et al*. (2003) revealed that potato plants were resistant to systemic CMV infection when grown at low temperature (24°C) and became infected at higher temperature (30°C). Temperature may have various effects on CMV infection. According to the above references and to Kyriakopoulou *et al*. (2000) the incubation period of a virus in a plant depends on many factors such as temperature, virus strain, inoculum dosage and plant genotype and development stage at infection time.

Almost all plants of BTH+CMV treatment were symptomless throughout both experimental periods. ELISA tests showed that in winter experiment only 20% of plants were infected in their uppermost leaves at 27 dpi. Actually one plant out of five

was infected in each of the two cabins in the glasshouse and remained infected till 38 dpi, while at 48 dpi and then both showed recovery. In the spring experiment, 20% of plants were infected at 28 dpi, increased to 30% at 48 dpi and reached 40% at 63 dpi till the end, without any recovery (**Figure 6.6**). At that time, infected plants were not equally divided between the two cabins, as one plant out of five was infected in the first cabin and three out of five in the second one. The induced or acquired resistance in plants by chemical compounds, like BTH, mainly reduces disease extent or severity and rarely prevents it from occuring (Hammerschmidt *et al*., 2001). So in both seasons, weekly BTH application enhanced plants to resist CMV infection at a rate of 80% (eight plants out of ten) on the 27^{th}-28^{th} dpi.

These results are in line with that of first year experiment of this study, where tomato seedlings of hybrid F1 Clodin were mechanically inoculated with the same strain of CMV and BTH was applied weekly. Then it was shown that 85.2% (23/27) of plants remained uninfected at 22 dpi, while all plants of CMV treatment had already been infected. As mentioned in previous chapters, the first evidence that BTH protected tomato plants against CMV-Y was provided by Anfoka (2000). Analogous results were obtained in that case, as it was demonstrated that one application of BTH, as a soil drench, reduced the disease incidence by 86.4% compared with inoculated but BTH untreated plants. In particular, 12.5% of inoculated and BTH treated plants were CMV-Y infected at 21 dpi, when the corresponding percentage of inoculated untreated plants was 91.7%.

Consequently, all the above mentioned experiments, refering to the effect of BTH application on CMV infection, concluded that BTH restricted CMV infection by 80% and above, for 21-28 dpi. However, in spring experiment the rate of uninfected BTH+CMV treated plants was reduced to 60% at 63 dpi. As it was mentioned above, in spring CMV was favoured from environmental conditions, its multiplication rate was higher and BTH effectiveness was still existent but lower compared to winter. Terry and Joyce (2004b) first considered the influence of growing conditions on efficacy of BTH to suppress grey mould (caused by *Botrytis cinerea*) on strawberry fruit. They concluded that BTH efficacy may depend on environmental conditions, as it was effective in reducing incidence of this fungus in winter experiment, but ineffective in two summer experiments.

An IC-PCR was performed as an alternative approach to verify the ELISA's results referring to CMV presence in BTH treated plants. IC-PCR, one of the most accurate and highly sensitive detection methods, was conducted, in order to avoid overestimation of the BTH effectiveness in case there was virus in new emerged leaves below ELISA detection limits. The results of both methods absolutely coincided, thus, estimation of CMV infection based mainly on ELISA and partly supported by IC-PCR was considered reliable. For this reason in all fruit samples derived from CMV and BTH+CMV treatments only ELISA tests were conducted, as it is comparatively inexpensive versus IC-PCR.

The three selected fruit samples, developed on different trusses, had marketable appearance for all treatments, in both experiments. So, ELISA tests were necessary to be conducted on samples of CMV and BTH+CMV treatments, in order to determine CMV presence. In winter cultivation ELISA showed that all plants of CMV treatment produced infected fruits, although 60% of them showed recovery. Thus, 63.3% (19/30) of samples from CMV treatment were infected and each plant had from one to three out of three infected fruits. This result was possible, as virus concentration tends to be much higher in fruit than in the other plant parts (Varveri, personal communication). Gallitelli (2000) mentioned that rarely farmers may be unaware that their tomato cultivation is CMV infected until the fruits ripen, because, although foliage and plant growth seem normal, fruits are unmarketable and have internal symptoms. On the other hand, none of the selected samples of BTH+CMV treatment was infected in winter, although 20% of plants were infected and then showed recovery. In spring cultivation when CMV infection was more severe, all harvested fruit samples (30/30) of CMV treatment were infected. In BTH+CMV treatment only 13.3% (4/30) of sample fruits were infected during spring. Each one of the four infected plants of this treatment produced one infected sample out of three, particularly the one derived from the first truss. In contrast to CMV treatment, where in both seasons a high percentage of infected fruits were produced, BTH+CMV treatment minimized the production of infected fruits. This means that BTH enhanced the resistance against CMV in tomato plants and in their produced fruits.

It is possible that the recovery observed in plants of CMV treatment in winter experiment might have been due to natural resistance of plants, but if that effect was present, it was relatively less than the acquired resistance derived from BTH

application. In the spring season, when virus fitness was higher than in the winter, the effect of natural resistance may have been short-lived, diminished or non-existent, allowing all plants to become systemically infected, while acquired resistance by the plant activator BTH again suppressed CMV.

6.4.2 Impact of BTH application and/or CMV infection on tomato plant growth, fruit morphology and production of hybrid Delos.

Plants were regularly monitored to record visible differences such as general plant appearance (plant height, number of leaves and stem diameter) or symptoms occurrence. Although it was prohibitive to use a completely randomized design, as already mentioned above (in section 6.2), care was taken to minimize differences in plant growth by placing plants of each treatment in different positions for each of the two experiments (**Figures 6.1** and **6.2**). Thus, there is little likelihood the results observed in plant morphology to be related to plant position in the greenhouse. Moreover, the recorded temperatures during all experiments were within the ideal range, 20-27°C per day and 14-20°C per night (Olympios, 2001), required for beneficial growth of tomato plants, with few exceptions in upper limits during August (30°C in winter cultivation) and June (32°C in spring cultivation).

In both seasons, the healthy control treatment had significantly taller plants than the other three treatments. The height of healthy control plants was almost uniform, leading in mean heights with the smallest s.d. numbers (ranged from ± 0.5 to ± 6 cm in winter, and from ± 0.9 to ± 5.9 cm in spring). During spring experiment plants of all treatments presented slower rate of growth in height, probably due to the received light quality (more vivid sun light in spring compared to winter, led to less auxin production and shorter internodes). So, in spring, plants were shorter than that of respective treatment in winter cultivation and healthy control plants needed ten more days in order to reach the height of 2.3 m and be topped compared to winter (**Figure 6.4 A** and **B**).

Plants of both experiments were provided with one more nutrient solution, based on potassium (K, in form of KPO_4), compared to the previous experiment (October 2008 - April 2009), in order to blossom earlier, with ultimate purpose of shortening time of the experiments duration. Indeed, Ryan *et al*. (1972) examined the

effects of varying K and nitrogen (N) fertilization on the growth of tomato plants under different seasons and found a positive and significant response of plant dry weight and creation of first truss flower to increasing K, but negative response to increasing N especially in winter. So, in these experiments plants of both cultivations obtained the necessary number of trusses one month earlier and were topped at 2.3 m in contrast with the plants of previous experiment, which were topped at 2.5 m.

In both experiments, the height of plants of CMV treatment was significantly lower than that of healthy control plants. Based also on results of previous experiment investigating the impact of CMV4 on tomato plants of hybrid Delos (October 2008 - April 2009, **Figure 5.6**) it emerges that plants of CMV treatment were significantly shorter than healthy control plants, which is in line with known CMV symptomatology (Miteva *et al*., 2005).

The application of BTH, regardless of the CMV inoculation, resulted in statistically significant reduction of plant height (**Figure 6.4 A, B**) and visible reduction of leaf area (**Figure 6.3**) compared to healthy control plants. Single BTH effect on plant growth was more pronounced in spring than in winter. Moreover, plants of BTH+CMV treatment presented significant lower height compared to healthy control plants and compared to BTH treated plants.

Anfoka (2000) equally demonstrated that CMV-Y significantly reduced the plant length of tomato plants versus both healthy control and BTH treated plants, but also showed that BTH did not affect the plant growth compared to healthy control plants. This discrepancy is probably due to the different application method of BTH, because Anfoka applied it once as soil-drench, while in current study it was applied continuously (weekly, twelve times) as a foliar spray.

