Effect of different forms of nitrogen nutrition on kiwifruit development and resistance to *Pseudomonas syringae* pv. *actinidiae*

Ana Patrícia Gonçalves Fernandes

Biologia Funcional e Biotecnologia de Plantas

Departamento de Biologia

2019

Orientador

Doutora Susana Maria Pinto de Carvalho, Professora Auxiliar, Faculdade de Ciências do Porto

Coorientador

Doutora Marta Wilton Vasconcelos, Professora Auxiliar, Escola de Biotecnologia da Universidade Católica

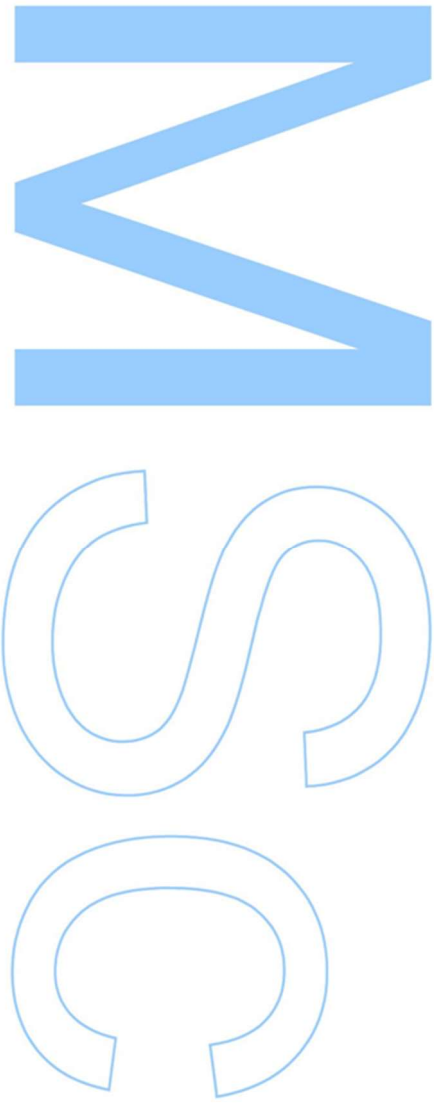
U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



Acknowledgments

I would like to begin by thanking my supervisor Susana Carvalho and my co-supervisor Marta Vasconcelos, for giving me this opportunity and trust me to integrate their team, sharing their knowledge and provide me with their time, concern and support throughout this important journey in my life.

A special thank you to Marta Nunes da Silva for always being there to help me, calm me when things didn't go the right way and guide me through every step of my thesis. Thank you for being my mentor and a friend.

To all the members of Plantech lab (Plant Biotechnology for Sustainability) for always being willing to help me in whatever I needed, for accepting me on their team and making my integration so easy and enjoyable and to the project 'Foundation for Science and Technology' (PTDC/AGR-PRO/6156/2014 and UID/Multi/50016/2019) for supporting and making it possible to perform this thesis.

To my friends, specially Daniel, Duarte and Ricardo, for accompany me and always support me in everything. Your role in this step of my academic journey was fundamental, thank you for always keeping up my spirit.

Finally, I would like to thank Tiago for taking care of me, motivate me and for being always available to comfort me when I felt the most anxious but also for celebrating with me the best moments, and to my family, specially my mother, for the emotional support and love, I couldn't ask for better, without you I would never had made it.

Thank you all very much.

Resumo:

A bactéria *Pseudomonas syringae* pv. *actinidiae* (PSA) é o agente causal do cancro bacteriano do kiwi (CBK), uma doença que afeta a produção deste fruto em todo o mundo, levando a acentuadas perdas económicas. Até ao momento, nenhum método curativo para o CBK foi desenvolvido e os tratamentos químicos existentes consistem apenas em evitar a entrada e a propagação do inóculo. É, portanto, necessária uma abordagem integrada que inclua uma nutrição equilibrada das plantas, medidas culturais para uma boa higiene do pomar e estratégias de controle biológico para reduzir a severidade desta doença. Existem evidências que apontam para o papel do azoto em aspetos relativos à resistência constitutiva e induzida, sendo que a forma de azoto também parece afetar a tolerância/suscetibilidade das plantas a vários patogénios. Posto isto, este estudo teve como objetivos: i) compreender as alterações biométricas, fisiológicas e metabólicas induzidas pela fertilização com diferentes formas de azoto em kiwis e ii) avaliar como é que diferentes formas de azoto influenciam a resposta do kiwi à infeção pela PSA. Para isso, plantas de *Actinidia chinensis* var. *deliciosa* cv. "Hayward" propagadas "in vitro" foram cultivadas hidroponicamente sob diferentes fontes de azoto (NO_3^-, NH_4^+ ou uma mistura de ambos) e (i) avaliadas 21 e 36 dias após serem transferidas para o meio hidropónico, ou (ii) avaliadas 15 dias pós-inoculação (dpi) com $1x10^7$ UFC.mL^{-1} de PSA através de avaliações biométricas, fisiológicas, produção de metabólitos secundários (clorofila total, carotenoides, polifenóis solúveis totais, flavonoides e lenhina), minerais e proteína total.

Quando comparadas com plantas tratadas com NO_3^-, as plantas tratadas com NH_4^+ apresentaram altura inferior (cerca de 0,6-vezes), menor comprimento das raízes (0,7 vezes), menor biomassa radicular (0,3-vezes) e valores inferiores de cálcio, magnésio e potássio (até 0,8-, 0,5-, e 0,6-vezes, respetivamente). Nestas plantas tratadas com NH_4^+, verificou-se ainda uma menor razão entre a raiz e a parte aérea, maior conteúdo de clorofilas, concentração de azoto e proteína total (1,2-, 1,7-, 2,2-vezes, respetivamente). As unidades formadoras de colónias (UFC), nas plantas mantidas com NH_4^+, foram 34- e 7-vezes superiores às dos tratamentos com NO_3^- e Mix, respetivamente. A inoculação das plantas tratadas com NH_4^+ resultou num aumento de lenhina nas raízes e de proteínas na parte aérea de 1,7- e 1,1-vezes, respetivamente. Adicionalmente, os teores de fósforo, potássio e ferro na parte aérea aumentaram 1,4-, 1,6- e 1,6-vezes, respetivamente, e o valor de zinco nas raízes diminuiu 0,5-vezes. Este estudo permitiu concluir que a suplementação com NH_4^+ resultou num menor desenvolvimento das plantas de kiwi e numa maior suscetibilidade à PSA do que os

tratamentos NO_3^- e Mix, o que pode ter sido devido a diferenças na assimilação e acumulação de nutrientes importantes na defesa das plantas como o cálcio, o magnésio e o potássio.

Palavras-chave: amónia, bactéria, carotenoides, clorofilas, fenólicos totais, flavonoides, lenhina, nitrato, nutrientes, proteínas totais, suscetibilidade, UFCs.

Abstract:

Pseudomonas syringae pv. *actinidiae* (PSA) is the causal agent of kiwifruit bacterial canker (KBC), a disease that affects the production of this fruit worldwide, leading to great economic losses. To date, no curative methods for KBC have been developed, and the existing chemical treatments only consist of avoiding inoculum loading and propagation. So, an integrated approach that includes balanced plant nutrition, cultural measures for good orchard hygiene and alternative biological control strategies to diminish disease severity. There is evidence pointing to the role of nitrogen in aspects of constitutive and induced resistance traits, and the form of nitrogen also appears to affect plant tolerance / susceptibility to various pathogens. Therefore, this study aimed to: i) understand the biometric, physiological and metabolic changes induced by the fertilization with different nitrogen forms in kiwifruit and ii) to evaluate how different forms of nitrogen influence the response of kiwifruit to PSA infection. For this, plants of *Actinidia chinensis* var. *deliciosa* cv. 'Hayward' propagated 'in vitro' were cultured hydroponically under different nitrogen forms (NO_3^-, NH_4^+ or a mixture of both) and (i) evaluated 21 and 36 days after transfer to hydroponic medium, or (ii) evaluated 15 days post inoculation (dpi) with $1x10^7$ $CFU.mL^{-1}$ of PSA through biometric, physiological evaluations, production of secondary metabolites (total chlorophyll, carotenoids, total soluble polyphenols, flavonoids and lignin), minerals and total protein.

When compared to plants treated with NO_3^-, NH_4^+-treated plants had lower height (by ca.0.6-fold), shorter root length (by 0.7-fold), lower root biomass (by 0.3-fold) and showed lower values of calcium, magnesium and potassium (up to 0.8-, 0.5-, and 0.6-times, respectively). In these plants treated with NH_4^+, was also observed a lower root to shoot ratio and a higher chlorophyll values, nitrogen and total protein (1.2-, 1.7- and 2.2-times, respectively). Colony-forming units (CFU) in NH_4^+-maintained plants were 34- and 7-fold higher than NO_3^- and Mix treatments, respectively. Inoculation of NH_4^+-treated plants resulted in an increase of lignin in the roots and protein in the shoots by 1.7- and 1.1-fold, respectively. Additionally, phosphorus, potassium and iron concentrations in the shoot increased 1.4-, 1.6- and 1.6-fold, respectively, and zinc values in the roots decreased by 0.5-fold. This study allowed to conclude that NH_4^+ supplementation resulted in a reduced development of kiwifruit plants and in a higher susceptibility to PSA than NO_3^- and Mix treatments, which may be due to differences in assimilation and accumulation of important plant defence nutrients such as calcium, magnesium and potassium.

Keywords: ammonia, bacteria, carotenoids, CFUs, chlorophylls, flavonoids, lignin, nitrate, nutrients, susceptibility, total phenolics, total proteins.

Index

i) Index of tables and figures

Figure 11: Concentration of Polyphenolics (A), Flavonoids (B) and Lignin (C) ($mg.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 12: Concentration of macronutrients: Nitrogen (A), Phosphorous (B), Potassium (C), Calcium (D) and Magnesium (E) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 13: Concentration of micronutrients: Boron (A), Zinc (B), Manganese (C) and Iron (D) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 14: Percentage of protein on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 24 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 15: Shoot and root length (cm) (A) and biomass (g) (B) of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 16: Colony Forming Units (CFUs) ($CFUs.g^{-1} \times 10^{11}$) of PSA in *A. chinensis* var. *deliciosa* cv. 'Hayward' inoculated plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 17: Root to shoot ratio (A); SPAD (B); Total chlorophyll ($mg.g^{-1}$) (C) and Carotenoids ($mg.g^{-1}$) (D) values in *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 18: Concentration of Polyphenolics (A), Flavonoids (B) and Lignin (C) ($mg.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 19: Concentration of macronutrients Nitrogen (A), Phosphorous (B), Potassium (C), Calcium (D) and Magnesium (E) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis var. deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 20: Concentration of micronutrients Boron (A), Zinc (B), Manganese (C) and Iron (D) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Figure 21: Percentage of protein on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

ii) List of abbreviations and acronyms

ANI - Average Nucleotide Identity

BCA - Biological Control Agents

CFU - Colony Forming Units

DGAV - Direção Geral de Agricultura e Veterinária

EPPO - European Plant Protection Organization

FAOSTAT - Food and Agricultural Organization – Statistics division

FW – Fresh Weight

HR - Hypersensitive Response

INE – Instituto Nacional de Estatística

KBC - Kiwifuit Bacterial Canker

LB medium - Luria Bertani medium

MDA - Malondialdehyde

MLS – Multilocus sequence

NH_4^+ - Ammonium

NO – Nitric Oxid

NO_3^- - Nitrate

NSA - Nutrient Sucrose Agar

PAL - Phenylalanine Ammonia-Lyase

PFM - *Pseudomonas syringae* pv. *actinidifoliorum*

PSA - *Pseudomonas syringae* pv. *actinidiae*

ROS - Reactive Oxygen Species

SA – Salicylic Acid

SPAD - Plant Analyzer Development

T3SS – Type III Secretion System

1. Introduction

1.1 Genus *Actinidia*

Originated from southwestern and central China, plants from the genus *Actinidia* are dioecious perennial vines, with an active-Y sex determination mechanism. Its vegetative growth gives origin to branches, succession of nodes and internodes and each node contains a leaf and a dormant axillary bud (Ferguson and Huang, 2007; Neves, 2008; Hanley, 2018). The annual growth can be originated from axillary buds and adventitious buds and the older semi lignified branches are generally reddish-brown and relatively erect, often with short linear lenticels. Leaves may vary in shape, but are simple and alternate, with hairs on the lower page. Flowers appear in the axillary part of the leaves, isolated or in small inflorescences, with at least five white, yellow or rose-coloured petals, depending on the species. The radicular system can reach great volumes and densities, especially comparing to other fruit trees (Neves, 2008a).

Actinidia fruits can be described as berries with numerous seeds embedded in a juicy pericarp, being able to contain up to 1 400 seeds (Ferguson, 1984; Hopping, 1990). Initially, this fruit was called “Chinese gooseberry”, but later was renamed as “kiwifruit” by the New Zealand fruit handling firm Turners & Growers Ltd. (Auckland, New Zealand). Although for many years the name “kiwifruit” referred only to the fruit of one specific cultivar of one of the several *Actinidia* species (*Actinidia chinensis* var. *deliciosa* cv. ‘Hayward’), nowadays it is used to refer the fruit of any *Actinidia* species (Ferguson, 2013).

The genus *Actinidia* is part of the Ericales order, being dicotyledonous flowering plants, and is comprised in the *Actinidiaceae* family, which, until now, includes over 54 different species (52 endemic to China, 1 from Japan and 1 from Nepal), 21 varieties and 75 taxa (Li *et al*., 2007; Huang, 2016; Hanley, 2018). However, kiwifruit production depends almost exclusively on two species: *A. chinensis* and, in a much lower degree, *A. arguta* (Hanley, 2018) (Fig.1).

A. chinensis species comprises the two most economically important varieties: *A. chinensis* var. *chinensis* (Fig.1A) and *A. chinensis* var. *deliciosa* (Fig.1B). *A. chinensis* var. *chinensis*, also known as the yellow-fleshed kiwifruit, has been increasingly growing in production with cultivar ‘Hort16A’, registered by Zespri under the trademark Zespri® Gold, being the second variety to have an international commercial dimension (Neves, 2008b; Hanley, 2018). The first place is occupied by *A. chinensis* var. *deliciosa,* green-fleshed kiwifruit, cultivar ‘Hayward’, which overwhelmingly dominates the market having as its main polliniser ‘Tomuri’, a late-flowering male cultivar (Neves, 2008b; Huang,

2016). Although these cultivars are the most commonly grown worldwide for commercial purposes, there are other *A. chinensis* var. *deliciosa* pistillate cultivars (female), such as 'Abbott', 'Allison', 'Bruno', 'Monty', 'Qinmei', and other staminated cultivars (male) with some degree of commercial interest, like 'Matua' and 'Chieftain' (Neves, 2008b; Huang 2016).

More recently *A. arguta* (Fig.1C), internationally known as kiwi berry, has gain some commercial interest, being present in several countries as USA, New Zealand, Chile, France, Germany, Italy and Japan. Although *A. arguta* fruits have a similar taste to of the green-fleshed kiwifruit, being inclusively sweeter in some cases, and a similar nutritional profile rich in antioxidants, vitamins and minerals, its cultivation continues largely limited mainly because harvesting the fruit is very labour intensive and its storage life is more limited than the other cultivars (Ferguson, 2013).

Figure 1: Fruits of: A- *Actinidia chinensis* var. *chinensis*; B- *Actinidia chinensis* var. *deliciosa*; C- *Actinidia arguta* (Adapted from Huang, 2016).

1.2 World and national commercialization of kiwifruit

A. chinensis var. *deliciosa* seeds were introduced for the first time from China to New Zealand in 1904, and in 1928 'Hayward' cultivar was developed by Professor Hayward Wright (Rubio *et al*., 2014; Huang 2016). The first commercial exportation of this cultivar took place in 1956, and in the 70's this culture definitively settled on the local market. Around the same time, the first orchards of kiwifruit began to appear in Italy, but it was during the 80's and 90's that there was further expansion and development of this crop. Although kiwifruit is native from China, from a commercial point of view, this country started to develop this culture more recently, about a quarter of a century ago (Franco, 2008). In Portugal, this culture has aroused a growing interest since the 1990s, due to the good prices achieved by the fruit at the market level, the productive potential, low

cost of production and also for presenting low phytosanitary problems (Félix e Cavaco, 2004; Franco, 2008).

Worldwide, kiwifruit production has been increasing along time since the exponential increment observed in 1999 (Fig.2). In 2017 was observed a decline on the production of kiwifruit, being the lower production observed in China in this year compared with 2016 (from 2 432 929 to 2 024 603 tonnes/year), a possible reason for this decrease (FAOSTAT, 2019). Still, the world's largest kiwifruit producer in 2017 was China (2 024 603 tonnes/year), followed by Italy (411 299 tonnes/year), New Zealand (332 153 tonnes/year), Chile (186 519 tonnes/year), Iran (146 583 tonnes/year) and Greece (93 631 tonnes/year), and these countries account for over 90% of the worldwide production (FAOSTAT, 2019; Hanley, 2018). However, countries like Turkey, Portugal, Spain and France also have high production rates of this fruit, with values ranging between 25.000 and 70.000 tonnes/year (FAOSTAT, 2019).

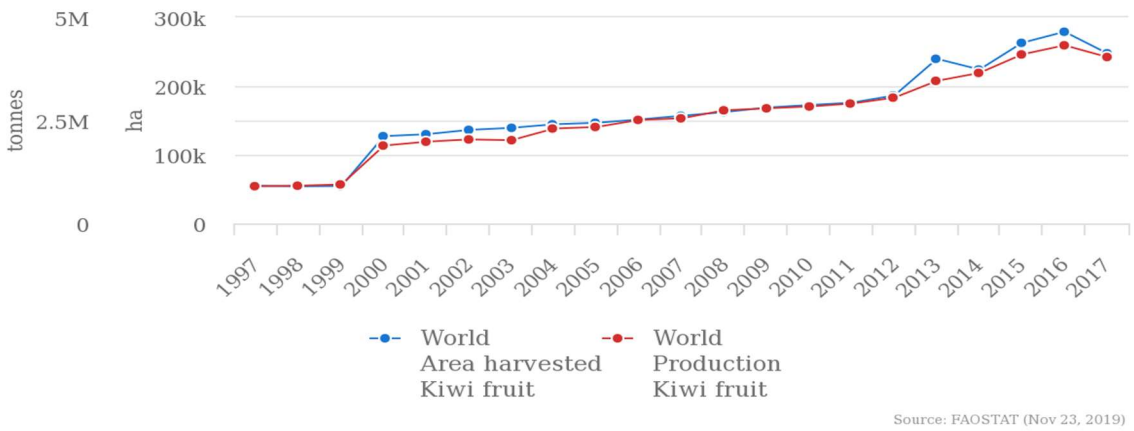


Figure 2: Production and yield quantities of kiwifruit worldwide from 1997 to 2017 (FAOSTAT, 2019).