On the other hand, and in accordance with results of this study, Godard *et al*. (1999) noticed plant growth reduction concerning the plant height and the leaf width and length of BTH sprayed and downy mildew inoculated cauliflower plants (F1 hybrid Billabong) compared to untreated and inoculated plants. They tested many different BTH concentrations (0.0015-0.25 mg a.i./ml) and in all experiments, it was recorded a dose-dependent growth reduction (5.9%-38.3%). Louws *et al*. (2001) also, observed that BTH treated tomato seedlings were smaller than untreated plants in greenhouse experiments. Furthermore, Mandal *et al*. (2008) noted that BTH caused significant reduction on flue-cured tobacco plant height, while Nischwitz *et al*. (2008)

assessed from 3 to 10 cm differences in height between BTH treated and untreated flue-cured tobacco plants. Gondim *et al*. (2008) examined three different concentrations of BTH on melon seedlings and all plants showed stunted growth in comparison with untreated.

The same trend as observed for plant height was recorded for stem diameter (**Table 6.1**). Healthy control plants had significantly thicker stems than plants of the other three treatments. Growth season did not significantly influence the stem thickness of healthy control and BTH treated plants, but influenced the stem thickness of CMV inoculated plants regardless of BTH application. In both seasons, virus inoculated plants, either of CMV or BTH+CMV treatment generally had the thinnest stems and during spring there was no significant difference between them. Besides, in spring experiment when the environmental conditions favoured CMV development, CMV and BTH+CMV treatments presented lesser values of stem diameter compared to the respective values in winter. These results coincide with those of previous experiment (October 2008 - April 2009, **Table 5.1**) and that of Balogum (2009) with tomato plants infected with PVX and/or TMV during winter and summer seasons.

The increase in the number of leaves per plant was recorded with passing of time and was almost the same in all treatments regardless of the growth season (data not shown). Hence, in spring cultivation plants of each treatment not only were shorter, but also had shorter internodes compared to plants of the same treatment in winter. Moreover, in both experiments plants of CMV treatment, especially the systemically infected, were characterised by a marked shortening of the internodes that gave a compact and bushy appearance compared to plants of all other treatments. Thereafter, BTH+CMV treatment had the shortest internodes, followed by plants of BTH treatment and finally healthy control plants were ranked with the highest internodes.

Comparison of the number of living leaves among treatments showed that plants of both treatments with BTH application had the lowest number of leaves, because around two to four leaves of the bottom wilted. This unsatisfactory result may have happened due to toxicity, although BTH is promoted as safe and non-phytotoxic plant protector (Benhamou and Bélanger, 1998) and rare reports of harmful BTH effect on plant growth are published (Terry and Joyce 2000; Louws *et al*., 2001; Abbasi *et al*., 2002; Perez *et al*., 2003). Ishii *et al*. (1999) in agreement with results of

current study, noticed phytotoxicity such as chlorosis, browning or mosaic formation on first BTH treated leaves of cucumber plants, reporting that the level of toxicity was variable according to the environmental conditions such as light or temperature. BTH, also, presented phytotoxicity in one week old cantaloupe seedlings (Smith-Becker *et al*., 2003) and phytotoxic symptoms such as white necrotic lesions on treated leaves of flue-cured tobacco (Mandal *et al*., 2008).

Moreover, BTH application and/or CMV infection led to reduced root mass regardless the growth season. Though the root reduction was not estimated, it was more pronounced in plants of CMV treatment compared to those of BTH or BTH+CMV treatments. The negative effect of CMV on tomato roots was also observed in the previous experiment (October 2008 - April 2009). Miteva *et al*. (2005) demonstrated that CMV infection limited the growth of tomato roots and they presented elements referring to root length and weight reductions of CMV infected tomato plants versus healthy control. Regarding the effect of BTH on root development, Abo-Elyousr and El-Hendawy (2008) determined the influence of BTH and/or bacterial spot (caused by *Xanthomonas axonopodis* pv. *vesicatoria*) treatments on shoots and roots of tomato plants. Similarly to current study, they showed that BTH reduced both shoots and roots compared to healthy control plants, but improved them compared to bacterial spot infected plants. In particular, they assessed the FW and DW of tomato plants shoots and roots. Contrarily, Benhamou and Bélanger (1998) reported that root system of BTH treated tomato plants appeared healthy. It is important to note that they applied BTH as a foliar spray to tomato plants as happened in current study, but once and not repeatedly.

Fruit yield based on total crop weight of ripened fruits, marketable and not, in plants of CMV treatment was reduced compared to healthy control for both seasons. In contrast, the number of all ripened fruits of CMV treatment for both experiments was increased compared to the other three treatments. This fact is in accordance with the first experiment (October 2008 - April 2009) where the fruit number of CMV treatment was slightly increased compared to healthy control. However, it is in contrast with literature, where it is reported that CMV infected tomato plants produce few fruits (Conti *et al*., 1996; Šutić *et al*., 1999; Cerkauskas, 2004; Agrios, 2005). Balogun and Daudu (2007) reported that the rate of flower abortion was higher in

CMV inoculated tomato plants and this led to poor yield, but a similar effect was not observed in experiments of this study.

Moreover, Gallitelli (2000) reported that tomato yield was drastically reduced due to CMV consisted of unmarketable, few, small and unevenly mature fruits. In the current study, the unmarketable fruits reached the 24% of all ripen fruits of CMV treatment in winter cultivation and the 46% in spring. This statement is reducing still more the yield and the number of marketable fruits compared to healthy control plants. In terms of the interactive effect of season and virus infection, the CMV treatment presented less marketable fruits in spring experiment compared to winter, although the total, marketable and not, fruit production was higher. Analogous values with those of the winter cultivation occured for the previous experiment (October 2008 - April 2009).

The number of ripened fruits in BTH treated plants and their weight were reduced compared to healthy control plants. The highest losses in total fruit number and in yield weight were generally recorded in both CMV inoculated and BTH treated plants. However, both BTH treatments produced marketable fruits, with exception of only two fruits of BTH+CMV treatment with CMV symptoms in spring cultivation. These fruits were produced in the first truss of two different plants out of the four infected, and were divided between the two cabins.

Taking into account only the marketable fruits the deduction is that CMV treatment produced less fruits compared to all the other treatments in spring experiment, less fruits compared to healthy control and more fruits compared to BTH and BTH+CMV treatments in winter.

The weight of sample fruits showed that the growth season had influence on fruit size. In spring, the mean weight and all measured fruit dimensions, as well as the total number of ripen fruits of each treatment were higher compared to the respective values in winter crop (**Tables 6.2**, **6.3** and **6.4**). Balogun (2009) reported the same trend of higher tomato yield and average fruit weight in summer cultivation compared to winter.

Analysis of variance of fruit measured parameters showed that fruits of healthy control plants significantly differed from those of virus inoculated and/or BTH treated, exactly as happened with plant growth parameters. That is, fruits of healthy control plants had higher values compared to the others. Considering the

CMV and/or BTH effect on fruit size, fruits of BTH treatment had the highest values in both seasons. In winter cultivation fruits of BTH+CMV treatment had the lowest values, while in spring fruits of CMV treatment had the lowest values.

Tests for correlations between mean values of weight, morphological measurements and analyte concentrations of tomato fruits were made using Spearman's rank correlation. In accordance with the results of previous experiment (October 2008 - April 2009) many significant ($P < 0.05$), strongly positive correlations (in the majority r^2 was more than 0.95) among fruit weight and morphological characteristics were found in both experiments (**Table 6.8**). Similarly to the previous experiment, there was not any significant treatment-fruit position interaction or treatment-glasshouse cabin interaction for all variables assessed and for both growth seasons.

BTH application and/or CMV inoculation do not cause any significant difference in the ratio of DW/FW for pericarp of tomato fruits in each experiment separately. In the three experiments fruits of CMV treatment had the lowest values of DW/FW compared to the other treatments, though without statistical significant difference.

Weather may have contributed to increased fruit size, yield and proportion of DW/FW of pericarp in spring experiment compared to winter. Indeed, Adams *et al*. (2001) demonstrated that temperature affected the rate of tomato fruit growth in volume; in particular low temperatures reduced fruit volume. Additionally, in spring cultivation, particularly in May and June, with higher temperatures and more intense sunlight, the required rates of water containing the essential nutrients were increased and according to Chen and Inbar (1993) this beneficial uptake, positively affected plant growth and physiology. Probably this happened because of the improved absorbance of water with nutrients, followed by enhanced photosynthetic process and consequently by intensive plant and fruit growth, leading also to increased DW/FW rate.

The days that tomato fruits needed to ripe after flower opening was measured as DAA and it was observed that in spring experiment fruits of healthy control plants and of BTH treatment ripened in almost 2.5 and one less days respectively compared to winter (**Table 6.5**). This is possibly explained by the slightly higher temperature that occurred at fruit formation in spring versus winter. According to Hurd and Graves

(1985) temperature has a considerable effect on the time of fruit maturation. Especially for tomatoes, Adams *et al.* (2001) demonstrated that low temperatures increased the time that flowers need to be converted into ripe fruits. They tested many diferent temperatures (14-26°C) and a temperature dependent fruit ripening (95-42 DAA) was recorded. Contrarily, fruits of CMV and BTH+CMV treatments ripened in almost two and 1.5 more days respectively in spring compared to winter. This probably occurred because the CMV infection was more pronounced in spring than in winter.

Observations of the average number of DAA among the examined treatments showed that fruits of CMV treatment ripened significantly later than fruits of all the other treatments in both seasons. In the previous experiment (October 2008 - April 2009) fruits of CMV treatment also needed more DAA than fruits of healthy control plants in order to ripen. From the results is obvious that the more infected fruits were selected as samples, the more DAA they needed in order to mature and consequently the higher differences in DAA between CMV treatment and healthy control occurred. Indeed, when 100%, 75% and 63.3% of fruit samples were CMV infected, the fruits of CMV treatment needed 59.8, 59.23 and 57,9 DAA in order to ripen respectively and the DAA differed from the respective healthy control by nearly 7, 4.5 and 2.5 days. So it could be assumed that the more intense CMV infection leads to a higher delay in fruit maturation.

In current study BTH induced SAR in tomato plants, as it managed to reduce CMV incidence in all experiments, but also caused them a fitness cost by leading to reduced plant growth and yield compared to healthy untreated plants, regardless of the season of growth and the CMV infection. There are studies showing that plants treated with appropriate concentrations of BTH had not detrimental effect on plant growth and yield, but do not clarify whether the comparison is against BTH untreated but diseased plants or untreated healthy plants. For instance, Friedrich *et al*. (1996) reported that diverse plants treated with BTH showed no negative crop appearance or yield but without citing specific published results.

On the other hand, there are studies mentioning the negative response of BTH application to plant development and yield. For instance, Hammerschmidt *et al*. (2001) reviewed that wheat plants treated with BTH produced fewer lateral shoots and yielded a smaller amount of grain compared to untreated, and BTH treated faba bean

plants were smaller, with fewer root nodules than controls, but without giving more details about the kind of control. Romero *et al*. (2001) reported that intensive and prolonged (seven to eight weekly applications) BTH treatment on different bell pepper genotypes resulted in negative effect on yield, as there is a cost when induced resistance is expressed constitutively.

In the present study in both experiments BTH+CMV treatment not only decreased CMV incidence in leaves and fruits, but also enhanced tomato plant growth and improved fruit appearance compared to CMV treatment. Moreover, in spring BTH+CMV treatment increased the yield versus CMV treatment. There are researchers reporting the same trend, like Görlach *et al*. (1996) who showed that BTH treatment on field protected wheat against powdery mildew infection, caused by *Erysiphe graminis f* sp. *tritici*, and led to ~18% increase in yield relative to untreated and infected plants. Hammerschmidt *et al*., (2001) cited that BTH was used succesfully against *Bemisa tabaci* on tomato crops and led to better yield compared to untreated and infected plants. Finally, Abbasi *et al*. (2002) showed that BTH reduced the incidence of bacterial spot or anthracnose on tomato fruit and increased marketable yield compared to untreated and infected plants.

6.4.3 Impact of BTH application and CMV infection on the quality of tomato fruits of hybrid Delos

6.4.3.1 Quantification of NSCs

Total NSCs profile of tomato fruits was different between the two seasons. In spring cultivation total NSCs content was increased compared to winter for all examined treatments. That was possibly due to increased photosynthesis in spring (fruits were harvested May - June 2010) versus winter (fruit were harvested late November 2009 - early January 2010), because of more favourable environmental conditions. More intense sunlight in spring versus winter, resulted in higher translocation of more photosynthetic compounds into the fruit and, therefore, increased sugar concentration. Mikkelsen (2005) reported that light has the most profound effect on the fruit sugar content of all the other environmental factors, because the more sunlight reaching the fruit, the higher sugar concentration results. As a consequence, Mikkelsen (2005) concluded that tomatoes grown during the winter months contain substantially less sugar than tomatoes grown in the summer. Similar results were observed by Hernández Suárez *et al*. (2008 b) who showed that tomatoes from four out of five examined cultivars, harvested from February to June 2005 had higher mean glucose and fructose content than those harvested from October 2004 to January 2005.

For both cultivations fructose was the most abundant sugar but with similar value to glucose. On the other hand, sucrose was below the detection limit or was detected in traces. All these agree with results of Loiudice *et al*. (1995), Hernández Suárez *et al*. (2008 b and c), and the previous experiment (October 2008 - April 2009) of current study. In the previous experiment the values of fructose and glucose of healthy control fruits were lower compared with the equivalent of these two experiments, although the harvest took place between winter and spring (February - April 2009). This might have happened due to different fertilization as concerns K that was additionally applied as KPO_4 in the second year's experiments. Studies have shown that K and phosphorus (P) nutrition have a positive effect on fruit sugar content (Lacatus *et al*., 1994; Mikkelsen, 2005).

Referring to CMV effect on NSCs concentration it was observed that virus infection did not affect sucrose content, significantly reduced fructose and total NSCs

in both seasons, while glucose was significantly reduced only in winter experiment versus healthy control. Sugars in plants are produced from photosynthetic activity and this activity was found decreased in CMV infected tomato fruits (Georgieva *et al*., 2000) and plants (Song *et al*., 2009) and CMV infected melon plants (Shalitin and Wolf, 2000). Therefore, the less photosynthetic process lead to less synthesis of NSCs on virus infected plants compared to healthy plants.

Many reports mention that leaves of virus infected plants have less chlorophyll content compared to healthy leaves (Haider and Hossain, 1994; Akanda *et al*., 1998; Muqit *et al*., 2007). Particularly, Akanda *et al*. (1998) investigated CMV infected tomato leaves and found that the virus infection caused decrease in photosynthesis process. Further investigation from Muqit *et al*. (2007) in ash gourd infected with three different viruses showed that organic carbon was reduced from 2.46% to 22.31% in the virus infected leaves. Indeed, there are studies which have indicated that carbohydrate metabolism in the source leaf was influenced by viral infection (Tecsi *et al*., 1994, 1996; Shalitin and Wolf, 2000). Particularly, as already mentioned in previous chapter (section 5.4.2.1), infected source leaves had reduced photosynthetic rate and high respiration rate leading to reduced contents of soluble sugars (Shalatin and Wolf, 2000). Islam *et al*. (2003) showed that overall sugar content was significantly reduced in tomato fruits infected with TYLCV. Gupta (2010b) investigated the effect of *Soybean mosaic virus* (SMV) infection on carbohydrate content in nodules of soybean, and showed reduction of all carbohydrate (reducing sugar, non-reducing sugar and starch) content in comparison to nodules of the healthy control plants. Similarly, fungus infection alters carbohydrate metabolism in plants and fruits. For instance Oke and Banjoko (1991) observed decreases in reducing sugars of pawpaw (*Carica papaya*) fruits infected with *Penicillium digitatum* or *Fusarium oxysporum*.

These results did not confirm those of the previous experiment, where fructose was increased compared to healthy control by approximately 8% on both DW and FW basis and total NSCs was increased by 6% only on DW basis. One possible explanation for the discrepancy between the results of the two different year's experiments could arise from the different number of CMV infected fruits and different severity of CMV fruit infection in each experiment. In previous experiment CMV treatment had double number of plants (20 plants versus 10 in the next two

experiments), 25% of fruit samples were not infected and had increased sugar content compared to infected fruits. Only in spring cultivation of second year's experiment all fruit samples were severely CMV infected. So, the existence of not infected fruits may have altered the sugar concentration of CMV treatment.

BTH treatment significantly reduced NSCs content in fruits of winter versus healthy control, while did not affect it in spring cultivation. This may have happened because in winter fruits of BTH treatment matured by more than one day earlier compared to healthy control, whereas in spring they matured about half day later than healthy control. Moreover, the reduction of leaf area due to BTH application in winter was more pronounced compared to spring and this obviously caused reduction in photosynthetic area and consequently reduction in carbohydrate content. There is no information on the effect of BTH application on plant or fruit sugar content to be compared with the results of current study.

Similarly to BTH treatment, in winter BTH+CMV treatment significantly reduced all NSCs contents in fruit samples compared to healthy control. These reductions were higher versus reductions of BTH or CMV treatments. This could have happened because fruits of BTH+CMV treatment matured earlier by one day versus fruits of BTH treatment and significantly earlier by two and 4.5 days compared to healthy control and CMV treatments respectively. Moreover, CMV infection and BTH application may have acted synergistically referring to NSCs contents and led to increased reduction.