In Portugal, the harvest area of kiwifruit has been continuously growing, from 983 ha in 1999 to 2.650 ha in 2017, and the production, although with some fluctuations, grew from 20 997 tonnes in 1999 to 35 411 tonnes in 2017 (Fig.3) (FAOSTAT, 2019). In 2018, Portugal produced a total of 34.057 tonnes in a harvest area of 2.736 ha, the second largest production of kiwifruit ever, being the North region of this country the main area of kiwifruit production and accounting for 80% of national production (27.097 tonnes) (INE, 2019). However, in this year, fruit load was very heterogeneous, mainly as a result of the intensity of the orchard exposure to adverse weather conditions (precipitation and low temperatures) that occurred at flowering / pollination times (INE, 2019).

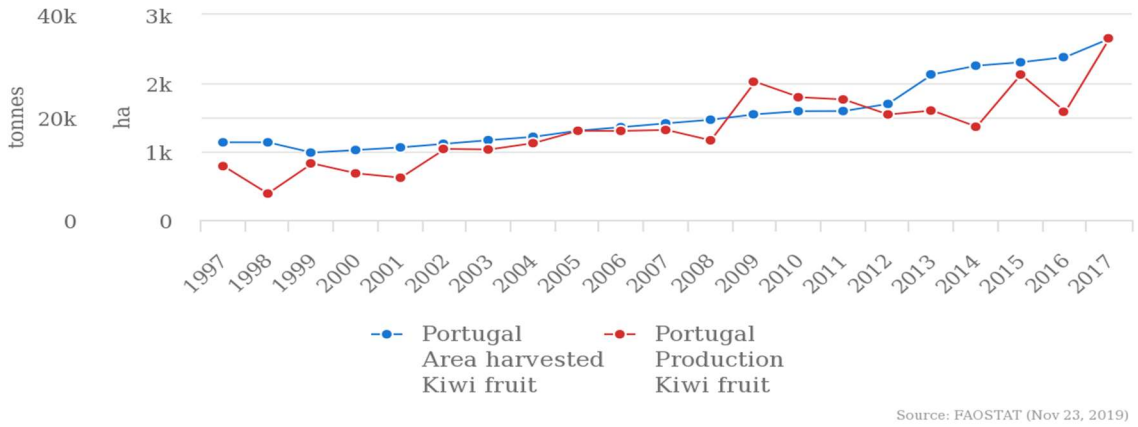


Figure 3: Production and yield quantities of kiwifruit in Portugal from 1997 to 2017 (FAOSTAT, 2019).

1.3 PSA geographical and taxonomical evolution

Pseudomonas syringae pv. *actinidiae* (PSA) is a bacterial pathogen responsible for the kiwifruit bacterial canker (KBC), one of the biggest threats to kiwifruit production worldwide, causing massive economical losses (Scortichini *et al*., 2012; Vanneste, 2012; SOPI, 2014). In terms of microbiological properties, PSA is a gram-negative, aerobic, motile bacteria, with rod-shaped and polar flagella. It is considered an oxidase-negative and arginine dihydrolase-negative strain and elicits hypersensitive response (HR) on *Nicotiana tabacum* (tobacco) leaves (Scortichini *et al*., 2012).

KBC was first described in Japan in 1989 on *A. chinensis* var. *deliciosa* cv. 'Hayward' (Serizawa *et al*., 1989; Takikawa *et al*., 1989). At the time, this disease was responsible for considered damage in Korea, China and Japan, and was later discovered in Latina Province (Lazio, Italy) in 1992, where damage was less severe (Scortichini, 1994). Until this date, PSA isolation had only been achieved from *Actinidia chinensis* var. *deliciosa* cv. 'Hayward' plants, but after 15 years of low incidence, PSA re-emerged in 2008 in Italy, as an especially aggressive and invasive strain, having been isolated from *A. chinensis* var. *chinensis* cv. 'Hort 16A' orchards (Scortichini *et al*., 2012; Donati *et al*., 2014). Since then, this bacterial outbreak begun to severely infect Italian kiwifruit orchards planted with cultivars of *A. chinensis* var *chinensis* such as 'JinTao' and 'Hort16A', as well as orchards of *A. chinensis* var. *deliciosa* cv. 'Hayward' present in the main producing regions of Italy, with important economic consequences (Balestra *et al*., 2009a; Balestra *et al*., 2009b).

Due to its large dispersion capacity, symptoms resembling those from KBC were first identified in the north of Portugal in March 2010, on *A. chinensis* var *deliciosa* cv. 'Summer' plants in the region "Entre Douro e Minho" (Balestra *et al*., 2010). Also in 2010, KBC was reported in France and New Zealand and by 2016 PSA had globally reached

all kiwi-producing countries such as Spain, Greece, Chile, Switzerland and Australia (Abelleira *et al*., 2011; Everett *et al*., 2011; Vanneste *et al*., 2011b Holeva *et al*., 2015; EPPO, 2018) (Fig.4).

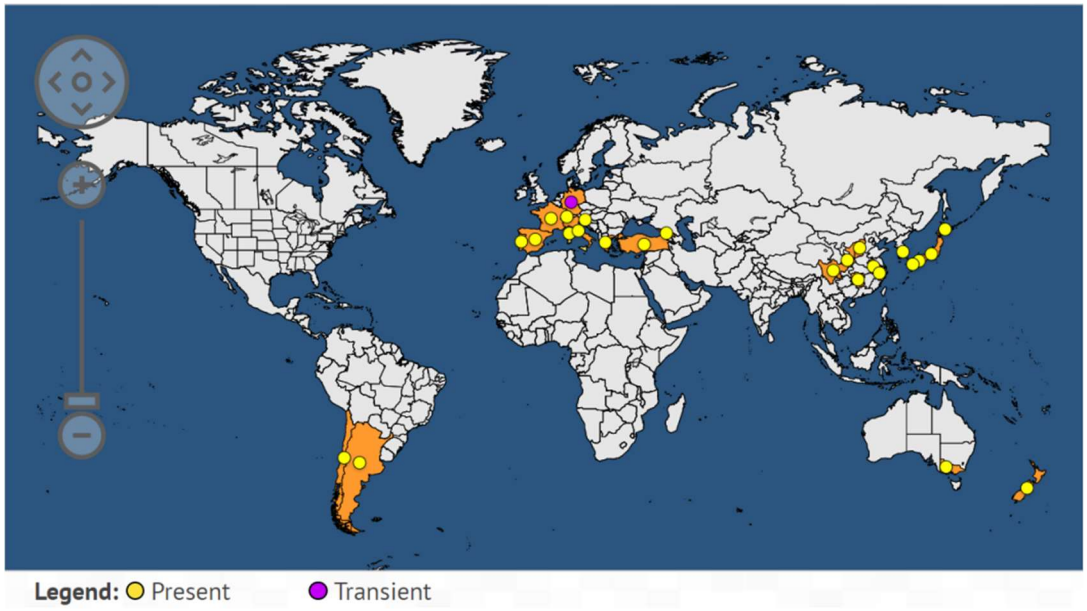


Figure 4: World distribution map of *Pseudomonas syringae* pv. *actinidiae* in 2019 (EPPO, 2019).

This bacterium is part of the order Pseudomonadales, family Pseudomonadaceae, genus *Pseudomonas,* and belongs to genomospecies 8 of the *Pseudomonas syringae* complex, together with *Pseudomonas avellanae* and *P. syringae* pv. *theae* (Gardan *et al*., 1999; Scortichini *et al*., 2002). More specifically, the *Pseudomonas syringae* complex comprises gram-negative pathogenic strains able to induce disease only on a limited number of host plants and are grouped into intraspecific taxa, called pathovars, based on their host adaption and disease symptoms, as is the case of PSA (Young, 2010; Cameron and Sarojini, 2014). The associated pathogenesis of this species in susceptible plants, is achieved through the injection of effector proteins into plant cells via the type III secretion system (T3SS), which is encoded by *hrp/hrc* genes and is required for HR elicitation in nonhost or resistant host plants (Cunnac *et al*., 2009).

The epidemic outbreaks that occurred in 2008 in Europe and in 2010 in New Zealand, caused by several highly virulent PSA strains, has prompted several research efforts to know and control this disease, including those involved in assessing the genetic diversity of PSA populations (Koh *et al*., 2014; Ciarroni *et al*., 2015). Since then, six distinct PSA populations (biovars), have been identified and named according to the chronological order that they were identified, and taking into consideration the degree of virulence, distribution patterns of virulence genes for type III effectors, multilocus

sequence (MLS) analysis of bacterial housekeeping genes and analysis of toxin production-encoding genes (Chapman *et al*., 2012; Vanneste *et al*., 2013; Cunty *et al*., 2014; Fujikawa and Sawada 2016; Sawada *et al*., 2016).

PSA1 and 2 are systemic, moderately aggressive PSA strains, which possess the genes associated with phaseolotoxin and coronatine production, respectively (Chapman *et al*., 2012). PSA1 was responsible for the first disease outbreak in Japan (1984-1989) and latter in Italy (1992) in *A. chinensis* var. *deliciosa* cv. 'Hayward' plants, but the damage was much more severe during the Japanese outbreaks, suggesting that different climatic parameters and agronomic techniques may play a fundamental role in the virulence of genetically similar bacterial species (Scortichini *et al*., 2012). Based on MLS analysis, PSA5 is closely related to PSA1 and 2, although it does not produce neither phaseolotoxin nor coronatine. PSA6 produce both these toxins and it appears to be an endemic strain such as PSA 2 and 5 (Sawada *et al*., 2014; Cunty, 2015; Fujikawa and Sawada, 2016; Sawada *et al*., 2016; Fujikawa and Sawada, 2019). "PSA4" was thought to be a PSA biovar until 2014, having been reclassified as *Pseudomonas syringae* pv. *actinidifoliorum* (PFM) (Cunty *et al*., 2014; Ferrante and Scortichini, 2014).

Although PSA1 and PSA2 revealed virulence factors, they are known for being less aggressive than biovar 3, that first emerged in Italy (Ferrante and Scortichini, 2009; Koh *et al*., 2010; Donati *et al*., 2014). PSA3 (referred only as PSA from this point forward), also known as PSAV, is a systemic, highly aggressive pandemic biovar. This population differs from PSA1 and PSA2 for lacking the gene fragments associated with production of phaseolotoxin and coronatine and for the presence of several effector genes, such as *hopA1,* related with the T3SS (Ferrante and Scortichini, 2010; Vanneste *et al*., 2011a). PSA is also more aggressive than PFM, that only causes leaf symptoms, not leading to plant death, and shows lower bacterial colonization on 'Hayward' plants (Abelleira *et al*., 2015; Ribeiro, 2018; Nunes da Silva *et al*., 2019). The strains belonging to this biovar, were first reported in Italy in 2008 (Ferrante and Scortichini, 2009) and are responsible for the most recent and severe outbreaks associated with PSA (Cunty *et al*., 2014), being present in all main kiwifruit producing regions, including New Zealand, Chile, China and Europe (Balestra *et al*., 2009; Ferrante and Scortichini, 2009; Everett *et al*., 2011; Scortichini *et al*., 2012).

1.4 Disease cycle and symptoms

Tissue infection of host plants by PSA can occur at any time of the year but KBC development and severity depends essentially on the crop cycle stage and physiological state, the kiwifruit cultivar, the bacterial strain concerned, as well as the temperature and

relative humidity conditions (Serizawa and Ichikawa, 1993; Scortichini *et al*., 2012; DGAV, 2013). KBC causes brown-black leaf spots in plant leaves, often surrounded by a chlorotic margin, twig die-back, blossom necrosis, reddening of lenticels and extensive cankers along the main trunk and leader, frequently with a whitish to orange ooze formed from bacterial exudates (Scortichini *et al*., 2012) (Fig.5).

Bacterial exudates appear during the end of autumn-winter/early spring and, along with the wind, are a very effective way of spreading the inoculum between and within the plants (Scortichini *et al*., 2012). In addition to these exudates, the presence of PSA epiphytic population in plant tissues and debris, is responsible for short distance dispersion of PSA, while for long distance dispersion the most likely source of inoculum is the movement of infected plant material (Vanneste *et al*., 2011b). PSA is able to survive on pollen grains of male flowers of various *Actinidia* species, which has also been considered a possible source of bacterial spread (Vanneste *et al*., 2011b; Scortichini *et al*., 2012).

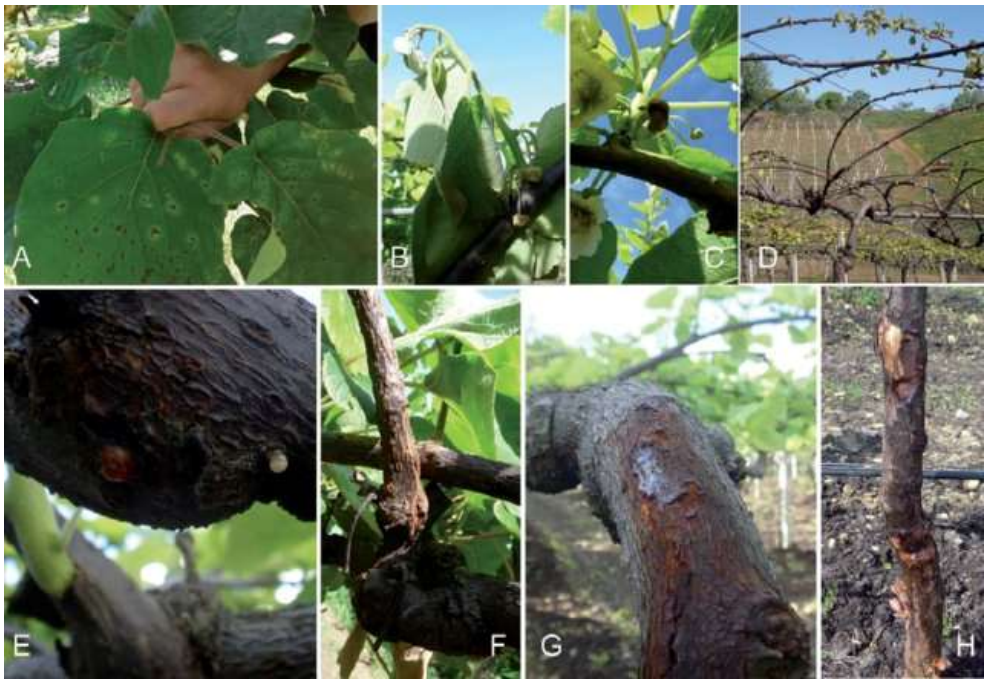


Figure 5: Symptoms of *Pseudomonas syringae* pv. *actinidiae* in *A. chinensis* var. *deliciosa* cv. 'Hayward'. Leaf spots with yellow halos (A); Death of the branches (B); Necrosis of flowers (C); Inhibition of new shoots (D); Red and white exudate on a branch (E); Reddening of lenticels (F); Bacterial cancer with exudate (G); Red-brownish discoloration under the bark of the trunk (H) (adapted from Donati *et al*., 2014).

KBC starts with the entrance of bacteria into plant tissues through natural openings, like stomata and lenticels, or through artificial injuries caused by wind or pruning tools. By springtime, PSA can move systemically from leaves to the young stem through the leaf petiole, which is the most critical point of the infection process (Ferrante

et al., 2012). This is due to the occurrence of frequent rains (high humidity) and optimal spring temperatures (12-18 °C), that culminate in rapid pathogen multiplication (Scortichini *et al*., 2012). At temperatures above 27 °C, which are normally reached during summer, PSA can survive inside the hosts tissues, but the infection is inhibited as the result of its low activity and low production of exudates (Vanneste *et al*., 2011b; Scortichini *et al*., 2012).

Autumn is also a period of high risk for infection spread due to mild temperatures and frequent rains. Also, due to the natural openings resulting from fallen leaves and fruits and to cultural operations that promote the occurrence of artificial wounds, the dispersion and colonization of lenticels and gems drastically increases (Vanneste *et al*., 2011b). In winter, PSA is thought to be maintained in the tissues of infected plants, where it is able to survive latently in infected branches and form new colonies. Bacteria can inclusively migrate to the roots of the infected plant, where it is kept inactive until the occurrence of frosts and hailstorms, which greatly enhances their ability to spread (Vanneste *et al*., 2011b; Scortichini *et al*., 2012). In fact, the occurrence of frosts and increased precipitation (of 30-35 %) during the year of 2008, were two of the most important contributors to the spread of the disease in the Italian kiwifruit producing regions (Ferrante e Scortichini, 2014). In late winter/spring, disease symptoms essentially comprise cankers in trunks and leaders, which can cause the contraction of whole branches and even kill mature vines. Thus, it is generally acknowledged that spread and severity of the disease in a certain moment is mainly dependent on the degree of infection in the previous fall and early winter (Donati *et al*., 2014).

In Mediterranean climates PSA infection can be divided into two phases according with the damage associated: the first phase takes place in winter and includes damage to the main vine structure and overwintering canes, having direct effects on yield by reducing the size of the vines; the second phase occurs during spring and involves damage to the new season's growths, such as leaves, flowers and canes. Unlike the former, the second phase does not have such a direct effect on yield, but is important for disease spread (Serizawa *et al*., 1989).

1.5 Metabolic and molecular response to PSA infection

KBC affects all plants of the genus *Actinidia*, but each species manifests different extent of disease symptoms. Field evidence revealed that cultivars of *A. chinensis* var. *chinensis* seemed the most susceptible, with higher disease incidence and symptom severity, compared with *A. chinensis* var. *deliciosa* (Balestra *et al*., 2009a). Thus, *A. chinensis* var. *deliciosa* appears much more susceptible to KBC than *A. arguta*, in which

visible disease symptoms are scarce and outbreaks do not lead to production losses (Vanneste *et al*., 2014). In fact, *A. arguta*, *A. polygama* and *A. macrosperma* were classified as tolerant to PSA based on field monitoring (Datson *et al.*, 2015). Furthermore, recent findings showed experimentally for the first time, that *A. arguta* is less susceptible to PSA than *A. chinensis* var. *deliciosa,* possibly due to a faster response regarding the activation of plant antioxidant system, translated by early expression of the enzyme catalase (EC 1.11.1.6) (Ribeiro, 2018; Nunes da Silva *et al*., 2019). This tolerance/susceptibility to pathogenic bacteria like PSA depends on a complex cascade of reactions, that culminates in certain biochemical and physiological responses (Petriccione *et al*., 2013a).