Contrarily, in spring cultivation glucose content of BTH+CMV treatment was significantly increased versus healthy, while fructose and total NSCs were not affected. In spring experiment samples of BTH+CMV treatment needed approximately two and 1.5 more days in order to mature compared to healthy control and BTH treatments respectively, probably this is an explanation why fructose and total NSCs contents of BTH+CMV treatment did not differ from healthy control and BTH treatment and why glucose was significantly higher than all the other treatments.

The results of sucrose were not considered since many of the values were below the minimum quantification limit, which was set at 4.5 mg g^{-1} on DW basis of sample.

6.4.3.2. Quantification of organic acids

In both experiments citric acid was the predominant organic acid followed by oxalic, while ascorbic (without dehydroascorbic acid; DHA) was in trace amounts. The season of cultivation differentiated the organic acids concentrations in a uniform way for the four treatments. Hernández Suárez *et al*. (2008 a) demonstrated that the sampling period is more important in the differentiation of the organic acid concentration of tomato fruits than the cultivar, cultivation method or production region.

Hence, in winter experiment citric and oxalic acid contents in fruit samples of all treatments were increased compared to spring, in both DW and FW bases. On the other hand, ascorbic acid content in winter was reduced compared to spring in DW and FW basis of all treatments, with the exception only of the DW basis of BTH+CMV treatment (**Table 6.10**). These results are comparable to findings of Hernández Suárez *et al*. (2008 a) who showed that tomatoes, of three different cultivars, harvested during October 2004 - January 2005 had higher mean concentrations of oxalic and citric acids and lower of ascorbic acid than tomatoes produced during February - June 2005. Similarly, the organic acids contents of the previous experiment (October 2008 - April 2009, **Table 5.9**) ranged more around the values of winter experiment than spring.

Sugars and organic acids mainly compose the dry matter of tomato fruit (Vermeir *et al*., 2007). Specifically, nearly half of the total dry matter consists of glucose and fructose (Shi and Le Maguer, 2000; Yin *et al*., 2010). In spring cultivation (January - June 2010) the main organic acid, citric, of all treatments was quite reduced (from 20% to 38% on DW) compared to the relative treatments of the other two experiments (October 2008 - April 2009 and August 2009 - January 2010), whereas the total NSCs content was quite increased (from 18% to 40% on DW) respectively. Perhaps, the most plausible explanation of this result is that during spring the use of organic acids as respiratory substrates and their conversion to sugars during ripening are increased compared to winter (Islam *et al*., 1996).

In spring cultivation and independently of treatment, ascorbic acid's concentration presented spatial difference on DW basis, similarly to that of the previous experiment (October 2008 - April 2009, **Table 5.11**). That is ascorbic content tended to be significantly higher on fruits derived from the upper plant parts,

where exposition to direct sunlight is better than in the middle and bottom plant parts, where shady conditions exist. According to Venter (1997) and Dumas *et al*. (2003) direct sunlight favours the accumulation of ascorbic acid, as already mentioned in the previous experiment. This differentiation may have happened only in spring experiment, because the sunlight was more intense than in winter.

CMV infection significantly increased the concentration of ascorbic acid in both cultivation periods versus healthy control. In spring, citric acid content in fruits of CMV treatment was significantly decreased compared to healthy control, while in winter it was increased but without significant difference. This may have happened due to the delay in fruit maturation that CMV infection provoked. This delay might have resulted in less total NSCs and higher ascorbic acid contents in fruits of CMV treatment compared to healthy control. In both seasons oxalic acid content was not altered by CMV treatment. This trend in general agrees with the earlier results (October 2008 - April 2009), where oxalic acid content in tomato fruits was not affected by CMV infection and ascorbic acid content was significantly increased compared to healthy control. But then, citric acid concentration was significantly increased against healthy control.

Organic acids concentrations of fruits of BTH treated plants did not vary considerably compared to healthy control fruits. Only in winter cultivation oxalic acid content on DW basis of fruits from BTH treatment was significantly increased against healthy control (**Table 6.10**). Referring to citric acid, in winter its content in tomatoes from BTH treatment was significantly increased only in DW basis while it did not differ in FW basis versus healthy control.

So, BTH application as a foliar spray on tomato plants did not affect any of the examined organic acids concentrations on FW basis of tomato fruits for both cultivation seasons. There is no information in literature about the effect of BTH on plant or fruit organic acids, except for ascorbic acid. Skłodowska *et al*. (2010) applied BTH as foliar spray on apple trees and studied its impact on ascorbic acid 2, 7 and 14 days after treatment. They found that the ascorbic content was significantly diminished (from 66% to 42%) over the examined period in comparison to control, while total ascorbic (with DHA) was significantly reduced (by 38%) only till the 7^{th} day after the BTH application. Although this decrease in ascorbic acid remained unclear, they assumed that BTH either caused enhanced utilization of ascorbic, or

disturbed its synthesis. Cao *et al*. (2011) showed that BTH treatment enhanced the ascorbate peroxidase (APX) activity. APX is an enzyme using ascorbic as a substrate, so the results of these two studies are in accordance. Taking into consideration results about ascorbic acid obtained by Skłodowska *et al*. (2010) and those of current study, it could be suggested that BTH may change ascorbic acid content only shortly after BTH application. It is known that BTH undergoes degradation in plants a few days after its application (Buonaurio *et al*., 2002), and for this reason dicotyledonous crops required repeated BTH applications to give long lasting induced resistance (Görlach *et al*., 1996; Cole, 1999; Morris *et al*., 1998; Louws *et al*., 2001; Romero *et al*., 2001). Particularly for tomato plants, a study was conducted by Scarponi *et al*. (2001) to investigate the fate of BTH in sprayed tomato leaves and it was found that BTH was rapidly transferred to apical leaves and totally disappeared three days after application. Since BTH application on current study took place on foliage of tomato plants, long before flower appearance and fruit formation, it could be presumed that BTH had no effect on fruit ascorbic acid concentration.

In BTH+CMV treatment, the organic acids contents varied with the season of cultivation. Thus, in winter all measured acids were significantly increased, on DW and FW basis, compared to healthy control, whereas in spring only citric was significantly reduced on DW basis versus healthy control. So, in winter experiment, the organic acids contents were the highest of all the other treatments. This could be due to synergistic action of BTH application and CMV inoculation, because in winter, BTH treatment also presented significantly increased citric and oxalic contents versus healthy control, while CMV treatment in both seasons had significantly increased ascorbic content versus healthy control. Another explanation for the highest organic acids concentrations of BTH+CMV treatment, in winter experiment, could be that fruits of this treatment matured (based on their colour) and were harvested earlier than those of BTH, healthy control and CMV treatments respectively. In spring experiment when few fruit samples of BTH+CMV treatment were infected ascorbic acid content was closer to values of BTH treatment, while citric and oxalic acids contents were between the contents of BTH and CMV treatments.

6.4.3.3 Quantification of carotenoids

Environmental conditions such as temperature, light and moisture variations affected the carotenoids content of tomato samples at harvest. In spring experiment, lycopene and β-carotene contents in fruits of all treatments were lower compared to winter (**Table 6.13**). This may be due to higher temperatures occurring during spring cultivation, because high temperatures have been reported to drastically reduce the lycopene content of tomatoes (Grierson and Kader, 1986; Ishida, 2000; Dumas *et al*., 2003). Toma *et al*. (2008) also demonstrated that tomatoes grown at lower temperatures than normal had much more lycopene accumulation. Moreover, it has been mentioned that tomato lycopene content decreases in moisture stress (Naphade, 1993). Though, plants were not stress irrigated, it is an absolute fact that in spring, especially during May and June, the depletion of the available soil moisture was higher than in winter.

The concentration of lycopene in these two experiments was lower compared to the previous experiment (October 2008 - April 2009, **Table 5.9**). The reason may also be the different temperatures that occurred during fruit maturation in each of the three experiments. In the previous experiment the samples were harvested during late February - early April 2009 (middle temperatures: ~18-26°C), while in winter season they were harvested during late November 2009 - early January 2010 (low temperatures: ~16-23°C) and in spring season during May - June 2010 (high temperatures: ~25-32°C). Dumas *et al*. (2003) reviewed that temperature strongly affects lycopene concentration, as temperatures below 12°C inhibit lycopene biosynthesis and temperatures above 32°C stop this process, while as favourable temperatures are considered 22-25°C. Thus, the temperatures occurring in the previous experiment were more beneficial for lycopene synthesis than those of the current experiments.

In both seasons, independently of any treatment, a spatial difference was found in lycopene concentration, with fruits derived from the lower parts of the plant having more lycopene (**Table 6.11**). This could be explained by the fact that lycopene production is inhibited by direct exposure to excessive sunlight (McCollum, 1954; Leoni, 1992; Dumas *et al*., 2003). Indeed, fruits derived from the top of the plant are more exposed to sunlight compared to those derived from the bottom, where foliage protects them from sun. On the other hand, in winter cultivation β-carotene content on

FW basis was more increased in fruits derived from upper plant parts. This probably implies that the exposure to more sunlight stimulates the conversion of lycopene into β-carotene.