PSA is a hemiobiotrophic pathogen, which means that at an early stage it behaves as a biotrophic agent, feeding on plant tissues but keeping it alive, and later a necrotrophic phase, causing degradation of plant tissues. This posterior phase is only possible if the pathogen is successful in overcoming the defence mechanisms of the host plant (Freeman and Beattie, 2008). During the biotrophic phase PSA does not induce the appearance of significant symptoms, but during the necrotrophic phase the bacterium can migrate systemically from the leaf veins to the shoots and, consequently, lead to the development of the characteristic disease symptoms previously described (Petriccione *et al*., 2013b).

As an initial response to bacterial colonization, plants produce reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), in order to restrict the entry and proliferation of the bacteria, through the activation of basal defence mechanisms (Petriccione *et al*., 2013a). Although ROS are important secondary messengers and a normal product of plant cellular metabolism, when in excess, they can pose a threat to plant cells, causing lipid peroxidation and damage to nucleic acids, which can lead to cell death. In this context, lipid peroxidation of plant cells has been widely used as an indicator of damage caused by ROS in cell membranes subjected to oxidative stress conditions. Malondialdehyde (MDA) is one of the final products of phospholipidic unsaturated fatty acids peroxidation, allowing to estimate the level of lipid peroxidation present in plant tissues (Sharma *et al.,* 2012).

ROS scavenging is then necessary for plants defence and normal function, requiring the action of several non-enzymatic and enzymatic antioxidants present in plant cells. Enzymatic antioxidants include enzymes such as superoxide dismutase (EC 1.15.1.1), involved in the dismutation of O_2^- ions to hydrogen peroxide molecules, catalase, which dissolves hydrogen peroxide into oxygen and water, ascorbate

peroxidase (EC 1.11.1.11), which is involved in the glutathione-ascorbate cycle and guaiacol peroxidase (EC 1.11.1.7), that oxidizes preferentially aromatic electron donors, such as guaiacol, at the expense of hydrogen peroxide. Guaiacol peroxidase is also associated with important biological processes such as cell wall lignification and defence against biotic and abiotic stresses (Sharma *et al*., 2012).

Non-enzymatic antioxidants comprise secondary metabolites such as phenolic compounds, including flavonoids, lignin, alkaloids and tocopherols. Besides their capacity to scavenge ROS, phenolic compounds function as signalling compounds, pigments, internal physiological regulators and are involved in tolerance mechanisms against pathogens (Lattanzio, 2013; Wang *et al*., 2018). In fact, a study by Miao *et al*. (2009) demonstrated that phenolic concentration in the annual twig and foliage of resistant kiwifruit cultivars to PSA were significantly higher than in susceptible cultivars. Flavonoids in particular, are one of the most abundant metabolites in plants tissues, being involved in the tolerance mechanisms against abiotic stresses, such as nutrient deficiency, temperature fluctuation, mechanical injuries or against pathogens (Kulbat, 2016). An antagonistic effect of flavonoids on plant bacterial pathogens has been reported, specifically against *Pseudomonas syringae* pv. *tomato*, with a decrease in pathogen mobility after exposure to these compounds, resulting from the loss of the flagellum and the inhibition of the T3SS associated with *Pseudomonas* spp. strains virulence (Vargas *et al*., 2013). Lignin is a branched polymer of phenolic compounds that constitutes plants secondary cell wall, and functions as a physical barrier against pathogenic attacks due to its insolubility, stiffness and indigestibility (Freeman and Beattie, 2008). Carotenoids, on the other hand, present important antioxidant abilities against ROS, being produced by plants under stress conditions. In fact, it was demonstrated by Wang *et al.* (2018) that after PSA infection in *Actinidia chinensis* var. *deliciosa* cultivar 'Jinkui', genomic expression of eight genes related to biosynthesis of carotenoids, which are tetraterpenoids, increased compared to control plants.

Most of the phenolic compounds are synthesized by the phenylpropanoid pathway, in which phenylalanine ammonia-lyase (PAL) plays a critical role, being involved in the production of secondary antimicrobial metabolites and proteins, and in the HR (Reglinski *et al*, 2013; Wang *et al*., 2018). The HR is a secondary line of plant defence that culminates in a rapid, localized necrosis of plant tissue observed when virulent bacterial strains infect tolerant varieties of susceptible plant species or nonhost plant species (Xiao *et al*., 1992). In susceptible plants, HR is a target of bacterial type III effectors from the T3SS. Indeed, a functional hypersensitive response and T3SS that delivers effector proteins into host cells has been revealed to be the key pathogenicity factor required for

P. syringae to colonize host plants. Moreover, hundreds of genes that encode proteins responsible for the induction of RH, have already shown to be increased after infection with PSA in *A. chinensis* var. *chinensis* cv. 'Soreli' (Petriccione *et al*., 2013a) and on *A. chinensis* var *deliciosa* cv. 'Jinkui' (Wang *et al*., 2018).

1.6 Control methods

Given the dramatic impact of KBC and the high facility for PSA dispersal, the development and enforcement of sustainable control methods is of upmost urgency. So far, no curative methods for KBC have been developed, and the existing chemical treatments consist only on preventive measures to avoid inoculum load and spread, being especially efficient in the early stages of disease development (Cameron and Sarojni, 2014; Vanneste *et al*., 2011b). Therefore, an integrated approach that includes a balanced plant nutrition, cultural measures that assure good orchard hygiene and biological control strategies alternative to chemical treatments are necessary to drastically reduce incidence and severity of the disease (Donati *et al*., 2014; Scortichini *et al*., 2012).

Chemical treatments depend greatly on administering copper-based sprays as well as antibiotics like streptomycin (Cameron and Sarojni, 2014; Koh *et al*., 1996; Nakajima *et al*., 2002; Lee *et al*., 2005; Vanneste *et al*., 2011b). Streptomycin is an aminoglycoside antibiotic that interferes with the translation of bacterial mRNA, subsequently leading to cell death, and is considered to be very efficient against PSA (Han *et al*., 2003; Cameron and Sarojni, 2014). However, the use of antibiotics for the control of plant pathogenic bacteria is illegal in Europe, being predominantly used in some Asian countries and in New Zealand in exceptional cases (Serizawa *et al*., 1989; Vanneste *et al*., 2011b).

Copper-based compounds such as sulphate, have been used as an alternative to antibiotic compounds to fight the disease. These formulations are suggested to be used at critical points where plants can be more susceptible to PSA, such as leaf fall, postharvest, after winter pruning and budbreak, in order to reduce the inoculum and protect the new receptive tissue (Donati *et al*., 2014; Vanneste *et al*., 2011b). Unfortunately, both antibiotic- and cooper-based compounds can induce phytotoxicity, leave residues on fruits and raise bacterial resistance events (Cameron and Sarojni, 2014; Donati *et al*., 2010). So far, all PSA strains collected in Italy during the epidemics of 2008/2009 are not resistant to antibiotic compounds, but evidence of the first PSA isolates resistant to copper sulphate was already demonstrated (Colombi *et al*., 2017). Also, it is important to note that once the pathogen is inside plant tissues, it cannot be reached by surface-protecting bactericides (Petriccione *et al*., 2013a).

Cultural methods to control PSA consist essentially on maintaining good orchard hygiene and performing good vine management. Several techniques of orchard management, such as irrigation, fertilization and pruning, have implications for PSA incidence and epidemiology, since they influence the vegetative and reproductive performance of the plant (Costa *et al*., 2013; Donati *et al*., 2014). In fact, late pruning or pruning during periods of high relative humidity should be avoided. On the other hand, high nitrogen (N) fertilization seems to increase plant susceptibility, probably by increasing plant vigour and sprouting new shoots, being also discouraged (Costa *et al*. 2013; Donati *et al*., 2014;).

In addition to regular antimicrobial-treatments, it is also necessary to remove all symptomatic material from the orchard, either by burning or burial, minimize the size and number of wounds inflicted in plants and disinfect all the material used for pruning. However, the removal of symptomatic plants only serves to destroy plants with visible infection symptoms, not protecting the orchard from plants recently affected by PSA or in which the bacterium is present as an epiphyte (Cameron and Saroijini, 2014; Vanneste *et al*., 2011b).

The use of biological control agents (BCA) against PSA may represent a great option for an integrated control strategy, but studies regarding these environmentally friendly alternatives are still very limited. Bacteriophages, viruses that are able to infect and replicate inside bacteria, leading to cellular lysis of the host, have shown promising results, but this approach was only tested in *in vitro* conditions (Di Lallo *et al*., 2014; Donati *et al*., 2014; Frampton *et al*., 2014; Yu *et al*., 2016). Other substances of natural origin, such as chitosan, mixture of essential oils, elicitors of the salicylic acid (SA) pathway and antimicrobial peptides showed promising results, but need further confirmations to ascertain the efficiency of this compounds against PSA (Ferrante and Scortichini, 2010; Cameron *et al*., 2014; Cellini *et al*., 2014; Scortichini, 2014; Song *et al*., 2016; Vavala *et al*., 2016). The development and identification of tolerant pollinators and cultivars is an ongoing line of investigation and may be one of the most promising ones, as in some places tolerant cultivars can be found in the field with positive results (Donati *et al*., 2014; Kisaki *et al*., 2018; Sawada *et al*., 2015; Vanneste, 2017). Kisaki *et al*. (2018) observed a high resistance from *A. rufa* cultivars, specially *A. rufa* cv. ‘Fuchu’ through the disease index and severity (measuring the diameter at the widest portion of each necrotic lesion) after inoculation by infiltration on leaves of PSA biovar 3.

1.8 Mineral nutrients in plant development and defence

For an element to be considered an essential nutrient for plants, three criteria must be met: i) the plant must be unable to complete its life cycle in the absence of the mineral element, ii) the function of the element must not be replaceable by another mineral element and iii) the element must be directly involved in plant metabolism (Arnon and Stout, 1939). According with this strict definition, the essentiality of 14 mineral elements was established for carbon (C), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) as macronutrients and iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo) and chlorine (Cl) as micronutrients (Kirkby, 2012; Pandey, 2018; White and Brown, 2010). These minerals play essential roles, such as being constituent of cellular structures and metabolites, in cell osmotic relations and turgor-related processes, energy transfer reactions, enzyme-catalysed reactions and plant reproduction (Pandey, 2018).

Nutrition has also been implicated in plant resistance and tolerance to several pathogens, mainly by altering growth and tissue composition, but also by regulating the expression of pathogen resistance genes (Gupta *et al*., 2017; Huber and Graham, 1999; Huber *et al*., 2012). Although the impact of nutrition on plant resistance is relatively small in highly susceptible or highly resistant cultivars, it can be substantial in moderately susceptible or partially resistant cultivars (Huber *et al*., 2012). The pathogen itself may interfere with nutrient translocation or utilization efficiency in plants causing nutrient deficiency or hyperaccumulation and toxicity. In fact, increased susceptibility to the invading pathogen may result specifically from a reduction in the availability of a unique or several nutrients caused by their utilization by the pathological agent (Dordas, 2008). Therefore, nutrients are important for both plant and microorganism development, and all essential nutrients can affect disease severity (Huber and Graham, 1999; Agrios, 2005; Huber *et al*., 2012; Gupta *et al*., 2017).

Berry *et al*. (1988) working with *Clavibacter michiganensis* subsp. *Michiganensis* in *Solanum lycopersicum* (tomato) resistant cultivar ‘Plovdiv 8/12’ and susceptible cultivar ‘Moneymaker’, reported that nutrient solutions with 100 ppm or higher concentrations of Ca reduced susceptibility to the pathogen in both resistant and susceptible cultivars by visual assessment of disease symptoms. On the other hand, Yamazaki and Hoshina (1995) demonstrated an antagonistic effect of increasing Ca concentrations (0, 8 and 16 mM) and disease severity of *Pseudomonas solanacearum* strain MAFF03-01487 on *Lycopersicon esculentum* cv. ‘Zuiei’ (a moderately resistant cultivar) but the same was not observed for the the highly susceptible cultivar ‘Ponderosa’ where the disease development was rapid at all Ca concentrations. Oliveira

et al. (2012) have shown that higher concentrations of Si (1.50 g of SiO 2 kg^{-1} of soil) did not affect disease incidence caused by *Xanthomonas citri* subsp. *malvacearumin* in *Gossypium hirsutum*, but decreased disease severity, probably due to the silicon role on cell wall lignification or activation of specific mechanisms, such as phytoalexin production (Fawe *et al*., 2001). Kiraly (1976) reported that K-deficient *Oryza sativa* (rice) had a higher susceptibility to *Xanthomonas oryzae,* likely because K-deficiency is related to thin cell walls and accumulation of unused N which encourages pathogenic attack (Graham, 1983). The effect of nutrition on the host plant regarding spread and multiplication of the bacterial pathogen, seems to be similar to that observed on facultative fungal parasites, where Ca and K deficiencies lead to an enhanced multiplication of the pathogen and disease severity (Kiraly, 1976; Huber and Thompson, 2007).

In the case of *P. syringae* bacteria, very little information is available on the effect of nutrients in its impact on disease susceptibility (Hoffland *et al*., 2010; Holmes, 2012; Gupta *et al*., 2013). In fact, most of the literature available of the effect of nutrition on plants disease, is still related to fungal disease (Huber and Graham, 1999; Huber *et al*., 2012; Gupta *et al*., 2017). K deficiency was reported to affect disease tolerance on both facultative and obligated fungal parasites but, beyond optimal K supply for growth, no increase in tolerance with increasing K supply was observed (Huber and Graham, 1999; Dordas, 2008; Huber *et al.,* 2012). Most studies do not correlate the individual effect of K on fungal diseases, studying the effect of fertilization with K along with N and/or P. For example, Srihuttagum and Sivasithamparam (1991) reported that in *Pisum sativum* (field pea) the percentage of root disease index decreased with the application of a mixture of P and K or of N, P and K fertilization when plants were infected with *Rhizoctonia solani*, and with a mixture of N, P and K fertilization when plants were infected with *Fusarium oxysporum*, possibly because K deficiency leads to thin cell walls and to the accumulation of low-molecular-weight organic compounds which may encourage pathogenic attacks (Graham, 1983). Ca can act as a secondary messenger helping in plant recognition of pathogenic invaders and in HR response, and its deficiency is related with leakage of metabolic products that can stimulate pathogen infections due to the role of Ca in the biological membranes (Huber *et al*., 2012; Gupta *et al*., 2017). Bateman and Lumsden (1965) demonstrated that in hypocotyl tissue of beans (*Phaseolus vulgaris* var. Red Kidney) Ca can contribute to cell wall stability through the middle lamella, countering the effect of pectolytic enzymes, including polygalacturonase, that pathogens such as *Rhizoctonia solani* use to dissolve the middle lamella and enter host cells (Huber et *al*., 2012). P was found to be effective specially against airborne pathogens, with foliar

application of phosphate conferring resistance, possibly due to the release of elicitor-active compounds or HR induction (Huber *et al*., 2012). In a study with *Cucumis sativus* (cucumber) plants, resistance against *Sphaerotheca fuliginea* was achieved with 20 ppm P in hydroponic solution before the occurrence of fungus establishment. After the establishment of the pathogen only foliar applications of 1 % of mono-potassium phosphate was effective in controlling the pathogen (Reuveni *et al*., 2000). Mg is related to plant photosynthesis, as it is a central chlorophyll constituent and a photosynthates (like sucrose) transporter, and its deficiency can cause the accumulation of these molecules, making the tissues more susceptible to pathogen attack (Gupta *et al*., 2017). Also, Mg increases resistance of tissues to degradation by pectolytic enzymes of macerating or soft rotting pathogens. In contrast, high rates of Mg that interfere with Ca uptake may increase the incidence of diseases such as bacterial spot of tomato and *Piper nigrum* (pepper) or *Arachis hypogaea* (peanut) pod rot (Huber and Jones, 2012). Moreira *et al*. (2015) reported that severity of brown spot disease, caused by *Bipolaris oryzae,* decreased with higher Mg concentrations (0.25 to 4.0 mM of magnesium sulphate) applied on nutrient solution to rice plants. The same study also observed an increase in total chlorophyll and better photosynthetic performance with the higher concentration of Mg. Other nutrients, such as B, Mn and Cu, play key roles in phenol metabolism and lignin biosynthesis, important secondary metabolites for plants defence (Huber *et al*., 2012).

Despite their undeniable importance in disease resistance, nutrients have dynamic interactions between them, and factors such as the growth stage of the plant, environmental conditions, and biological activity can influence the effect of a nutrient on disease development (Fageria 2011; Gupta *et al*., 2017).

1.8.1 The role of nitrogen

N is the most important nutrient for plant growth, and its role on disease resistance has been target of research in the past years, yet with non-conclusive findings, which may be due to differences in the type of pathogen, plant species, form of N nutrition or application timing (Engelhard, 1989; Huber and Watson, 1974; Büschbell and Hoffmann, 1992; Marschner, 1995; Hoffland *et al*., 2000; Huber *et al*., 2012; Gupta *et al*., 2017). Generally, facultative fungal parasites grow better on low N concentration, but it is commonly the form of N accessible to the plant or pathogen that affects disease severity rather than the amount of N *per se* (Huber and Watson, 1974; Chase, 1989; Blachinski *et al*., 1996).

The main forms of N assimilated by plants are nitrate (NO_3^-) and ammonium (NH_4^+), but different plants have different adaptabilities to absorb and utilize these two N sources, resulting in different physiological responses within the plant (McCrimmon *et al*., 1993). The assimilation of this two ionic forms of N are distinct: while NH_4^+ may be promptly assimilated into amino acids and other organic compounds after absorption, the use of NO_3^- by plants requires the presence of the enzyme nitrate reductase (EC 1.7.1.2) (for NO_3^- reduction to nitrite in the cytoplasm) and nitrite reductase (EC 1.7.2.1) (for the reduction of nitrite to NH_4^+, which happens on the chloroplast). Thus, NO_3^- assimilation as N form results on a more demanding process in terms of time and energy (Haynes and Goh, 1978). However, high NH_4^+ concentrations on plants adapted to assimilate NO_3^- can cause alterations in intracellular pH and osmotic balance and uncouple photophosphorylation from electron transport, following the accumulation of NH_4^+ in leaves (Gerendás *et al*., 1997). Also, it can induce nutrient deficiency, especially of cations like Ca, K and Mg, arising from the impaired uptake of metal ions, competition with these cations for assimilation and transport sites, and inhibition of secondary growth from the acidification of the rooting medium (Rayar and van Hai, 1977; Haynes and Goh, 1978; Gerendás *et al*., 1997; Hoopen *et al*., 2010). This acidification of the nutrient medium by NH_4^+ is well documented on soil and water culture experiments and it seems to be correlated to the fact that uptake of cationic N (as NH_4^+) is not fully compensated by a reduced uptake of mineral cations, and so, the charge balance is maintained by the release of protons. Kawasaki *et al*. (1995) demonstrated that in rice, corn, *Hordeum vulgare* (barley), cucumber and tomato, Ca concentration was higher with NO_3^- as N source, compared to NH_4^+ and antagonistic relations between NH_4^+ and bivalent cations Ca and Mg were reported on other studies as a result of the mechanism of charge balance in ion uptake, since N ionic form controls cation and anion uptake (Gloser and Gloser, 2000; Borgognone *et al*., 2013; Na *et al*., 2014; Tsabarducas *et al*., 2017). Also, several studies reported a negative relation between NH_4^+ and K (Ashraf and Sultana, 2000; Lu *et al*., 2005; Na *et al*., 2014), although other reports supported a positive interaction between N and K when K is in high levels, but are often associated with NO_3^- rather with NH_4^+ (Adams *et al*., 1980; Fageria, 2001). In fact, adequate K supply is essential for the efficient use of NO_3^- by crop plants, perhaps because it may be involved as an accompanying cation in NO_3^- uptake by co-transport in the xylem from plant roots to shoots. In addition, K seems to have an influence on the translocation of photosynthetic assimilates, needed to support the active uptake process of NO_3^- by plant roots (Fageria, 2001).

On the contrary, the reduced soil pH due to NH_4^+ assimilation can lead to increased uptake of some micronutrients, while NO_3^- uptake is associated with the proton consumptions via $2H^+$/ NO_3^- symport, leading to an increase in the pH of the outer solution and consequently lower uptake of micronutrients (Fageria, 2001; Borgognone *et al*., 2013). For example, Fe uptake in rice grains at various N levels were highly significant and indicated that Fe increased with the addition of N as NH_4^+. Higher P uptake is also generally associated with NH_4^+ since a decrease in soil pH induces higher solubility of Ca-phosphates in the rooting medium and increases the phosphate ion that is preferentially absorbed by roots ($H_2PO_4^-$) (Hoffmann *et al*., 1994; Ashraf and Sultana, 2002; Pedersen *et al*., 2019). Although it has been assumed that most plant species normally uptake N in the NO_3^- form on their normal nutrition, being NH_4^+ nutrition usually an artificial situation, there are some plants such as many species of *Pinus* that prefer NH_4^+ as their N form and the use of NH_4^+ on N supplementation is widely used (Haynes and Goh, 1978; Cao *et al*., 2018). In fact, when the pH is controlled many field crops can grow well under NH_4^+ culture (Osaki *et al*., 1995).

Studies documenting the effect of N on plants tolerance or susceptibility to fungal diseases tend to report that higher N concentration is related to higher susceptibility to obligate parasites and higher tolerance to facultative parasites (Dordas, 2008; Gupta *et al*., 2017). For example, Howard *et al*. (1994) noted an increase of disease severity caused by *Puccinia graminis* on *Triticum aestivum* L. (wheat) with increasing concentrations of N rates (0, 30, 45, 60, 90, and 120 lb.acre^{-1}) while Chase (1989) observed a decrease of disease severity (% of leaf area with symptoms) of Xanthomonas blight-infected *Syngonium podophyllum* plants with increasing N rate supply (50, 150 and 250 mg per pot per week). Differences in disease severity between obligate and facultative fungal parasites rely basically on their nutritional requirements (Dordas, 2008; Huber *et al*., 2012; Gupta *et al*., 2017). Despite this, most studies fail to take into consideration the effect of N form supplied and sometimes do not mention if the supply given to the plants is low, optimal or excessive (Huber *et al*., 2012).

N, particularly NO_3^-, is involved in constitutive and induced resistance traits, by enhancing nitric oxide (NO) and salicylic acid (SA) accumulations, which are implicated in the effector triggered immunity by manipulating HR defence response (Dietrich *et al*., 2004; Durner and Klessing, 1999; Gupta *et al*., 2013). On the contrary, NH_4^+ seemed to compromise tobacco plant resistance to *Pseudomonas syringae* pv. *phaseolicola* by encouraging metabolic reprogramming of HR-defence towards sugar and 4-aminobutyric acid production (Gupta *et al*., 2013). In tomato plants, increased susceptibility to *Pseudomonas syringae* pv. *tomato* was observed with higher N concentrations (Hoffland

et al., 2000). Regarding kiwifruit plants in particular, very few information on how N nutrition affects plants ability to resist PSA invasion is available at the moment. The only two reports that attempted to explore the impact of N nutrition in *A. chinensis* var. *deliciosa* cv. 'Hayward' susceptibility to PSA utilized different methodologies and one of them was very preliminary where control plants were not available, and few biological replicates were used for a strong statistical analysis (Holmes, 2012; Mauri *et al*., 2016). Also, both had controversial results with one not reporting significant differences between the different forms of N utilized and the preliminary report indicating that NO_3^- fertilization had higher plant survival rate. Therefore, it is not clear whether PSA infection altered the balance of leaf nutrients observed in such a study (Holmes, 2012).

1.8.2 Fertilizers

A fertilizer consists in any natural or manufactured solid or liquid material that improves the levels of available essential nutrients for the proper development and growth of a plant and/or the chemical and physical properties of soil (Gowariker *et al*., 2009; Kiiski *et al*., 2016a). Their application can influence plant disease development in a direct manner, by affecting the nutritional status of the plant, or indirectly by altering the environmental conditions which can influence the development of the disease, such as changes in light interception and humidity within the crop (Dordas, 2008).

There are two great general divisions of fertilizers: standard fertilizers, which are used in large quantities in agricultural practices and can be divided into solid and liquid fertilizers, and special fertilizers, which include foliar fertilizers among others (Kiiski *et al*., 2016b).

Solid fertilizers are of great importance as nearly 90 % of all the N applied is in solid form (Kiiski *et al*., 2016b). Factors including the plant species, the type of soil and fertilizer, determine the timing and method of fertilizer application in order to ensure that nutrients remain in the active rootzone, thus avoiding losses, and that fertilization is timed with the peak of nutrient uptake demand, optimizing both plant yield and quality (Roy *et al*., 2006; Jones and Jacobsen, 2009). Fertilizers can be applied prior to seeding (preplant), at the time of seeding (starter), or during the cultivation period (Jones and Jacobsen, 2009; Roy *et al*., 2006).

In the case of kiwifruit, fertilization is one of the cultural practices with greater influence on plant yield, being N a worth to note mineral on that regard as well as on fruit size and storage (Tagliavini *et al*., 1995; Pacheco *et al*, 2008). N is easily loss, especially through leaching, because of its mobility in soil, and so, N fertilizers must be applied during periods of high N demand (Alcoz *et al*., 1993; Jones and Jacobsen, 2009). On

that note, kiwifruit orchards generally need 190 lb of N per acre per year and its solid (granular) supplementation is usually divided into two application: the first one at budbreak, providing the N needed for early-season growth and fruit set, and the second one after full bloom (Costa *et al*., 1992; Strik, 2005). Liquid fertilizers containing 10 lb of actual N can substitute dry fertilizers and be used along irrigation, while higher rates of N can injure roots on lighter soils. In fact, the roots of kiwifruit vines burn easily with fertilization, and so, concentrating the fertilizers near the trunk can have that negative effect (Strik, 2005). Although higher rates of N fertilization are often related to greater fruit yield, some articles also associate higher rated of N with less fruit firmness and consequently less marketable fruits (Tagliavini *et al*., 1995; Costa *et al*., 1997; Pacheco *et al*., 2008). This occurrence can be explained when N is present as NH_4^+ form and consequently diminish Ca uptake concentrations (Tagliavini *et al*., 1995). P and K fertilization are also usually applied on kiwifruit orchards maintenance (Costa *et al*., 1992; Strik, 2005). P at amounts around 55 lb and before budbreak due to its low mobility, and 80 to 130 lb of K in late winter/early spring, depending on soil type (Strik, 2005; Jones and Jacobsen, 2009). When the soil is deficient in zinc or iron it is also important to use chelates to overcome these issues (Costa *et al*., 1992). It is of great concern to not use fertilizers which contain chloride (i.e. KCl), as kiwifruit vines are very sensitive to this nutrient (Strik, 2005). Depending on the region of kiwifruit production different nutrients can be complemented with those above and pH levels also oscillate. For example, in Italy the soils for kiwifruit production usually have pH levels between 7 and 8.5 while in France kiwifruit orchard are established on acidic soils with pH around 6.5 (Costa *et al*., 1992).

2. Aims

Given the negative impact of KBC, it is important to understand how plant grow factors can be manipulated, namely regarding plant nutrition, in order to reduce the severity and/or incidence of this disease. Since N is one of the most important nutrients required for optimal plant growth and fitness, the objectives of this dissertation were to:

i) Evaluate the impact of different sources of N - NO_3^- and NH_4^+ - on the growth and development of *Actinidia chinensis* var. *deliciosa* cv. 'Hayward' plants.

ii) Elucidate how NO_3^- and NH_4^+ supplies influence the accumulation of several plant secondary metabolites and plant mineral nutrition.

iii) Exploit how different sources of N affect the susceptibility/tolerance of *Actinidia chinensis* var. *deliciosa* cv. 'Hayward' to PSA.

iv) Understand how several secondary metabolites and mineral accumulation are regulated in response to PSA infection under different N sources.

3. Materials and Methods

This study included two distinct experimental trials. The first one (Trial I) aimed at understanding how different N sources (NO_3^-, NH_4^+ or a mixture of both) interfere with kiwifruit plant growth parameters, production of several secondary metabolites and mineral accumulation in plant tissues. This trial started in the moment plants were transferred from *in-vitro* conditions into a hydroponic system, and plant sampling was performed 21 and 36 days post transfer (dpt). The second trial (Trial II) aimed at evaluating the potential of different N supplies (NO_3^-, NH_4^+ or a mixture of both) in increasing kiwifruit plant tolerance to PSA and at understanding the biometric, metabolic and nutritional changes induced by plant inoculation 15 days post inoculation (dpi), which corresponded to 36 dpt (Fig. 6).

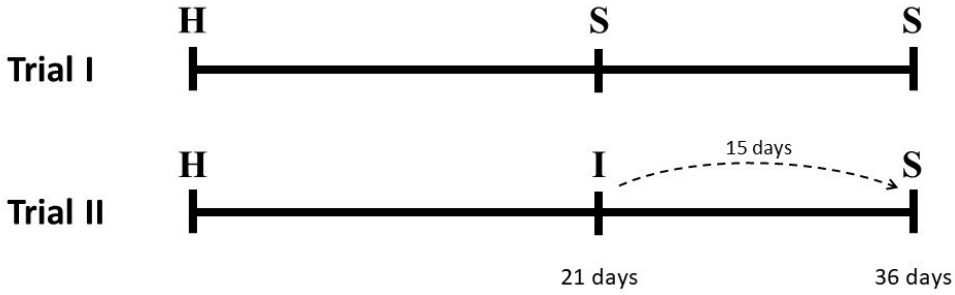


Figure 6: Schematic representation of Trial I and Trial II where: H – Plant transfer from '*in vitro*' conditions to hydroponic solution, S – plant sampling and I – plant inoculation with PSA.

3.1 Plant material

Micropropagated plants of *A. chinensis* var. *deliciosa* cv. 'Hayward' were obtained from QualityPlant – Investigação e Produção em Biotecnologia Vegetal, Lda (Castelo Branco, Portugal) and kept in a climate chamber (Aralab Fitoclima 10000EHF, Aralab, Rio de Mouro, Portugal) with a 16 h light photoperiod at 22 °C providing 325 µmol $s^{-1}m^{-2}$ of photosynthetic photon flux density, and 8 h dark at 19 °C. Plants were grown under ambient CO_2 (400 ppm) and relative humidity was maintained at 75 % throughout day and night periods (Fig.7).

Plants were transferred to rooting medium (Murashige and Skoog medium with agarose and complemented with 20 $g.L^{-1}$ of sucrose and 0.3 $mg.L^{-1}$ of indole-3-butyric acid, adjusted to a pH 5.7 with KOH) (Bourrain, 2018), where they were kept for a month (Fig.5).

Figure 7: Maintenance of *A. chinensis* var. *deliciosa* plants in vitro in rooting medium.

Thereafter, plants were transferred to hydroponic solution (Fig.8) with optimal nutrient solution (1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.2 mM $MgSO_4$; 0.3 mM; $NH_4H_2PO_4$; 25 mM $CaCl_2$; 25 mM H_3BO_3; 0.5 mM $MnSO_4$; 2 mM; $ZnSO_4$; 0.5 mM $CuSO_4$; 0.5 mM MoO_3; 0.1 mM $NiSO_4$) and seven days later to a hydroponic system with three distinct hydroponic solutions, differing in the type of N supply (NO_3^-, NH_4^+ or a mixture of both) according to Gupta *et al*. (2017) (Table 1). For each N solution treatments 2 containers with 5 plants were prepared (biological replicates).

Figure 8: Maintenance of *A. chinensis* var. *deliciosa* plants in hydroponics.

Table 1: Final concentrations (mM) of nutrients in the different nutrient solutions (NO_3^-, NH_4^+ and Mix).

Nutrient	**Formula**	**NO_3^- Solution**	**NH_4^+ Solution**	**Mix Solution**
Potassium nitrate	KNO_3	3	0	1.5
Ammonium chloride	NH_4Cl	0	3	1.5
Calcium chloride	$CaCl_2$	1		
Magnesium sulfate	$MgSO_4$	1		
Potassium phosphate dibasic	K_2HPO4	0,5		
Potassium phosphate monobasic	KH_2PO_4	1		
Boric acid	H_3BO_3	92.5		
Manganese chloride	$MnCl_2$	18.3		
Zinc sulfate	$ZnSO_4$	1.5		
Copper sulfate	$CuSO_4$	0.64		
Sodium molybdate	Na_2MoO_4	0.24		
Na-FeEDTA	Na-FeEDTA	0.025		
MES (pH = 6.3)	$C_6H_{13}NO_4S$	1		

3.2 Plant sampling

Trial I

Twenty-one and 36 dpt to the hydroponic system, plants were removed from the solution, carefully washed with deionized water and separated into shoots and roots. After biometric analysis plants were flash frozen in liquid N and stored at -80 °C for further metabolomic and nutritional analysis.

Trial II

Twenty-one dpt, plants were mock-inoculated or inoculated with PSA, and 15 days post inoculation (dpi) they were removed from the hydroponic system, carefully washed with deionized water and separated into shoots and roots. After biometric analysis, a portion of plant leaves was used for PSA colony forming units (CFU) determination, and the remaining plant was flash frozen in liquid N and stored at -80 °C for posterior metabolomic and nutritional analysis.

3.3 Biometric analysis

Biometric analysis was performed by measuring shoot height and root length, with a common ruler, and shoot and root biomass (fresh weight) in an analytical scale. Data on shoot height and root length data were used to extrapolate root:shoot ratios.

3.4 Preparation of bacterial suspension and inoculation

For the artificial bacterial inoculation, PSA biovar 3 (CFBP 7286, isolated in Italy) was grown in Luria Bertani medium (LB) at 27 °C with shaking (75 rpm) for 16 h.

On the day of inoculation, bacterial colonies were re-suspended in sterile Ringer's solution and a $1x10^7$ $CFU.mL^{-1}$ inoculum was prepared by measuring inoculum absorbance at 600 nm in a nanophotometer (Implen GmbH, Munich, Germany). Plants were inoculated with PSA or with Ringer's solution alone (non-inoculated control), by dipping a sterile swab on the respective solution and rubbing the lower page of each plants leaf three times.

For each type of N solution and inoculum, five biological replicates were prepared.

3.5 Determination of Colony Forming Units (CFU)

In order to evaluate the degree of bacterial colonization in plant tissues, the tip of every leave of PSA-inoculated plants was collected with sterile scissors and surface sterilized by washing in 70 % ethanol for 1 min, 1 % sodium hypochlorite for 1 min and twice in sterile water for 1 min (Cellini *et al*., 2014). Plant tissues were macerated in aseptic conditions in 10 mL of Ringer's solution, and sequentially diluted ten times until 10^{-5}. One hundred microliters of each dilution of each sample was plated in Nutrient Sucrose Agar (NSA) medium in triplicate. Samples were incubated for 48 h at 27 °C, after which the number of colonies in each plate were counted, and CFU determined according to the following formula:

$$\mathrm{CFU/g} = \frac{(\mathrm{n°\ colonies} \times \mathrm{dilution\ factor})}{volume \times biomass}$$

3.6 Quantification of secondary metabolites

For the analysis of metabolites related with photosynthesis (total chlorophylls and carotenoids) and plant defence (total phenolic compounds, flavonoids and lignin), 100 mg of freeze-dried sample (both root and shoot) were extracted with 1 mL of methanol at 80 % (vol:vol). Samples were placed in an ultrasonic bath for 20 min for the extraction of cellular metabolites, after which they were centrifuged at 15 000 g for 15 min. The

resulting supernatant was used for the quantification of total chlorophylls, carotenoids, total soluble phenolics and flavonoids, while the sedimented biomass was used for lignin quantification.

3.6.1 Total soluble phenolics

Total soluble phenolics quantification was preformed according to the Folin-Denis method adapted from Marinova *et al*. (2005). To 4.5 mL of ultrapure water were added 500 µL of Folin-Denis reagent and 100 µL of methanolic extract obtained as previously described. After incubation for 5 min at room temperature, 5 mL of sodium carbonate at 7 % (w/v) were combined with the mixture, which was incubated in the dark for 1 h at room temperature. Finally, 2 mL of ultrapure water were added and absorbances recorded at 750 nm on a nanofotometer. The concentration of soluble phenolic compounds in each sample was carried out taking into consideration a gallic calibration curve.