In previous experiment (October 2008 - April 2009) there was a trend towards increased lycopene and β-carotene in tomato fruits of CMV treatment but with significant difference only for β-carotene, while in current experiments there were significant increases for both carotenoids in fruits of CMV infected plants versus healthy control, except for the β-carotene content in winter and only on FW basis, where the difference was not significant (**Table 6.13**). Therefore, CMV infection induced a higher production of carotenoids on tomato fruits and this may be related to the stress that virus infection causes and increases the biosynthesis of carotenoids or to the increase of fruit maturation duration of infected plants compared to healthy control. Petrova *et al*. (2009) who also found increased carotenoids content in leaves of CMV infected pepper plants mentioned that probably the carotenoids serve in a protective way against virus infection.

BTH treatment significantly increased the concentrations of lycopene and β-carotene for both cultivation seasons and both DW and FW bases compared to healthy control. Moreover, BTH treatment significantly increased the β-carotene content in both experiments and the lycopene content in spring experiment compared to CMV treatment. Most of the available information about BTH impact on tomato nutritional value focuses on the concentration of potential health-related compounds rather than taste-related compounds, such as sugars and acids. So, there is at least one available article for the BTH effect on lycopene and especially on tomato fruits.

Iriti *et al*. (2007) applied BTH (0.3 mM) post-harvest as a spray on ripened red tomatoes, three times during a week, and two days after the last spray lycopene content was measured by HPLC analysis. Lycopene was significantly increased in BTH treated fruits by 15.7% versus untreated. Furthermore, microscope observation showed that in BTH treated tomatoes the cell area occupied by lycopene crystals was higher and lycopene crystals appeared larger and more brilliant than in untreated fruits, even though these differences were not significant. The authors also concluded that lycopene as the major antioxidant in tomato fruits is possibly responsible for the resistance to grey mould that they demonstrated as a result of the post-harvest BTH treatment.

Hence, Iriti *et al*. (2007) demonstrated that BTH post-harvest application on tomato fruits significantly increased lycopene content in them, whereas in current study it was established that preharvest BTH application on tomato plants, long time before fruit formation, had the same effect on tomato fruits. It could be deduced that the alteration on plant metabolism that BTH caused to tomato plants, with regard to lycopene synthesis, was long lived. Although there is no equivalent information in literature for β-carotene, it is presumable for it to have the same fate with lycopene, because β-carotene is biosynthesized from it.

Fruits of BTH+CMV treatment had considerably higher levels of lycopene and β-carotene in winter experiment on both DW and FW bases, compared with the other treatments. This happened, although fruits of BTH+CMV treatment were harvested earlier than fruits of all the other treatments and lycopene concentration is known to be increasing sharply over time during ripening process (Dumas *et al*., 2003). This indicates that in plants treated with BTH and then challenged with CMV, a synergistic action took place in respect of carotenoids accumulation resulting to their higher concentration compared to BTH or CMV treatments. In spring experiment the carotenoids contents were also significantly increased compared to healthy control, but β-carotene was significantly reduced compared to BTH or CMV treatments and lycopene was significantly reduced only compared to BTH treatment. So in spring experiment, BTH+CMV treatment led to diminished carotenoids content versus to BTH or CMV treatments, though few fruit samples of BTH+CMV treatment were CMV infected and the DAA that they needed to mature were increased compared to fruits of BTH treatment.

Spearman's rank correlation showed that mainly the FW of ascorbic acid was closely correlated with lycopene and β-carotene in winter experiment and with antioxidant capacity in both experiments (**Table 6.12**). Ascorbic acid contributes to the antioxidant activity of tomato fruits so it is normal to be a high correlation (r^2~0.7) between them regardless of the season. Besides, in each season, lycopene and β-carotene were strongly and possitively correlated for both FW and DW basis (**Table 6.14**). This was prospective, considering that β-carotene is produced from lycopene.

6.4.3.4 Antioxidant capacity

Many studies have indicated that the antioxidative system of plants under stress is affected leading to oxidative burst (Apel and Hirt, 2004; Gondim *et al*., 2008; Song *et al*., 2009; Skłodowska *et al*., 2010). Then plants activate the antioxidants, enzymes to protect their cells against oxidative damage (Shigeoka *et al*., 2002; Hafez *et al*., 2004; Gondim *et al*., 2008; Radwan *et al*., 2010).

The season of cultivation had a strong influence on antioxidant content of tomatoes, as in spring cultivation the measured antioxidant capacity was quite increased in all treatments compared to winter. According to Dumas *et al*. (2003) it would be more appropriate to express the antioxidant content relative to the DW basis of fruits, because the main factors involved in its formation (temperature, light, water availability, mineral nutrients and stage of fruit development and ripening) often also affect the DW of the examined tissues. In the previous experiment (October 2008 - April 2009) the antioxidant capacity values were in nearly equal amounts with those of the winter experiment, since environmental conditions between them matched better than with those of the spring experiment.

Some reports describe contradictory results with both induction and inhibition of antioxidant enzyme activities involved in free radical scavenging (Baker and Orlandi, 1995; Clarke *et al*., 2002; Hernández *et al*., 2001; Dumas *et al*., 2003). So, it is dangerous to justify the seasonal differentiation in antioxidant capacity of tomato fruits and also difficult to define the optimum environmental conditions for increased biosynthesis of antioxidants. Dumas *et al*. (2003) reviewed the environmental impact on antioxidant content of tomato fruits and reported that some of the factors related with the development of various antioxidants seem to be contradictory. For instance, as was shown in the current study, direct sunlight favours ascorbic acid's accumulation (**Tables 5.11, 6.11**), whereas on the other hand, it blocks lycopene's synthesis (**Table 6.11)**. The formation of phenolic compounds requires light, like ascorbic acid does. Wilkens *et al*. (1996) demonstrated that cherry tomato plants grown under high light had about twofold greater phenols content than plants under low light. Thus, in spring experiment perhaps higher phenols accumulation occurred, because the natural light (sunlight) was without doubt higher than in winter and in previous experiment, leading to increased antioxidant capacity.

CMV infection substantially affected the antioxidant activity of fruits in both seasons. Fruits of CMV treatment had significantly higher antioxidant capacity than fruits of the other three treatments (**Table 6.13**, **Figure 6.8**). The result obtained in the previous experiment (October 2008 - April 2009) for antioxidant capacity is in accordance with those. Thereby, in all three experiments CMV infection provoked a statistical significant increase in tomato pericarp antioxidant activity compared to healthy control fruits, with respect to DPPH radical scavenging. Many reports examined the antioxidant status in several virus infected plants and concluded that virus infection caused increased antioxidant capacity in comparison with healthy plants (Técsi *et al*., 1996; Riedle-Bauer, 1997; Huang *et al*., 2006; Radwan *et al*., 2006; Song *et al*., 2009; Radwan *et al*., 2010).

No significant change of total antioxidant content due to BTH treatment was observed in both experiments, either on DW basis or on FW. Since in both cultivations lycopene and β-carotene contents in fruits of BTH treatment were significantly increased versus healthy control, there was a possibility for total antioxidant activity to also be enhanced, but this was not definite considering that antioxidant capacity involves many other compounds apart from carotenoids. As already mentioned in previous chapter the antioxidant activity in tomatoes except for lycopene, β-carotene, and ascorbic acid (vitamin C), which were separately measured in current study, arises from γ-carotene, lutein, phytoene, phytofluene, vitamins A and E and various phenolic compounds too (Wang *et al*., 1996; Martinez-Valverde *et al*., 2002; Dumas *et al*., 2003; Chang and Liu, 2007). All these compounds are thought to be health-promoting factors with antioxidant properties in tomato fruits.

Little information is available on the impact of BTH on antioxidant capacity in diverse fruits, while more is available referring to vegetation tissues. BTH has been described to be associated with accumulation and activation of phenolic compounds (Benhamou and Bélanger, 1998; Karjalainen *et al*., 2002; Terry and Joyce, 2004b). Soylu *et al*. (2003) demonstrated BTH induced resistance in tomato seedlings against bacterial canker. They associated the expression of induced resistance with a significant enhanced POX and glutathione peroxidase (GPX) activities, measured till ten days after BTH treatment. Skłodowska *et al*. (2010) examined the antioxidant system of BTH treated apple trees for a fortnight after treatment and found that CAT activity was significantly increased versus untreated control, while APX activity was

not affected. Cao *et al*. (2010) showed that during ten days of storage, postharvest BTH treated strawberries presented higher levels of anthocyanin and increased activities of enzymes correlated with the anthocyanin, flavonoid and phenylpropanoid pathways compared to untreated fruits. Furthermore, Cao *et al*. (2011) examined for interval of ten days, the effect of postharvest BTH treatment on radical scavenging capacity in strawberry fruits against DPPH and they showed that the capacity was significantly increased compared to untreated fruits. They also, measured the total phenolics and the activities of main antioxidant enzymes and demonstrated that BTH enhanced the antioxidant system of strawberry fruits by increasing the content of total phenolics, total anthocyanins and the activities of SOD, APX and glutathione reductase (GR).