3.6.2 Flavonoids

Total flavonoids were quantified through the aluminium chloride method adapted from Zhishen *et al*. (1999). Two millilitres of ultrapure water were combined with 150 µL of 5 % $NaNO_2$ and 100 µL of methanolic extract. After incubation for 5 min at room temperature, samples were added 150 µL of 10 % $AlCl_3$ and 1 mL of 1 M NaOH, as well as 1.2 mL of ultrapure water. Sample absorbances were recorded at 510 nm in a nanofotometer and flavonoid concentration in each sample was estimated taking into account a catechin calibration curve.

3.6.3 Total chlorophylls and carotenoids

Total chlorophylls and carotenoids were quantified through a protocol adapted from Sumanta *et al*. (2014). Using the methanolic extract obtained as described above, the absorbance of each sample was recorded at 470, 652 and 665 nm in a nanofotometer. Total chlorophyll was estimated according to the following formulas:

$$Chlorophyll\ a = (16.72Abs_{665} - 9.16Abs_{652}) \times volume/biomass$$

$$Chlorophyll\ b = (34.09Abs_{652} - 15.28Abs_{665}) \times volume/biomass$$

$$Carotenoids = (1000Abs_{470} - 1.63Chla - 104.96Chlb)/(221 \times biomass)$$

3.6.4 Lignin

Lignin quantification was performed according to a protocol adapted from Fukushima and Hatfield (2001). The sedimented biomass that remained in the

microcentrifuge tube after methanol extraction was subjected to three 20-minute washes in an ultrasound bath with distilled water, acetone and hexane. Afterwards samples were dried at 60 °C for 72 h, and 10 mg of sample was added 1 mL of 12.5 % acetyl bromide (in acetic acid), followed by incubation at 50 °C for 2 h with vigorous stirring. The digested samples were centrifuged for 5 min at 15 000 g, and 100 µL of the supernatant was combined with 200 µL of acetic acid, 150 µL of 0.3 M NaOH and 50 µL of 0.5 M hydroxylamine hydrochloride and 500 µL of acetic acid. The absorbance of each sample was recorded using a nanofotometer at 280 nm and lignin concentration in each sample was carried out taking into consideration a lignin calibration curve.

3.7 Mineral analysis

Mineral composition of plant samples was evaluated by inductively coupled plasma optical emission spectrometry (ICP-OES). One hundred mg of dried plant tissues (both root and shoot) were mixed with 5 mL of 65 % HNO_3 and 1 mL of H_2O_2 in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps, consisting of different temperature and time sets: 130 °C for 10 min, 160 °C for 15 min, 170 °C for 12 min, 100 °C for 7 min, and 100 °C for 3 min. After digestion, the resulting solutions were diluted with ultrapure water until a final sample volume of 20 mL. Mineral concentration determination was performed using the ICP-OES Optima 7000 DV (PerkinElmer, USA) with radial configuration.

3.8 Total nitrogen and protein

N and total proteins were quantified in 80 mg of sample (roots and shoots) in a Leco N analyser machine (Model FP-528, Leco Corporation, St. Joseph, USA) controlled by an external PC using Windows® software. Total protein was determined using N analysis by sample combustion in an oxygen-rich high temperature environment (Valente *et al*., 2019).

3.9 Statistical analysis

Data were analysed with GraphPad Prism version 6.0 (GraphPad Software, Inc., California, USA). Significant differences between treatments were determined by analysis of variance (ANOVA) followed by Fisher's LSD test ($p < 0.05$).

4. Results and Discussion

4.1 Trial I – Effect of different nitrogen nutrition on kiwifruit plants fitness

4.1.1 Biometric measurements

For the evaluation of the effect of different N sources on kiwifruit plants development, biometric measurements (biomass and shoot and root length) were taken at 21 and 36 dpt plants (Fig.9).

Throughout the experimental period, a significant increase was observed in shoot and root lengths in plants maintained with NO_3^- solution, in which shoot length increased by 1.8-fold (reaching 14.1 ± 1.5 cm at 36 dpi) and root length by 2.4-fold (attainting 35.8 ± 1.4 cm) (Fig.9A). Plants treated with Mix solution also showed a significant increase in shoot length, which increased from 6.9 ± 1.0 to 13.1 ± 3.6 cm (i.e. 1.9-fold) while comparing the two sample dates.

By the end of the experimental period, plants maintained with NO_3^- and Mix solutions were significantly higher than plants under NH_4^+ supplementation (by ca. 1.5-fold), with NO_3^- plants also showing the longest roots, with 35.8 ± 1.4 cm, representing a 2.1- and a 3.1-fold increment compared with NH_4^+ and Mix plants, respectively.

In general, shoot and root fresh weight (FW) biomasses significantly increased from 21 to 36 days in all treatments, with exception of roots of NH_4^+ and Mix-treated plants, in which only a tendency for this increase was observed (Fig.9B). Mix-treated plants had the highest increase in shoot biomass, reaching 11.9 ± 1.3 g by the end of the experimental period, which was 1.4 and 2.1-fold higher than NO_3^- and NH_4^+, respectively. In turn, in consistency with observed for root length, NO_3^- showed the highest increase in root biomass (8.0-fold), attaining 12.2 ± 0.8 g at 36 days. This represents a 3.0- and a 2.3-fold increment, compared NH_4^+ and Mix-treated plants, respectively.

Although *Actinidia chinensis* var. *deliciosa* plants grow better in acidic soils and, for that reason, would be expected to grow better in NH_4^+ condition, the rapid absorption and assimilation of NO_3^- ions is important for plants growing in acidified areas, because these processes are coupled with substantial increases in pH values around the roots. Consequently, roots may be less threatened by nutrient disharmony and toxic aluminium ions (Gloser and Gloser, 2000). Indeed, Rinallo and Modi (2002) on their study of oxalate concentration, also reported that NH_4^+ supplementation on kiwifruit plants, as both ammonium chloride and ammonium nitrate, resulted in smaller plants with a smaller leaf

area compared to NO_3^- supplementation, possibly due to lower concentration of ascorbic acid, for its role on cell division and expansion and in photosynthesis. On the other hand, kiwifruit plants high sensitivity to NH_4^+, reported by Lionakis and Schwabe (1985), could also have led to the decreased growth of NH_4^+-treated plants observed in the present work. Besides, lower growth is often associated with an acidification of the rhizosphere or the intracellular medium due to the NH_4^+ assimilation dependent excretion of protons via the H^+-ATPase, although some authors indicate that cytoplasmic pH is well regulated under normal conditions, and other processes might be responsible for the plant responses to NH_4^+ nutrition, like reduced rates of net photosynthesis (Horchani *et al*., 2010; Borgognone *et al*., 2013).

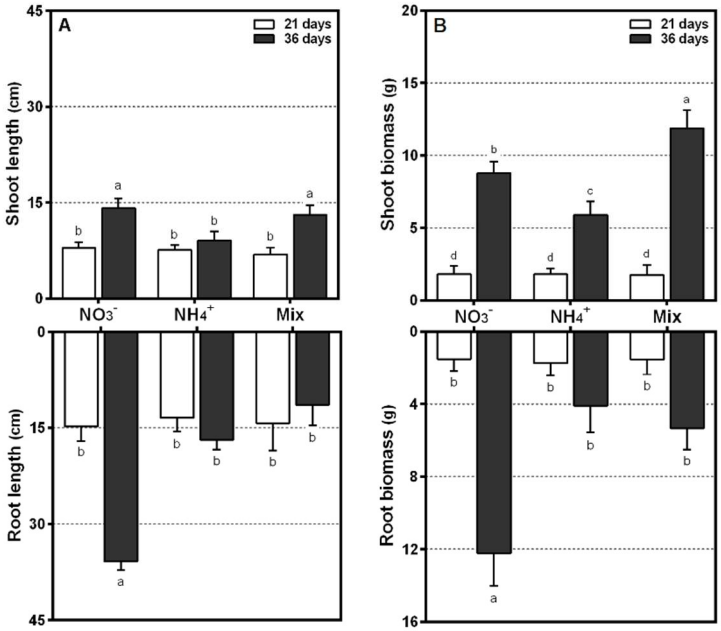


Figure 9: Shoot and root length (cm) (A) and FW biomass (g) (B) of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Several other works reported that when NO_3^- is applied as N source, one of the adaptative responses employed by plants is the promotion of lateral root growth, which could support the increased root biomass in plants treated with this mineral observed in the present study (Hackett, 1972; Forde 2002; Zhang *et al*., 2007; Na *et al*., 2014; Tsabarducas *et al*., 2017). This phenomenon may also result from the fact that, as Zhang *et al.* (1999) reported, NO_3^- behaves as a signalling molecule in roots, overlapping auxin pathway in plant growth.

4.1.2 Physiological measures

Both root to shoot ratio and chlorophyll levels are useful indicators of plant health state in two different ways. Evaluation of chlorophyll concentration provides information on leaf senescence and N status in plant tissues, which can vary in response to environmental stresses (Nauš *et al*., 2010). Total chlorophyll quantification in plant tissues is a destructive but more established process for assessing chlorophyll values, while soil-plant analysis development (SPAD) provides an alternative approach for the measurement of relative leaf chlorophyll levels and photosynthetic capacity, being a non-destructive method based on the analysis of leaf transmittance (Ling *et al*., 2011).

Root to shoot allocation it is a process in which plants optimize their resource use in response to environmental aspects. Generally, when nutrient availability increases plants tend to lower their investment on root growth and, on the other hand, when under stress situations that limit the use of nutrients, their allocation to roots tends to be grater, increasing root to shoot ratio (Agren and Franklin, 2003). In the present work, a significant increase of root to shoot ratio from 0.7 ± 0.1 to 1.4 ± 0.1 (i.e. 2.0-fold) was observed on plants treated with NO_3^- throughout the experimental period of Trial I (Fig. 10A). At the end of the experimental trial a significant difference was observed on root to shoot ratio between NO_3^- treated plants and plants maintained with NH_4^+ (0.6 ± 0.1) and Mix solutions (0.4 ± 0.1) (by ca. 2.6-fold). High values of root to shoot ratio of plants with NO_3^- may be due to the fact that NO_3^- assimilation requires more time and energy from the plant, compared with NH_4^+, having to invest more energy towards the roots to obtain the N levels needed for normal plant growth and development (Haynes and Goh, 1978). In fact, a study by Garbin and Dillenburg (2008) reported that N deficiency symptoms, which include the increase of root to shoot ratio and decreased chlorophyll concentration, were induced when NO_3^- was provided alone as N source in *Araucaria angustifolia*. Also, similarly to what was observed in the present work Tsabarducas *et al*., (2017) reported an increase on root to shoot ratio *Olea europaea* L. (cv. 'Kalamon') supplemented with NO_3^- and associated this event to the influence of NO_3^- as a signalling molecule on auxin biosynthesis, transport and accumulation and on cytokinin biosynthesis and transport to the shoot.

Carotenoids are involved on several important roles in the plant such as development, antioxidant capacity, production of phytohormones and photosynthesis along with chlorophylls (Hirschberg, 2001; Yoo *et al*., 2003; Cazzonelli and Pogson, 2010). A significant decrease of total chlorophyll was observed in plants supplemented with NO_3^- (from 2.0 ± 0.2 to 1.3 ± 0.1 $mg.g^{-1}$, i.e.0.4-fold) (Fig.10B). Along with this reduction, plants treated with NO_3^- also showed a 0.5-fold significant decrease in

carotenoids concentration (from 518.2 ± 47.9 to 266.7 ± 35.4 μg.g^{-1}) (Fig.10C). In addition, carotenoids concentration significantly decreased from 346.2 ± 51.3 to 214.7 ± 30.2 μg.g^{-1} (i.e. 0.4-fold) in Mix-treated plants.

Zeaxanthin and antheraxanthin are abundant plant carotenoids belonging to the xanthophyll cycle and are responsible for the protection of photosynthetic apparatus by enabling on site energy dissipation, as excess energy can lead to photo-oxidative damage (Demmig-Adams and Adams, 1995). This greater concentrations of carotenoids in younger plants treated with NO_3^- and Mix solutions could have happened as a result of acclimation by young leaves to cope with excess irradiance present during the initial stages of leaf expansion (Jiang *et al*., 2006). Higher concentrations of these specific carotenoids on young leaves were already reported by some articles (Schindler *et al*., 1994; Jiang *et al*., 2006).

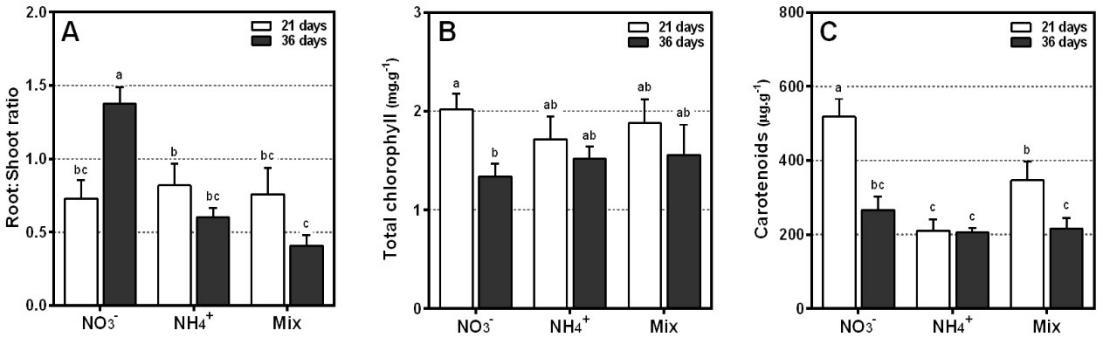


Figure 10: Root to shoot ratio (A); Total chlorophyll (mg.g^{-1}) (B) and Carotenoids (mg.g^{-1}) (C) values in *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.1.3 Secondary metabolites

Secondary metabolites play important roles in regulation of plant growth and defense to pests and pathogens (Wang *et al*., 2018). Phenols specifically, work as signal compounds, internal physiological regulators, pigments or chemical messengers, and are part of some resistance mechanisms of plants against pathogens (Wang *et al*., 2018). After the contact with a pathogenic microorganism plants initiate a cascade of biochemical changes in their cells, including an increase in ROS concentration. In order to protect their own tissues, plants have developed efficient antioxidant system that includes both enzymes, which catalyse ROS decomposition, as well as non-enzymatic antioxidants, including phenolic compounds like flavonoids, lignin and carotenoids (Lattanzio, 2013; Kulbat, 2016). Maintaining high antioxidant activity allows plants to efficiently capture toxic ROS, determining their tolerance to stress (Kulbat, 2016). On

that note, the concentration of polyphenols, flavonoids and lignin were measured between 21 and 36 days-old plants, to verify the difference in the development of these important components on each experimental situation (Fig.11).

On the first trial, comparing 21 dpt with 36 dpt plants, there were no significant differences on polyphenolics (Fig.11A). There was, however, a significant decrease of 0.2-fold in polyphenols concentration on the roots of plants supplemented with NO_3^- solution, which decreased from 4.5 ± 0.6 to 3.7 ± 0.8 $mg.g^{-1}$. Despite this decrease, polyphenols concentration on roots of NO_3^- treated plants remained reasonable high, with a 4.9-fold and a 4.5-fold increment compared with NH_4^+ and Mix solutions, respectively, at 21 dpi, and 1.5-fold between NO_3^- and Mix treatment at 36 dpt.

Articles focused on N and secondary metabolites are often correlated with the amount of N instead of the N form *per se (*Hakulinen *et al*., 1995; Jones and Hartley, 1999; Ibrahim *et al*., 2011). Total phenols and flavonoids increasing under limiting N fertilization was reported from some studies (Koricheva *et al*., 1998; Ibrahim *et al*., 2011). Ibrahim *et al*. (2011) also noted on *Labisia Pumila* Blume, that as less N was provided (from 270 to 0 kg N/ha), the more total phenols and total flavonoids were produced. This event may be due to the fact that low N fertilization increases the availability of phenyl alanine due to restriction in protein production, and so, more phenyl alanine is available for the production of secondary metabolites (Jones and Hartley, 1999; Ibrahim *et al*., 2011).

Although no significant changes on flavonoids concentrations were observed in this experiment (Fig. 11B), Zhu *et al*., 2014 reported, on *Prunella vulgaris* plants, that NO_3^- fertilization resulted in a higher concentration of flavonoids then NH_4^+ fertilization, however, the mechanisms by which such metabolic changes occur is not yet understood.

From 21 to 36 dpi, lignin concentration significantly increased on both shoots and roots of Mix-reated plants by 2.1-fold and 1.5-fold, respectively, and on shoots of NH_4^+ treated plants by 1.8-fold (Fig.11C). At the end of the experimental trial, higher values of lignin were observed on Mix treated plants, with 6.4 ± 1.0 $mg.g^{-1}$ on shoots and 6.2 ± 0.3 $mg.g^{-1}$ on roots. In addition, significant increases of 2.1-fold and 1.5-fold were observed in shoots and roots of plants maintained with Mix solution in comparison with NO_3^- solution, and of 1.4-fold (shoots) and 1.7-fold (roots) comparing with NH_4^+ solution. *Actinidia* species are characterized by having ligneous stems with semi-lignified branches (Neves, 2008a). In fact, a study Nemli *et al*., 2003 on *A. chinensis* Planch. reported that these plants have more lignin content on their stalks (about 27%) then hardwood species (17-26%) and similar lignin content with softwood species (25-32%). *Actinidia* formation of vascular tissue on the roots is also typically of that of woody roots,

with lignified cell wall of vessels, tracheids and fibers (Lemon, 1993). Thus, higher lignification on kiwifruit plants shoots and roots throughout the experiment was expected as they are woody plants with lignified shoots and roots (Lemon, 1993; Yang *et al*., 2002; Nemli *et al*., 2003 Neves, 2008).

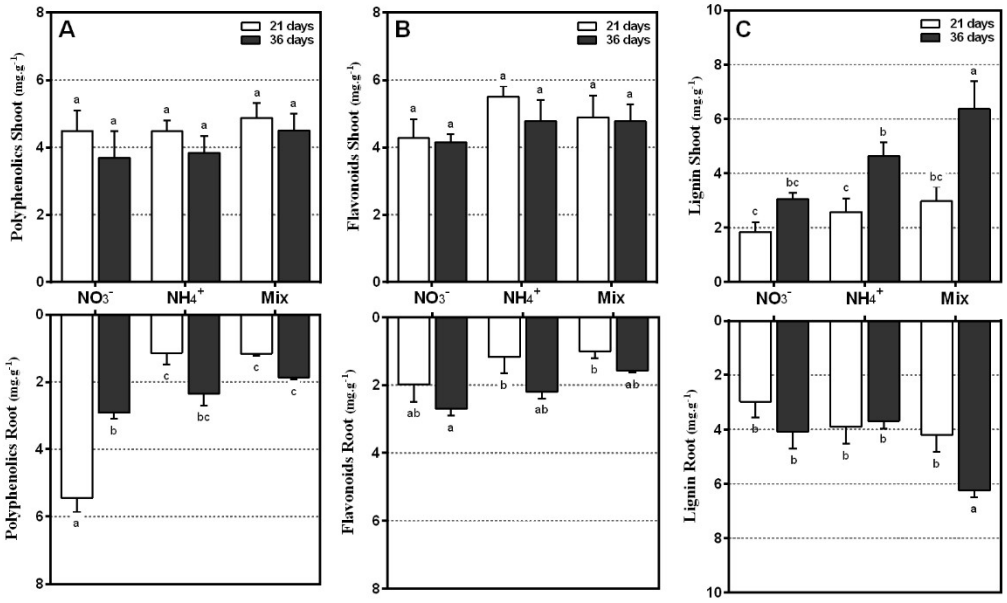


Figure 11: Concentration of Polyphenolics (A), Flavonoids (B) and Lignin (C) (mg.g^{-1}) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.1.4 Mineral concentration

Several studies have shown that the form of N affects nutrient availability, either due to differences in soil pH or to the induction of NH_4^+ toxicity, which lead to antagonism in cation uptake, and/or alterations in osmotic balance (Magalhaes and Wilcox, 1983; Therios and Sakellariadis, 1988; Ashraf and Sultana, 2000; Borgognone *et al*., 2013). Regarding kiwifruit plants, there are some reports about the influence of N fertilization on plant mineral composition, but not on N form specifically (Pacheco *et al*., 2008; Peticila *et al*., 2015).

In the present work, five essential macronutrients (N, P, K, Mg, Ca) and four micronutrients (Bo, Zn, Mn and Fe) showed different accumulation patterns according to each N form (Fig.12 and 13), suggesting that the form of N affects the mineral composition of kiwifruit plants through their development.

N is part of 1–5% of total plant dry matter and is an integral constituent of nucleic acids, proteins, co-enzymes, chlorophyll, phytohormones and secondary metabolites being a decisive factor for plant growth (Hawkesford *et al*., 2012). In the case of inorganic

N concentration, a significant decrease was observed over time in shoots of plants treated with NO_3^- (from 216 ± 37 to 153 ± 3 μg.g^{-1}, i.e. 0.3-fold) and Mix solution (from 360 ± 15 to 240 ± 9 μg.g^{-1}, i.e. 0.3-fold). Moreover, at the end of the experimental period significant differences in inorganic N accumulation were observed between NO_3^- treatment and NH_4^+ (0.5-fold) and Mix (0.4-fold) (Fig.12A). In roots, no significant differences in N concentration were observed within each treatment during the experimental period, but at 36 dpt significant differences were registered between N treatments, with NO_3^- treated plants having the least N concentration (153 ± 3 μg.g^{-1}) and Mix treated plants the highest (240 ± 9 μg.g^{-1}).

In a similar manner, Borgognone *et al*. (2013) reported higher N concentration in tomato plants supplemented with NH_4^+, in comparison with NO_3^-. The lower concentration of inorganic N in NO_3^- treated plants is probably due to the fact that NO_3^- assimilation demands more time and energy from the plant to be assimilated leading to decreased N accumulation in plant tissues, and may explain why root to shoot ratios are higher in plants from this treatment (Fig.10A) (Haynes and Goh, 1978).

Energy transfer and P as a component of macromolecular structures, such as ribonucleic acids, are examples of the importance of this specific macronutrient on plants development (Hawkesford *et al*., 2012). Throughout the experimental period, a significant decrease in P concentration was observed on both shoots and roots of plants treated with NH_4^+ solution (from 2 788 ± 300 to 1 732 ± 160 μg.g^{-1}, i.e. 0.4-fold and from 6060 ± 234 to 4399 ± 350 μg.g^{-1}, i.e. 0.3-fold, respectively), and on roots of Mix-treated plants (from 8810 ± 577 to 6204 ± 258 μg.g^{-1}, i.e. 0.3-fold) (Fig.12B). At the end of experimental period, NO_3^- treated plants showed a significant increment in P concentration in shoots compared to plants treated with NH_4^+ (1.5-fold) and Mix solutions (1.8-fold), and in roots compared with NH_4^+ (1.4-fold).

Despite the higher P concentration in NO_3^--treated plants observed in the present work, previous findings regarding P uptake under different N sources often report to an increase in P concentration in plants treated with NH_4^+. This probably happens due to a decrease in soil pH that induces higher solubility of Ca-phosphates in the rooting medium and higher $H_2PO_4^-$:HPO_4^2 ratio (increasing the phosphate ion that is preferentially absorbed by roots) (Magalhaes and Wilcox, 1983; Hoffmann *et al*., 1994; Pedersen *et al*., 2019).

In general, K is characterized by high rates of uptake due to the high permeability of plant membranes to K, and high mobility within the plants (Hawkesford *et al*., 2012; Pandey, 2018). K is responsible for important roles in plant tissues like enzyme activation, osmoregulation and protein synthesis (Hawkesford *et al*, 2012). Regarding K

concentration on this study, significant decreases were observed along the experimental trial in both shoots and roots after plant treatment with NH_4^+ and Mix (by ca. 0.5-fold on shoots and by 0.4- and 0.2-fold respectively on roots) (Fig.12C). At 36 dpt, NO_3^- supplementation represented the treatment with highest values of K on shoots (25795 ± 1807 $\mu g.g^{-1}$) and roots (55348 ± 979 $\mu g.g^{-1}$), whereas NH_4^+-treated plants had lower amounts of K in both shoots (9785.3 ± 487.1 $\mu g.g^{-1}$) and roots (28581 ± 1069 $\mu g.g^{-1}$) having a 2.6- and 1.9-fold significant difference between these two treatments on shoots and roots, respectively.

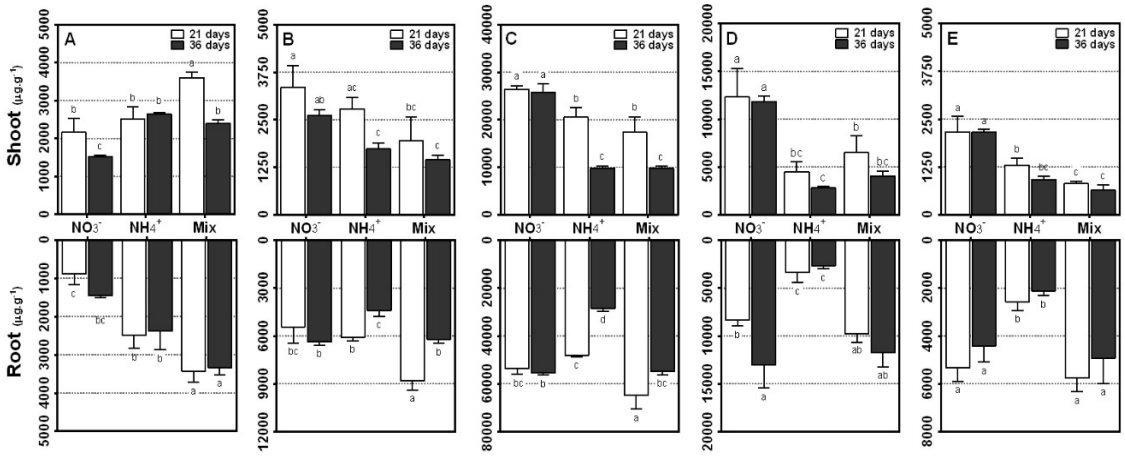


Figure 12: Concentration of macronutrients Nitrogen (A), Phosphorous (B), Potassium (C), Calcium (D), Magnesium (E) and ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Some studies have already reported deficient K uptake with NH_4^+ supplementation because NH_4^+ and K have similar charges and hydrated diameters resulting in competition for the site of transportation (Wang *et al*. 1996; Gerendás *et al*., 1997; Roosta and Schjoerring, 2007; Hoppen *et al*., 2010; Na *et al*., 2014). Also, in concordance with these results, Lu *et al*. (2005) reported a decrease in K uptake on tobacco plants supplemented with NH_4^+ and Ashraf and Sultana (2000) a decrease of K on leaves of sunflower plants supplied with NH_4^+, both suggesting a competition in the uptake between these two cations and the last reporting a retranslocation of K from the leaves to the xylem when NH_4^+ was the N source. NO_3^- uptake has been shown to stimulate net K^+ uptake in various crop species, suggesting that the NO_3^- ion serves as an accompanying anion during K^+ uptake and/or transport (Zhang *et al*., 2010). In addition, K seems to have an influence on the translocation of photosynthetic assimilates needed to support the active uptake process of NO_3^- by plant roots (Fageria, 2001). Therefore, in present work, the different K accumulation after the supply of different N

forms may result from the co-transportation of NO_3^- and K on ion uptake as well as from the competition for the uptake of NH_4^+ and K.

The various functions of Ca go from membrane and cell wall stabilization to osmoregulation and acting as a second messenger in plant signalling (Hawkesford *et al*., 2012). At 36 dpt, plants with NO_3^- supplementation showed significantly increased Ca concentration in roots, which reached 13 039 ± 2 400 $\mu g.g^{-1}$, representing a 1.6-fold increase compared with 21 dpt, (Fig.12D). Ca concentration was also significantly higher in plants treated with NO_3^- solution compared with NH_4^+ supplementation, by 4.2-fold in shoots and 4.9-fold in roots at 36 dpt.

Mg plays essential roles in processes like chlorophyll and protein synthesis as well as on enzyme activation and carbohydrate partitioning (Hawkesford *et al*., 2012). Mg did not vary significantly within N treatments along the experimental trial, but significantly differences were observed between treatments, with NH_4^+ treated plants showing, in general, lower values (Fig.12E). Plants treated with NO_3^- had higher values of Mg concentration in shoots, reaching 2166 ± 405 $\mu g.g^{-1}$, whereas Mix treated plants had the lowest, with 641 ± 141 $\mu g.g^{-1}$. At the end of experimental time, roots of NH_4^+-supplemented plants had significantly lower values of Mg compared to NO_3^- (by 0.5-fold) and Mix (by ca. 0.6-fold) treatments.

Accordingly, Tsabarducas *et al*. (2017) on *Olea europaea L. (cv. "Kalamon"),* Borgognone *et al*. (2013) on tomato plants cv. 'Moneymaker' and Gloser and Gloser (2000) on *Acer pseudoplatanus* and *Calamagrostis villosa* all noted that Ca and Mg concentrations decreased when N was exclusively provided as NH_4^+ on the nutrient solution. Although this decrease in cation uptake on NH_4^+ supplemented plants was often associated with the acidification of the rooting medium by NH_4^+ uptake (release of H^+) (Borgognone *et al*., 2013), and Gloser and Gloser (2000) reported on *Acer pseudoplatanus* and *Calamagrostis villosa* seedlings, that low pH (3.5 pH) could indeed cause a more pronounced negative effect of NH_4^+ on base cation uptake, these authors also observed that at steady 5.5 pH, this effect steal unfolded, suggesting that the influence of NH_4^+ ions on uptake rates of other cations was not entirely due to media acidification. The antagonistic effect between Ca, Mg and NH_4^+ can also be due to competition in uptake of this cations through the mechanism of charge balance in ion uptake, since N is a dominant macronutrient and its ionic form controls cation and anion uptake (Ashraf and Sultana, 2000; Borgognone *et al*., 2013).

In general, several studies reported that plants grown in NH_4^+ medium, without NO_3^-, contain lower concentrations of Ca, Mg and K, and higher levels of P (Magalhaes and Wilcox, 1983 Ashraf and Sultana, 2000; Borgognone *et al*., 2013; Na *et al*., 2014;

Tsabarducas *et al*., 2017). The present work seems to be in concordance with these studies, except regarding P concentration, which was still grater on NO_3^- treated plants. In this study case, higher root growth and biomass showed by kiwifruit plants treated with NO_3^- (Fig.9) could also have helped the higher uptake of macronutrients showed by these plants since different root growth system can affect mineral nutrient up-take (Na *et al*., 2014). It is also important to note, that medium acidification by NH_4^+ uptake could result on lower cation uptake not in a direct form, but through root growth inhibition (Liu *et al*., 2013).

B has important functions on normal development of primary cell walls, bio membranes, reproductive growth and development of root and shoot growth (Broadley *et al*., 2012). Regarding B concentration, results varied significantly with time in Mix-treated plants, whose shots presented 9.7 ± 3.4 $\mu g.g^{-1}$ at 21 dpt and 20.0 ± 3.0 $\mu g.g^{-1}$ at 36 dpi and roots 16.1 ± 1.4 $\mu g.g^{-1}$ at 21 dpt and 9.6 ± 1.8 $\mu g.g^{-1}$ at 36 dpt, representing a 2.0-fold increment and a 0.4-fold decrease, respectively (Fig.13A).

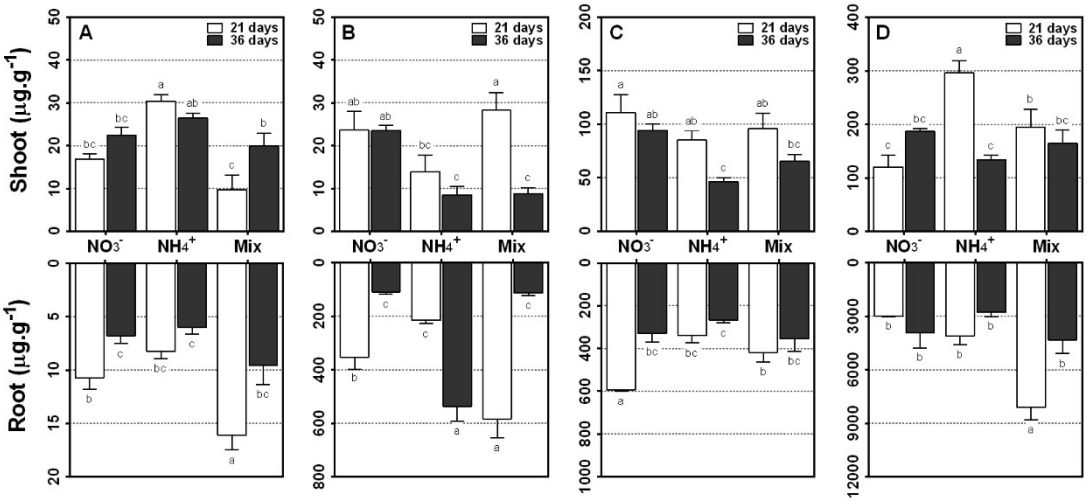


Figure 13: Concentration of micronutrients Boron (A), Zinc (B), Manganese (C) and Iron (D) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 21 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Zn is the only metal that is present in enzymes of all six enzyme classes, acts as an enzyme activator and is involved in protein synthesis (Broadley *et al*., 2012) Concerning Zn concentration in plant tissues, a 0.8-fold significant decline was observed from 21 to 36 dpt in roots of Mix-supplemented plants, as well as in NO_3^--treated plants (0.7-fold) (Fig.13B). On the contrary, Zn concentration in roots of NH_4^+-supplemented plants increased significantly until 36 dpt, by 2.5-fold, reaching a value of 535 ± 54 $\mu g.g^{-1}$. By the end of the experimental period, NH_4^+-treated plants had significantly higher Zn concentration in roots comparing with NO_3^- and Mix-treated plants (ca. 4.9-fold). In

addition, in shoots, there was a significant decrease of Zn in plants maintained with Mix solution (from 28 ± 4 to 8 ± 1 $\mu g.g^{-1}$, i.e. 0.7-fold) reaching, at the end of the experiment, along with NH_4^+ solution, a significant difference in relation to plants maintained with NO_3^- solution (by ca. 2.8-fold).

Mn is also part of several enzymes, plays a role in activating enzymes, production of macromolecules, photosynthesis and cell division (Broadley *et al*., 2012). A significant decrease of Mn concentration was observed in plant shoots maintained with NH_4^+ treatment (from 85 ± 9 to 46 ± 4 $\mu g.g^{-1}$, i.e. 0.5-fold) and in roots of NO_3^- treated plants (from 595 ± 6 to 329 ± 41 $\mu g.g^{-1}$, i.e. 0.5-fold) (Fig.13C). At the end of the experimental trial, plants supplemented with NO_3^- had 94 ± 6 $\mu g.g^{-1}$ of Mn, which represents a 2.0-fold significant increment compared to NH_4^+-treated plants.

Components of redox systems like heme proteins are constituted of Fe and several enzymes require Fe for their normal function. In addition, Fe is implicated in the normal functioning of photosynthesis (Broadley *et al*., 2012). During the experimental trial, plants maintained with NH_4^+ exhibited a significant decrease of Fe content on shoots that decreased from 296 ± 23 at 21 dpt to 133 ± 8 $\mu g.g^{-1}$ at 36 dpt (i.e., 0.6-fold) (Fig.13D). Similarly, Mix supplementation also induced a 0.5-fold decrease in Fe concentration in plants roots over time. At 36 dpt, Fe concentrations in the shoots and roots were not affected by N form which could be due to the supply of Fe exceeding plant needs, as the Fe concentration in the roots was higher than in the shoots (Wang and Below, 1998).

Reports on the effect of the N form on micronutrients accumulation in plant tissues are inconclusive. For example, a linearly increase of Fe, Mn, Zn and B concentrations with NH_4^+ (23.0 mM) were reported by Borgognone *et al*. (2013) in tomato plants cv. 'Moneymaker' and on a study by Tsabarducas *et al*. (2017) on *Olea europaea* L. (cv. 'Kalamon'), where Fe, Mn and Zn also increased with increasing NH_4^+ concentrations (from 1 to 16 mM) as the N source compared to NO_3^- increasing supplementation. NH_4^+ acidified the external medium, increasing the availability and uptake of these micronutrients and, in contrast, NO_3^- caused an alkalization of the external medium and reduced Mn and Zn availability and uptake. (Tsabarducas *et al*., 2017). On the other hand, strong antagonistic effects between Mn and Zn shoot concentrations and NH_4^+ were observed in a study by Wang and Below (1998) on *Triticum aestivum* L. cv. 'Len' and *Triticum durum* Desf. cv. 'Inbar' harvested after 21 days of growth. In the present study, the N form did not influence significantly micronutrient concentrations at 36 dpt except for Zn root concentrations, which were higher on plants subjected to NH_4^+ treatment, and Mn shoot concentrations, which were higher on plants subjected with NO_3^- supplementation.