The main and essential difference between experiments of current study and that conducted by afore-mentioned scientists is the time of BTH application as regards the antioxidant capacity assessment. For instance, Cao *et al*. (2011), who also studied antioxidant capacity in fruits against DPPH, applied BTH postharvest in strawberry and they measured DPPH scavenging activity 2 to 10 days afterwards, whereas in current study BTH was applied as a foliar spray long time before tomato fruit formation and DPPH scavenging activity assessment. Soylu *et al*. (2003) and Skłodowska *et al*. (2010) also estimated the antioxidant alteration 5 to 10 and 2 to 14 days after BTH treatment respectively. As already mentioned BTH is characterised by rapid degradation in plant tissues (Buonaurio *et al*., 2002), thus the accumulation and activation of antioxidant enzymes may be temporary. Indeed, Cao *et al*. (2011) showed that activities of SOD, CAT and GR enzymes and contents of total phenolics and anthocyanins increased rapidly in BTH treated strawberries till the fourth day after BTH application, and thereafter decreased gradually until the tenth day of the experiment, but still remained significantly increased compared to the relative measurements of untreated fruits. Similarly, Skłodowska *et al*. (2010) reported that BTH treatment substantially affected certain of the examined enzyme activities the first days after treatment and thereafter the activities diminished, while some of them on the 14^{th} day did not differ any more from the control value.

In the same way as in BTH treatment the antioxidant activity of BTH+CMV treatment was significantly reduced versus CMV treatment regardless of the growth season. In winter cultivation none of fruit samples of BTH+CMV treatment was

infected, so fruits were not under virus stress conditions which could alter their antioxidant capacity. Perhaps for this reason in winter experiment the antioxidant content of BTH+CMV treatment was closer to that of BTH treatment rather than CMV. On the contrary, in spring cultivation few fruit samples of BTH+CMV treatment were CMV infected. In that case there was virus effect on antioxidant capacity of tomato fruits but not as intense as in CMV treatment where all fruit samples were infected, resulting in significant statistical difference between BTH+CMV and CMV treatments.

6.5 Conclusions

The aim of current study was to determine the influence of BTH application in different seasons on CMV disease, plant growth, fruit production and fruit quality.

Analysis of the effect of season shows that in spring cultivation, CMV infection was more severe and prominent (100% infected tomato fruit samples and plants), with more pronounced symptoms and impact on fruit quantity and quality traits versus winter (63.3% infected fruits and 40% infected plants). On the other hand, in spring BTH was more effective with positive influence on fruit weight, and quality traits versus winter. In both seasons the acquired resistance derived from BTH+CMV treatment suppressed CMV, as in the end of winter experiment BTH+CMV treatment had no sample or plant infected, while in spring experiment only 13.3% of fruit samples and 40% of plants were infected.

There was also seasonal fluctuation of fruit size and carbohydrate composition regardless of treatment. For instance, fruit samples of winter experiment were smaller than those of spring. Moreover, variation in total NSCs content even on healthy control samples was observed. Thus, fruits harvested in winter season (late November 2009 - early January 2010) had lower sugar concentrations than those harvested in late spring season (May - June 2010), regardless the treatment. The opposite occurred with antioxidant capacity, carotenoids, citric and oxalic acids contents of all treatments, whose values were increased in winter versus spring. Ascorbic acid concentrations differed between the two cultivation seasons, but not in a uniform way for all treatments. Pieper and Barrett (2008) demonstrated that nutritional and quality parameters of tomato fruits vary greatly by year for the same tomato cultivar. Hence,

the sampling period is an important influential factor in the differentiation of the tomato samples according to their morphological and chemical characteristics.

During CMV-tomato plant interactions, host metabolism might be strongly altered. This includes an increase in the rate of respiration, a decrease in the rate of photosynthesis and differentiation in the accumulation of various substances (Balachandran *et al.*, 1997; Shalatin and Wolf, 2000). The results of these experiments referring to CMV treatment demonstrated higher total fruit number, diminished total weight of yield and plant growth (severe stunting, reduction of leaf area, stem diameter and root mass), significant delay in fruit ripening, significant reduced fruit mean weight and size, significantly increased ascorbic acid, lycopene, β-carotene contents and significantly enhanced total antioxidant capacity compared to healthy control.

Sugars and organic acids are important determinants of taste in fruits and vegetables, while carotenoids content and total antioxidant capacity indicate fruits and vegetables nutritional value. Taking into account that only in spring experiment all fruit samples from CMV treatment were infected, the conclusion could be that CMV infection caused deterioration in fruit taste by the significant reduction of total NSCs and citric acid concentrations, but also raised their nutritional value by the significant increase of antioxidant capacity, ascorbic acid, lycopene and β-carotene contents compared to healthy control.

BTH treatment significantly reduced fruit sample weight and size only in winter experiment against healthy control. Concentration of essential nutrients such as ascorbic acid and total antioxidant capacity in fruits of BTH treated plants did not differ from those in fruits harvested from healthy control plants. Furthermore, for both cultivation seasons, the accumulation of carotenoids in fruits of BTH treated tomato plants was found higher compared to healthy fruits.

BTH, as a systemic inducer of resistance in plants, resulted in reduction of disease severity. Both foliar and fruit CMV disease was significantly lower in BTH+CMV treated plants than in the CMV treatment regardless the growth season. In winter the effect of BTH+CMV treatment on tomato plants ranged more around the relative values of BTH treatment, while in spring approached those of CMV treatment. The opposite occurred for the tomato fruit morphological characteristics.

This study clearly showed that BTH application, independently of any kind of infection, reduced the growth of the hybrid Delos tomato plants. Nevertheless, there is a hypothesis saying that there is an enhanced fitness in BTH treated plants if parasites are present but a reduced fitness when parasites are absent (Baldwin, 1998; Romero *et al*., 2001), suggesting that induced resistance has unavoidably an inherent cost.

Indeed, the results of both experiments indicated that BTH could be used to reduce CMV incidence in tomato, however BTH application is not without its risks as the induced resistance showed negative effect on the tomato plants with respect to growth inhibition as well as reduction of yield and some quality traits of harvested fruits compared to healthy control. The metabolic processes which are changed due to BTH application might be the cause of yield reduction and quality deterioration of tomato. On the other hand, it is worth mentioning that BTH+CMV treatment not only decreased CMV incidence in leaves and fruits, but also slightly enhanced tomato plant growth and improved fruit appearance compared to CMV treatment.

CHAPTER SEVEN

General discussion and conclusions

7.1 General discussion

Greek horticulture is characterized by a great diversity of annual crops of high commercial value, among which tomato (*Lycopersicon esculentum* Mill.) is particularly important. The aphid borne *Cucumber mosaic virus* (CMV) is one of the most important factors limiting tomato production worldwide, Greece included, as it can completely destroy the crop. A direct control method for the suppressing viral diseases is not yet available and their control is only based on preventive measures of questionable effectiveness.

This study was carried out to determine the efficacy of pyraclostrobin and BTH in reducing the mechanically or aphid-transmitted CMV incidence in tomato under greenhouse conditions. The results comprise the first evidence that BTH plant activator could be valuable to the tomato cultivation against a Greek CMV isolate. So it was confirmed that BTH was capable of inducing SAR in tomato seedlings against CMV, even under the virus natural mode of transmission by aphids. On the other hand pyraclostrobin was ineffective.

Therefore BTH was chosen for further investigation and it was established beyond doubt that BTH repeated application, as a foliar spray suppressed CMV infection on tomato plants and fruits during either winter or spring cultivation. Results clearly demonstrated that independently of growth season, carotenoids content was substantially increased in fruits of BTH and/or CMV treated plants than healthy ones. It was also shown that BTH induced resistance, in the absence of virus pressure, did not modify the antioxidant capacity, NSCs and organic acids profiles in tomato fruit. Thus, tomato growers would be interested to use BTH, as it can play an important role in suppressing CMV and reducing economic losses from this virus infection to tomato, with increasing the health-related compounds of fruits and additionally without affecting the taste-related compounds.