4.1.5 Protein concentration

All vital processes in plants are associated with proteins, of which N is an essential constituent (Jahan *et al*., 2016). N can be assimilated by plants in two major ionic forms, NO_3^- and NH_4^+, which have different types of assimilation (Xu *et al*., 2012). NH_4^+ has a more direct assimilation process where it can be promptly assimilated into amino acids and other organic compounds after absorption while NO_3^- has to undertake reductions to be transformed in NH_4^+ and then be assimilated (Masclaux-Daubresse *et al*., 2010; Xu *et al*., 2012). On that note, the protein concentration was measured between 21 and 36 dpt kiwifruit plants (Fig.14).

In shoots, from 21 to 36 dpt a significant decrease in total protein was observed in NH_4^+ and Mix-supplemented from 26.1 ± 1.1 % to 20.5 ± 0.3 % (i.e. 0.2-fold) and from 28.1 ± 1.1 % to 18.7 ± 0.7% (i.e. 0.3-fold), respectively. Throughout the experimental period a significant decrease from 22.2 ± 1.1 % to 11.9 ± 0.2 % (i.e.0.5-fold) and from 22.3 ± 1.9 % to 11.3 ± 0.3 % (i.e. 0.5-fold) was also observed in shoots and roots, respectively, of plants treated with NO_3^- solution. At the end of the experimental trial, protein content was significantly different between N treatments, with plans maintained under NH_4^+ solution presenting 1.1-fold and 1.7-fold higher protein contents comparing to Mix and NO_3^- supplementation, respectively. In roots, lower values of protein content were observed in plants treated with NO_3^-, which exhibited a 2.6-fold and a 2.4-fold difference in relation with NH_4^+ and Mix supplementation, respectively.

A considerable amount of reports observed increases in amino-acids synthesis in NH_4^+ fed plants, compared to plants supplemented with NO_3^- (Dinev and Stancheva, 1995; Osaki *et al*., 1995; Ruan *et al*., 2007; Atanasova, 2008; Horchani *et al*., 2010; Borgognone *et al*., 2013). As NH_4^+ has a more rapid and less time-consuming incorporation into organic N then NO_3^- and taking into consideration the higher N content of NH_4^+ treated plants (Fig.12A), it was expected that plants fed with NH_4^+ based supplementation presented a higher percentage of protein as it was observed (Atanasova, 2008; Horchani *et al*., 2010). Also, NH_4^+ assimilated in the roots is transported in the plant as amino acids, proteins and other organic N compounds to avoid toxic concentrations of free NH_4^+ in the xylem sap (Borgognone *et al*., 2013). A decrease in protein content through the experimental trial went accordingly with the expected, as plants become more mature, did not needed to keep their metabolism so active, and accumulate biomass, diluting protein content throughout the growing tissues. This

phenomenon was especially evident in NO_3^- treated plants, which developed faster, thus showing the lowest protein content 36 dpt.

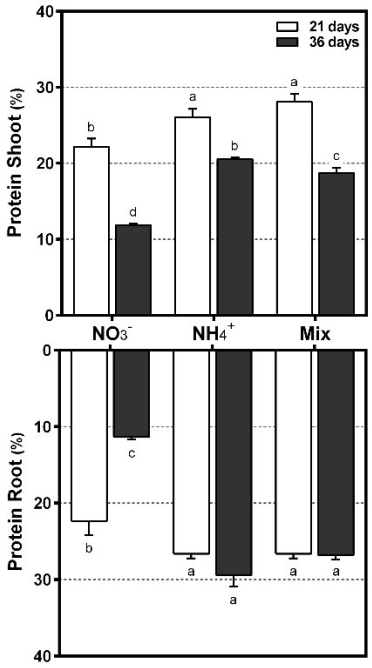


Figure 14: Percentage of protein on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 24 and 36 days. Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

.

4.2 Trial II - Effect of different nitrogen nutrition on tolerance against PSA

4.2.1 Biometric measurements

Pathogenic attack induces several differences on the host, such as activation of defense metabolites, signaling pathways and protein synthesis (Wang *et al*., 2018; Petriccione *et al*. 2013a). One of the cues that can be easily spotted upon systemic infection is on growth and architecture of the plant (Calonnec *et al*., 2012). As pathogens add an additional stress to plants, generally, affected plant are characterized by lower growth and reproduction (Ditommaso and Watson, 1995). On the other hand, plants can alter allocation to growth and reproduction in response to epidemics by an increase in susceptible tissue (dilution effect) (Calonnec *et a*l., 2012).

PSA can utilize sucrose and fructose which could lead to energy and nutrient stress and a study by Block *et al*. (2010) demonstrated that the type III effector HopG1 from *Pseudomonas syringae* pv. *tomato*, when constitutively expressed in *Arabidopsis*

thaliana, tabacco and tomato plants, dramatically altered plant development resulting in dwarfism (Nardozza *et al*, 2015; Lastdrager *et al*., 2014). On trial II, inoculation with PSA did not result in any significant difference on the biometric parameters evaluated (Fig.15). The significant difference occurred only between treatments as expected after the previous discussion of trial I where NH_4^+ plants were smaller and had low biomass probably due to medium acidification or competitivity for the uptake site with other cations. This demonstrated that PSA infection was not the cause for the low values of shoot and root length nor the low biomass numbers but the N source.

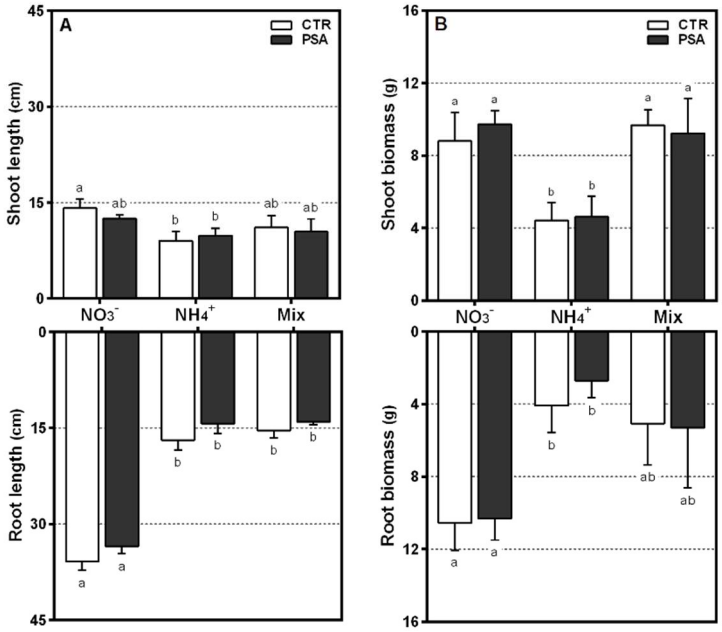


Figure 15: Shoot and root length (cm) (A) and biomass (g) (B) of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.2.2 CFUs

In order to understand the impact of NO_3^- and NH_4^+ fertilization on bacterial colonization, CFUs were determined 36 dpt and 15 dpi with PSA.

Plants maintained with NH_4^+ solution presented the highest CFUs count, reaching 23.7 ± 4.7 $\times 10^{11}$ CFUs.g^{-1} (Fig.16). That represents a significant 7.2-fold increment compared with Mix solution, which accounted for 3.3 ± 1.7 $\times 10^{11}$ CFUs.$g^{-1}$and a 33.9-fold increment in relation to NO_3^- treated plants, with 0.7 ± 0.4 $\times 10^{11}$ CFUs.g^{-1}. Although there was no significant difference between Mix and NO_3^- treatments, a tendency for a lower colonization in the case of plants maintained with NO_3^- was observed.

The lowest colonization value observed in plants treated with a NO_3^- solution and the highest value recorded in plants treated with NH_4^+ solution agrees with several studies that demonstrated that the form of N can affect disease development and plant resistance (Mur *et al*., 2016). For example, symptoms of black root rot (*Rhizoctonia solani*) of sugar beets (Afanasiev and Carlson, 1942) or *Fusarium* wilt on tomato (Borrero *et al*., 2012) were reported to increase following NH_4^+ nutrition. In fact, a study performed by Gupta *et al*. (2013) in tobacco plants, in which NH_4^+ was correlated with a compromised resistance to *Pseudomonas syringae* pv. p*haseolicola*, while NO_3^- appeared to have an important role in plant resistance. This is probably because NO_3^- seemed to augment HR mediated resistance in tobacco plants through NO and SA generation, which explained the faster cell death response compared to NH_4^+. On the other hand, NH_4^+ could have compromised resistance not only through reduced NO generation, but also by encouraging metabolic reprogramming of HR-defence towards sugar and 4-aminobutyric acid production (Gupta *et al*., 2013).

The results observed in the present work also seem to be in line with a preliminary report by Holmes (2012) that suggested a higher survival rate when PSA-inoculated cv. 'Hayward' plants were fertilized with NO_3^- and a detrimental effect on survival rate when fertilized with NH_4^+.

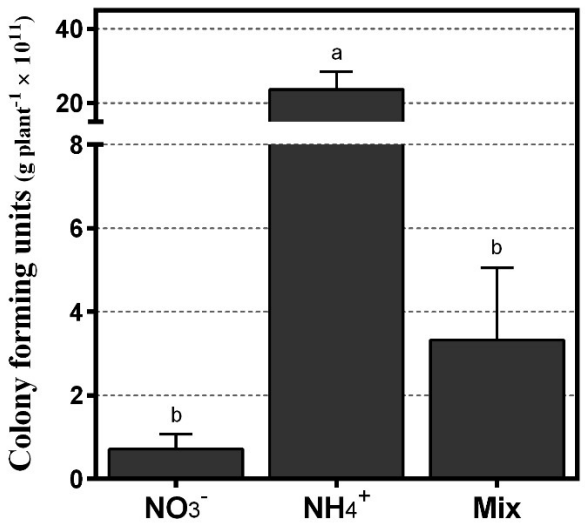


Figure 16: Colony Forming Units (CFUs) (CFUs.g^{-1}×10^{11}) of PSA in *A. chinensis* var. *deliciosa* cv. 'Hayward' inoculated plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.2.3 Physiological measurements

Root to shoot ratio of plants treated with NO_3^- were significantly higher compared to NH_4^+, but no significant difference was found between control and inoculated plants (Fig. 17A). However, a significant decrease of SPAD values was observed when comparing control and inoculated plants maintained with NH_4^+ solution (from 48.5 to 34.2, i.e. 0.3-fold) (Fig. 17B). Although control plants under NH_4^+ solution showed the highest SPAD values (48.5 ± 1.6), in inoculated plants SPAD values were higher under Mix supplementation (44.0 ± 3.0). Decreased photosynthetic activity, is generally observed during pathogenic attack to plant leaves, along with under-expression of genes related to photosynthesis (Berger *et al*., 2007; Petriccione *et al*., 2013a). On *Actinidia* specifically, this decrease is notable as one of the symptoms of KBC is damage to the leaves through extensive leaf spotting (Petriccione *et al*., 2013a). So, the lower photosynthetic activity (translated by lower SPAD values) noted on plants treated with NH_4^+ could be due to the damage to the leaves provoked by the high PSA colonization observed previously on this experimental situation (Fig.16).

However, contrarily to what was observed for SPAD values, total chlorophyll concentration did not show significant differences with PSA inoculation (Fig.17C). This discrepancy between SPAD values and total chlorophyll concentration may be due to a non-linear correlation between these two parameters, as already reported by Uddling *et al*. (2007), which results from a non-uniform chlorophyll distribution across the leaf surface and multiple scattering.

Like other secondary metabolites, carotenoids have antioxidant activity important for plant defence against pathogens and are also involved in plants photosynthetic capacity (Lattanzio, 2013; Yoo *et al*., 2013; Kulbat, 2016). The response of carotenoids concentration to PSA inoculation did not show significant differences, however, a tendency for lower values on inoculated plants treated with NH_4^+ was observed in comparison to the remaining experimental situations (Fig.15D). Low values of carotenoids in NH_{4+} treated plants (172.3 ± 17.6 $\mu g.g^{-1}$) could be one of the reasons leading to the significantly higher bacterial colonization in this treatment due to carotenoids role in scavenging ROS (Lattanzio, 2013; Kulbat, 2016). In fact, Wang *et al*., 2018 reported an upregulation of 8 carotenoid related genes succeeding PSA infection.

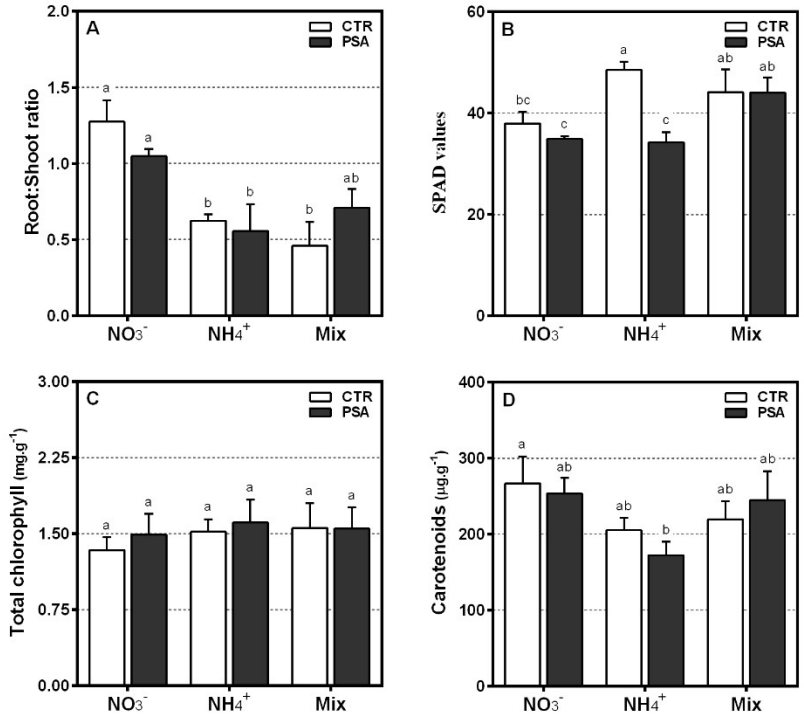


Figure 17: Root to shoot ratio (A); SPAD (B); Total chlorophyll (mg.g^{-1}) (C) and Carotenoids (mg.g^{-1}) (D) values in *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.2.4 Secondary metabolites

In the case of kiwifruit plants inoculated with PSA some investigation articles already noted an upregulation of phenolic compounds upon the infection (Miao *et al*., 2009; Wang *et al*., 2018). Wang *et al*. (2018) analysed the transcriptomic profile of PSA-infected kiwifruit plants in order to explore the species-specific interaction between PSA and these plants. The results of this study showed that phenol pathway-associated metabolism was upregulated upon bacterial infection suggesting that phenolic compounds may play an important role in the resistance of kiwi plants to PSA. Also, an antagonistic effect of flavonoids on plant bacterial pathogens has been reported, specifically against *Pseudomonas syringae* pv. *tomato*, with a decrease in pathogen mobility after exposure to these compounds, resulting from the loss of the flagellum and the inhibition of the T3SS associated with *Pseudomonas* spp. strains virulence (Vargas *et al*., 2013). Considering that, the concentration of polyphenols, flavonoids and lignin were measured between control and inoculated plants (Fig.18) to assess their influence on kiwifruit plants resistance to PSA.

On the second trial, comparing control plants with inoculated plants, no significant differences were observed on polyphenols and flavonoids concentration in shoots (Fig.18A and B). Nonetheless, in roots of NH_4^+ treated plants a significant increase in lignin concentration was observed between control plants, which had 3.7 ± 0.3 $mg.g^{-1}$, and inoculated plants, with 6.4 ± 0.8 $mg.g^{-1}$ (i.e. 1.7-fold) (Fig.18C).

Increased lignification of NH_4^+ supplemented plants may have been a strategy employed by plants to counteract the high bacterial colonization observed in this treatment, by mechanically reinforcing its cell walls. Indeed, articles have shown that, when plants are infected by pathogenic bacteria, lignin is synthesized in large quantities at the infection site, strengthening the lignification of plant cell walls and resisting further infection by the pathogenic bacteria (Song *et al*., 2019). When *Gossypium hirsutum* was inoculated with pathogenic bacteria, a large amount of lignin was synthesized, which improved its resistance to verticillium wilt (*Verticillium dahlia*) (Xu *et al*., 2011). In wheat, it was demonstrated that chemical inhibition of cell lignification resulted in decreased resistance to *Puccinia graminis,* indicating that lignin is an important macromolecule in terms of plants defence (Moershbacher *et al*.,1990). Finally, on kiwifruit, 35 genes involved in lignin biosynthesis were differentially expressed between a resistant ('Huate') and susceptible cultivar ('Hongyang') to PSA, where most of them were upregulated on the resistant cultivar demonstrating the importance of lignin biosynthesis against PSA (Song *et al*., 2019).