In current study, the suppressive effect induced by BTH against CMV declined during the winter season and enhanced during the spring season. The protective effects of BTH against diverse pathogens cannot be taken for granted, because the SAR efficacy induced by BTH on a particular crop depends on a number of variables such as environmental conditions, dose and frequency of BTH application, the pathogen, the host genotype, and in some cases, the growth stage of the plant (Vallad and Goodman, 2004). Moreover, growers, who have the intention to use chemical inducers of SAR, like BTH, should know that this kind of plant activators rarely prevent a disease from occurring but usually reduce its extent or severity.

BTH treated and CMV inoculated plants presented enhanced tomato plant growth and improved fruit appearance compared to CMV treatment. However the BTH treatment, regardless virus infection, caused reduction in vegetative tissue and crop as compared with the healthy control plants. Although more studies needed to more accurately determination of BTH effect on plant growth and fruit production, from the present results it can be concluded that the induction of resistance by this abiotic elicitor reflected an inherit cost in overall plant growth. This cost is probably caused by a competition between *de novo* production of compounds leading to pathogen resistance and those proteins that are involved in growth related processes (Heil *et al*., 2000). Indeed, defence against several pathogens associated with constitutive production of secondary metabolites. Production of these compounds not only diverts carbon from primary growth functions, but has also a cost of synthesis in terms of energy input and storage (Hammerschmidt *et al*., 2001).

The production of decreased yield which was demonstrated could not be acceptable by growers. But in agricultural production, the losses caused by the induction of resistance may be minor when compared to those observed in non-induced plants subjected to disease pressures especially under epidemic conditions. So a future research, in the field that time, could examine whether BTH (either soil or foliar application) based management of CMV in tomato plants results in higher or less yield and total cost (including implementation cost) compared to the virus protection of tomato cultivation by traditional preventive means. Further study is needed to define the limited set of conditions under which allocation cost that result from the production of defence related substances lead to fitness cost, even if small.

To conclude, this research project represents an important step in illuminating the antiviral effect of BTH and the impact of BTH application and/or a Greek CMV isolate inoculation on tomato plant and fruit. The attractive characteristics of SAR (powerful mechanism for enhancing the overall resistance in plants) make the use of BTH an approach for managing viruses in a sustainable manner, within the scope of a conventional agriculture system. Though induced resistance by BTH elicitor has an unavoidable inherent cost, nevertheless, it can be considered a valuable tool in the frame-work of integrated management of plant virus control, as no viable alternative is yet available.

7.2 Project conclusions

The project objectives were set out in Chapter 1, section 1.2.2. A brief summary of the conclusions of the project in terms of the objectives is shown below:

- *To investigate BTH and pyraclostrobin efficacy against CMV after aphid transmission or mechanical inoculation in tomato plant-seedlings.*

Independently of the used inoculation method BTH treatment suppressed CMV. In contrast to BTH, pyraclostrobin was ineffective against mechanically inoculated or aphid transmitted CMV (Chapter 4).

- *To examine if BTH and/or pyraclostrobin, under different incubation periods and number of applications, induce resistance in tomato seed plants to mechanically inoculated TSWV and PVY.*

BTH induced resistance in tomato plant-seedlings to TSWV, whereas did not induce to PVY. The best protection against TSWV disease was obtained when BTH was applied seven days before virus inoculation irrespective of the number of BTH applications. Pyraclostrobin induced resistance may act against some host-virus isolate combinations under certain conditions as it was effective against one PVY isolate during the immediate period after inoculation (Chapter 4).

- *To investigate BTH efficacy against CMV in produced tomato fruits under greenhouse cultivation.*

BTH resulted in reduction of CMV disease severity in tomato fruit regardless the growth season (Chapter 6). In particular none of the selected samples of BTH treated and CMV inoculated plants was infected in winter cultivation and only 13% (4/30) of sample fruit were infected during spring.

- *To determine the impact of BTH treatment and/or CMV infection on plant growth, fruit morphology and fruit yield production.*

CMV infection had profound effects on plant and fruit morphology. Virus infection stressed the growth of plants, decreased the fruit size and reduced the total yield compared to healthy control plants (Chapters 5 and 6).

BTH application, independently of any kind of infection, reduced the growth of tomato plants and the yield production versus to healthy control plants. BTH treatment significantly reduced fruit sample size only in winter experiment against healthy control, while in spring there was no significant difference in the fruit morphological characteristics between the two treatments. BTH treated and CMV inoculated tomato plants presented slightly enhanced plant growth and improved fruit appearance compared to CMV treatment regardless the season of growth (Chapter 6).

- *To determine the impact of BTH treatment and/or CMV infection on quality traits of marketable fruits, as regards to nonstructural carbohydrates (NSCs), organic acids, carotenoids and antioxidant capacity.*

BTH did not significantly affect the nutritional status of tomatoes regarding sugars, organic acids and total antioxidant capacity, with the exception of carotenoids content which was significantly higher against healthy control. Marketable fruits produced from CMV infected tomato plants were of equivalent or even higher value from a dietary perspective compared to healthy control.

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APPENDIX

An overview of the results of all physical and biochemical assessments

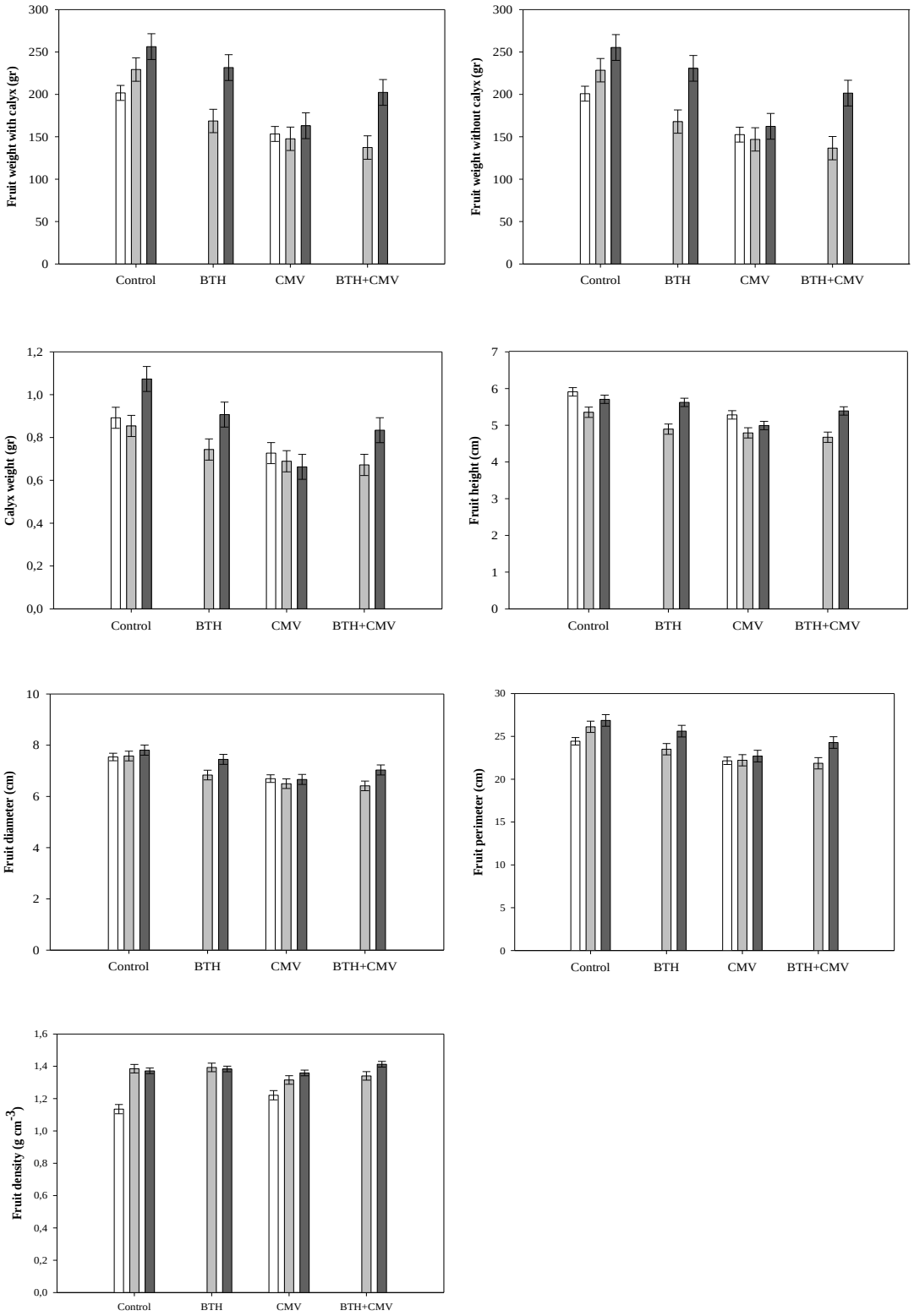

Figure 1 Effect of BTH application and CMV infection on the fruit weight with and without calyx, the calyx weight, the fruit height, diameter, perimeter and density of Delos tomatoes cultivated in October 2008 - April 2009 (▭), August 2009 - January 2010 (▬) and January - June 2010 (■). LSD bars (*P*=0.05) are shown.

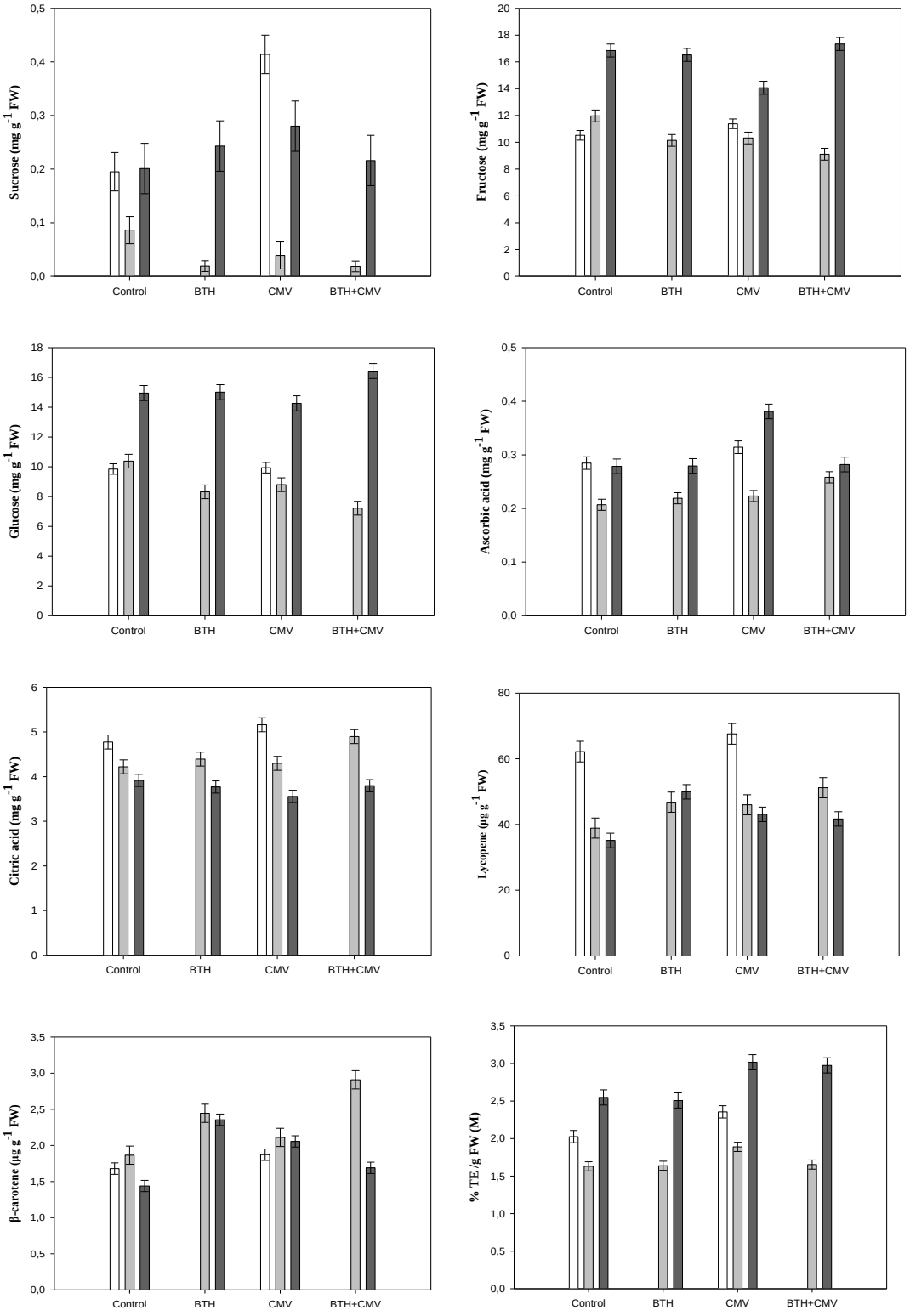

Figure A.2. Effect of BTH application and/or CMV infection on sucrose, glucose, fructose, ascorbic, citric, lycopene, β-carotene concentrations and antioxidant activity expressed per fresh weight (FW) of Delos tomatoes cultivated in October 2008 - April 2009 (▭), August 2009 - January 2010 (▬) and January - June 2010 (■). LSD bars (P=0.05) are shown.

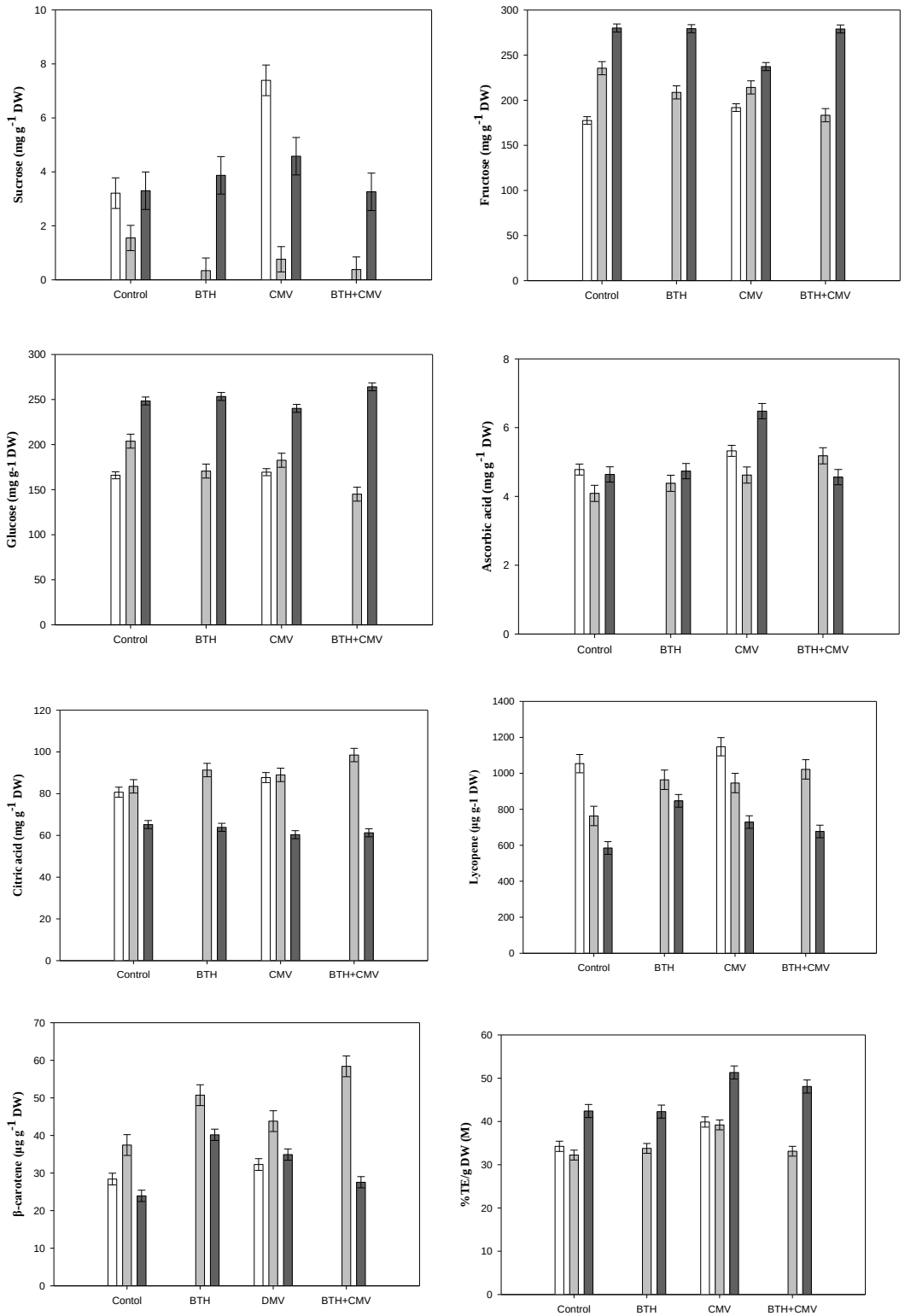

Figure A.3. Effect of BTH application and CMV infection on sucrose, glucose, fructose, ascorbic acid, citric acid, lycopene, β-carotene concentrations and antioxidant activity expressed per dry weight (DW) of Delos tomatoes cultivated in October 2008 - April 2009 (▭), August 2009 - January 2010 (▬) and January - June 2010 (■). LSD bars (P=0.05) are shown.