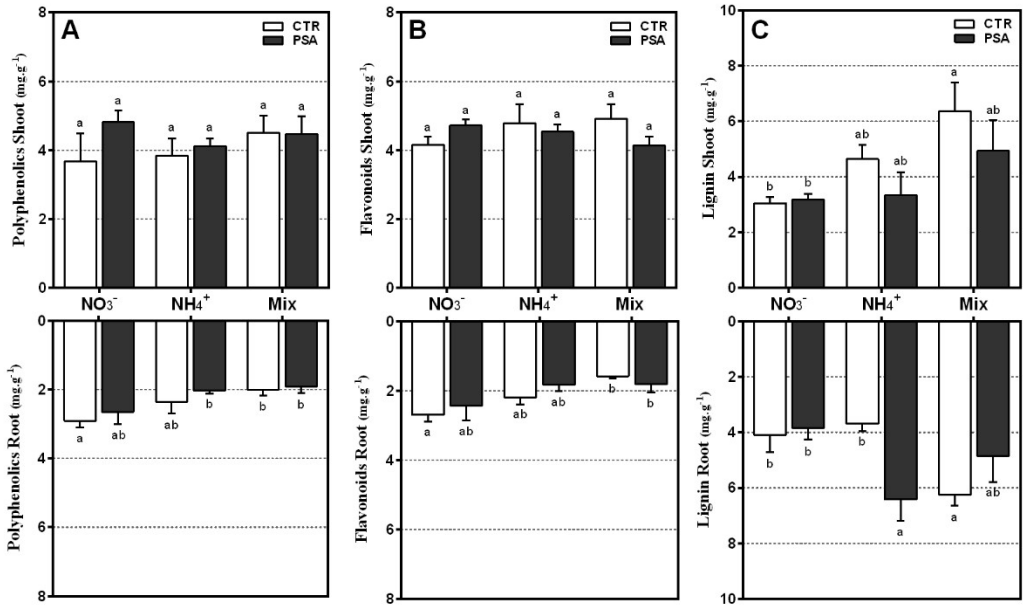


Figure 18: Concentration of Polyphenolics (A), Flavonoids (B) and Lignin (C) ($mg.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of

six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4.2.5 Mineral concentration

Pathogenic invasions can alter plant nutrient composition, since the pathogen itself needs and utilizes these nutrients, thus rendering the host more susceptible due to decreased nutrient availability. On the other hand, nutrients are also involved in plants resistance against pathogens by modulating the expression of defence genes and by altering growth and tissue composition (Gupta *et al*., 2017; Dordas, 2008, Huber and Graham, 1999). On that note, the comparison between values of five macronutrients (Fig.19) and four micronutrients (Fig.20) was evaluated between inoculated and control plants.

N abundance in plants is one of the most important factors influencing disease development, yet, its role seems contradictory because it was shown to increase or decrease disease severity depending on the situation (Engelhard, 1989; Huber and Watson, 1974; Büschbell and Hoffmann, 1992; Marschner, 1995; Hoffland *et al*., 2000). Generally, but not always, facultative fungal parasites and bacterial pathogens thrive with high N content, with higher multiplication and disease severity often associated with K and Ca deficiencies (Kiraly, 1976; Huber and Thompson, 2007). Nevertheless, it is generally the form of N available to the host or pathogen that affects disease severity or resistance rather than the amount of N (Huber and Watson, 1974; Chase 1989; Blachinski *et al.* 1996). In the present work, a significant difference was observed in N concentration in shoots of NO_3^--treated plants, comparing with the other two solutions, in both control and inoculated plants (Fig.19A). This difference was also reported on trial I and is probably due to a slower and more energy cost assimilation of NO_3^-, leading to decreased N accumulation in plant tissues (Haynes and Goh, 1978). However, a 1.3-fold significant difference between NH_4^+ and Mix supplementation was observed among inoculated plants. High levels of N in NH_4^+ treated plants could underpin the higher PSA colonization observed in this treatment, suggesting that this bacterium thrives better with higher N levels. Actually, on a study with tomato plants, it was noted that an increase in the N leaves concentration reaching 2.53 $mol.kg^{-1}$ of dry weight) resulted in a more intensively colonization of *Pseudomonas syringae* pv. *tomato* (Hoffland *et al*., 2000). Gupta *et al*. (2013) also reported that in tobacco plants infected with *Pseudomonas syringae* pv. *phaseolicola* the form of N influenced the HR response, with NO_3^- being associated with greater resistance through NO and SA accumulation in plant tissues, whereas NH_4^+ compromised plant resistance to the pathogen.

P is also an important nutrient for plant metabolism, participating in several biochemical and physiological processes (Prabhu *et al.,* 2007). In disease resistance the role of P is also inconsistent and contradictory, but foliar treatments with phosphate seem to be efficient against several airborne pathogens by conferring local or systemic resistance to the host plant (Huber *et al*., 2012; Gupta *et al*., 2017). In this experiment, P shoot concentrations significantly increased in inoculated plants maintained with NH_4^+ and Mix solutions by 1.4-fold (from 1732.3 ± 160.3 to 2413.6 ± 256.3 $\mu g.g^{-1}$) and 1.6-fold (from 1447.8 ± 124.5 to 2341.5 ± 247.0 $\mu g.g^{-1}$), respectively, in regard to control plants (Fig.19B). On roots, although NH_4^+ treated plants showed a tendency for increase their P values, only significant differences between treatments were observed indicating that PSA inoculation did not give rise to major differences in P content of kiwi plant roots. As PSA was inoculated on the leaves, it was normal that a response from the kiwifruit plants happened on the shoots first. Also, given that P is involved in many metabolic processes, such as energy transfer, and is a component of several organic molecules like ribonucleic acids, the upregulation of P concentrations on inoculated plants treated with NH_4^+ showed that these plants were probably under stress and were trying to activate their metabolic pathways in order to fight their high bacterial colonization (Hawkesford *et al*., 2012; Huber *et al*., 2012).

K concentrations on plant tissues regulates several aspects of plants primary metabolism (Amtmann *et al*., 2008). Symptoms of K deficiency like smaller and shorter roots, thin cell walls and accumulation of sink N, encourage pathogenic attack, however, K addition is only effective in disease control if it alleviates K deficiency (Huber *et al*., 2012). In this work, K values were significantly higher in inoculated plants supplemented with NH_4^+ solution on both shoots (17361 ± 1734 $\mu g.g^{-1}$) and roots (38900 ± 2146 $\mu g.g^{-1}$) compared to control plants (9785 ± 487 and 28581 ± 1069 $\mu g.g^{-1}$, respectively) under the same N treatment, translated in significant differences of 1.8 and 1.4-fold, respectively (Fig.19C). In addition, inoculated plants maintained with Mix solution displayed a significant higher value of K with 14478 ± 387 $\mu g.g^{-1}$ on shoots in regard to control plants, with only 9791 ± 358 $\mu g.g^{-1}$ (i.e. 1.5-fold). K nutrition can also influence hormonal defence pathways at several levels including the jasmonic acid pathway (Amtmann *et al*., 2008). A report by Armengaud *et al.* (2004) showed, on *Arabidopsis*, that K deficiency induces a large number of genes related to jasmonic acid. In recent findings was also observed that elicitation of jasmonic acid pathway resulted on higher PSA colonization on 'Hayward' plants, demonstrating that K deficiency could aggravate KBC by elicitation of jasmonic acid pathway (Pinto, 2018). So, it can be assumed that low concentrations of K on NH_4^+ supplemented plants could have prompted PSA

colonization through accumulation of low-molecular weight organic compounds and by elicitation of the jasmonic acid pathway.

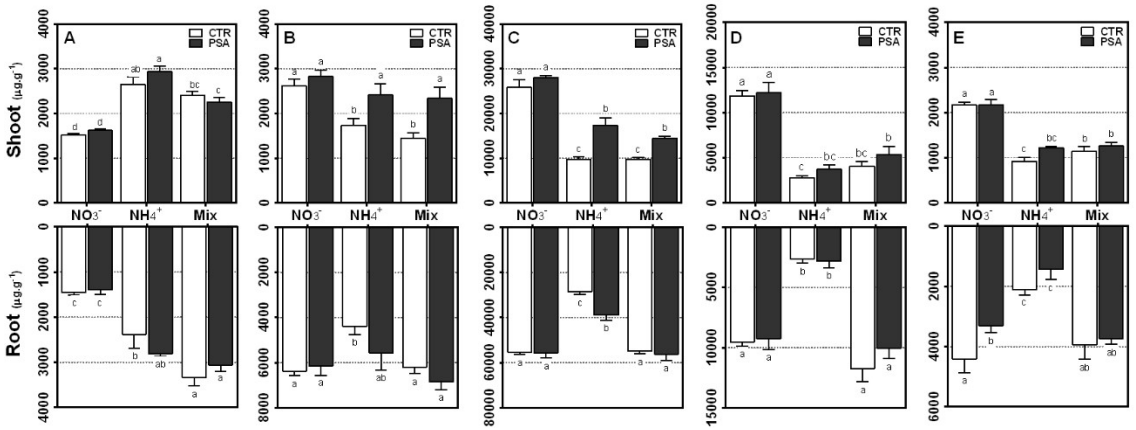


Figure 19: Concentration of macronutrients Nitrogen (A), Phosphorous (B), Potassium (C), Calcium (D) and Magnesium (E) (µg.g^{-1}) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

Ca seems a very important nutrient to plants defence because it is involved on the recognition of invading pathogens as secondary messenger, on the stability of biomembranes, in the inhibition of pectolytic enzymes produced by fungi and bacteria and on the HR against bacterial infections (Huber *et al*., 2012; Gupta *et al*., 2017). In the current work, Ca concentration did not vary significantly between inoculated and control plants, but in plants maintained with NH_4^+ were recorded low values of this nutrient, compared to the other N treatments, possibly due to NH_4^+ competition with and inhibition of cation uptake and translocation, which may have compromised the plants response to the pathogen (Haynes and Goh, 1978; Ashraf and Sultana, 2000; Tsabarducas *et al*., 2017) (Fig.19D). In general, the effect of nutrition on the host plant regarding spread and multiplication of bacterial pathogen, seems to be similar to that observed on facultative fungal parasites, where Ca and K deficiencies lead to an enhanced multiplication of the pathogen and disease severity (Huber *et al*., 2012). Specifically, on tobacco plants, *Pseudomonas syringae* induced hypersensitive reactions require a strong influx of Ca from the apoplast into the cytoplasm through Ca channels in the plasma membrane, demonstrating the importance of Ca in defence responses against this bacteria (Atkinson *et al*., 1990). Yamazaki and Hoshina (1995) also demonstrated an antagonistic effect of increasing Ca concentrations (0, 8 and 16 mM) and disease severity of *Pseudomonas solanacearum* strain MAFF03-01487 on *Lycopersicon esculentum* cv. 'Zuiei' (a moderately resistant cultivar). The same was not observed for the highly susceptible cultivar 'Ponderosa' where the disease development was rapid at all Ca concentrations. Along with the available bibliography exposing the

important role of Ca on plants defence responses it is possible to assume that low values of Ca concentrations expressed on NH_4^+ treated plants could have been one of the factors determining the high PSA bacterial colonization noted on these plants (Yamazaki and Hoshina 1995; Atkinson *et al*., 1990; Huber *et al*., 2012; Gupta *et al*., 2017).

Mg plays important roles in terms of photosynthesis, being part of chlorophyll molecules and by transporting photosynthates through the phloem. Mg deficiency can increase sucrose and amino acids levels in leaves, which may favour the attack of various disease-causing pathogens, including PSA, but little is known of the direct effect of Mg on pathogenic attacks (Gupta *et al*., 2017; Huber *et al*., 2012; Na *et al*., 2014). Also, Mg is involved in resistance against tissue degradation from pathogenic enzymes (Huber and Jones, 2012). In this work, in NO_3^--supplemented plants roots, Mg content was significant lower on inoculated plants with 3308.0 ± 223.4 $\mu g.g^{-1}$ comparing with control plants which had 4415.4 ± 458.4 $\mu g.g^{-1}$ (i.e. 0.3-fold) (Fig.19E). In general, roots and shoots of NH_4^+ treated plants presented significant lower values compared with NO_3^- supplementation as reported on trial I probably from NH_4^+ inhibition of cation translocation and uptake through medium acidification and competition with Mg as with Ca and K cations (Haynes and Goh, 1978; Ashraf and Sultana, 2000; Tsabarducas *et al*., 2017). In fact, a study by Melakeberhan *et al*. 2000 reported that decreasing soil pH (from 5.5 to 3.9) resulted in low availability of Mg and Ca which were associated with predisposition of *Prunus avium* L. 'Mazzard' seedlings to infection by *Pseudomonas syringae* pv. *syringae.* This discovery is in concordance with the present study, once Mg and Ca levels are significant lower on NH_4^+ treated plants and these plants demonstrated a higher susceptibility to PSA.

Nutrition may affect plant resistance or tolerance, but the outcome ultimately depends on the nutrient in question, the general nutritional status of the plant, the plant species and the type of pathogen or pest (Huber *et al*., 2012). On this experiment, macronutrients did not vary greatly between control and inoculated plants, but the already low values of macronutrients in NH_4^+ treated plants may have compromise plant tolerance to bacterial colonization once nutrient deficiency, specially Ca and K, generally aggravates susceptibility to bacterial pathogens (Huber *et al*., 2012; Gupta *et al*., 2017). Additionally, plant inoculation with PSA seemed to enable P and K accumulation in NH_4^+ treated plant, which may demonstrate that these plants were under stress due to high bacterial colonization, thus trying to activate defensive pathways dependent on P, related to energy transfer, and K, responsible for enzyme activation (Hawkesford *et al*., 2012).

Micronutrients are involved in almost all metabolic and cellular functions during normal plant development and are, in general, linked to disease resistance by maintenance of cell membranes integrity and cell wall rigidity and their role on generation and detoxification of oxygen radicals and hydrogen peroxide (Marschner, 1995; Huber *et al*. 2012; Gupta *et al*., 2017).

Micronutrient B confers rigidity to cell walls and has an important role on phenol metabolism as well as on lignin synthesis, being widely used against wood decay fungi (Huber *et al*., 2012; Gupta *et al*., 2017). B showed no significant differences between control and inoculated plants, but significant differences were observed between shoots of plants treated with NH_4^+ and those maintained with Mix solution, as anticipated by the previous trial, both in inoculated and control plants (Fig.20A). Although a slightly higher value of B was recorded on NH_4^+ plant shoots in comparison with NO_3^- and a significant higher values in comparison to Mix solution, NH_4^+ plants did not obtained the defense benefit provided by this micronutrient since these plants did not show higher phenolic compound concentrations on the shoots (Fig.18). This may be due to the fact that the concentration of phenolics is reduced at high N supply and plants treated with NH_4^+ presented the higher N concentrations (Gupta *et al*., 2017) (Fig 19A).

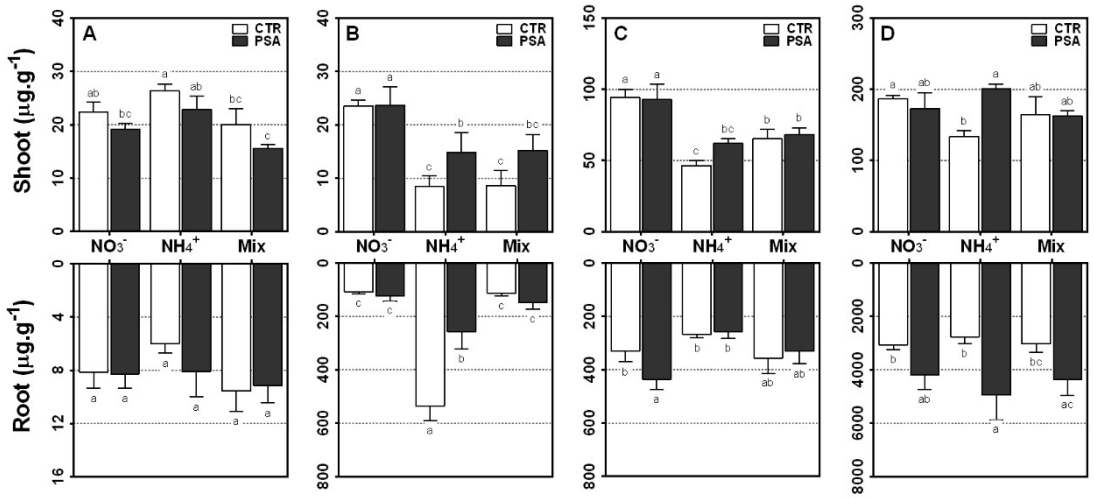


Figure 20: Concentration of micronutrients Boron (A), Zinc (B), Manganese (C) and Iron (D) ($\mu g.g^{-1}$) on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) for 36 days with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

A significant decrease of 2.1-fold was observed in Zn concentration between roots of NH_4^+-treated plants inoculated and control plants of, while on shoots only a tendency for higher values of Zn on inoculated plants supplemented with NH_4^+ and Mix solutions was found (Fig.20B). Despite the decrease of Zn concentration in roots, both control and inoculated plants treated with NH_4^+ exhibited significantly higher values (535.8 ± 54.8

and 255.8 ± 66.0 μg.g^{-1}, respectively) compared to the other treatments, while in shoots, the highest values were recorded for the NO_3^- treated plants (23.6 ± 1.2 and 23.6 ± 3.5 μg.g^{-1}, respectively). Zn plays an important role in enzyme activation and membrane integrity, and so, Zn deficiency can cause membrane leakage of low-molecular-weight compounds, providing a more suitable environment for pathogens growth and development (Marschner 1995; Huber *et al*. 2012; Gupta *et al*., 2017). In fact, a study in *Hevea brasiliensis*, showed that Zn deficiency, with subsequent increased leakage of sugars to the leaf surface, increased the severity of infection by *Oidium* spp. (Bolle-Jones and Hilton, 1956).

Mn plays an important role in biosynthesis of lignin and phenol compounds and although Mn fertilization can alter disease resistance, its use is restricted due to the ineffectiveness and weak residual effect of Mn fertilizers on most soils (Gupta *et al*., 2017). Roots of inoculated plants treated with NO_3^- exhibited significantly higher Mn concentration (436.6 ± 36.4 μg.g^{-1}) in comparison with control plants subjected to the same treatment (329.2 ± 40.5 μg.g^{-1}) and to inoculated plants treated with NH_4^+ (256.0 ± 26.4 μg.g^{-1}), which represents s difference of 1.3-fold and 1.7-fold, respectively (Fig.20C). Additionally, NO_3^- supplementation resulted in significantly higher Mn concentration in shoots, compared with the remaining treatments, as was also observed in Trial I. Indeed, several studies have already correlated Mn with decreased disease severity, including in tomato plants infected with *P. syringae* (Conlin and McCarter, 1983; Heckman *et al*., 2003; Gupta *et al*., 2017).

Inoculation of plants treated with NH_4^+ resulted in higher amounts of Fe, reaching values of 200.8 ± 6.8 μg.g^{-1} in shoots and 4940.2 ± 926.3 μg.g^{-1} in roots, which represents a 1.5-fold and 1.8-fold increase in comparison with control plants of the same N treatment (Fig.20D). Iron has a dual effect in plants defence because it can create dangerous free radicals, but at the same time the plant host can use Fe to increase local oxidative stress in defence responses against pathogens or use it as a constituent of defensive cell wall appositions (Greenshields *et al*., 2007; Gupta *et al*., 2017).

Similarly to what was observed for macronutrients, in NH_4^+ treated plants several changes were observed regarding micronutrient concentrations with inoculation of PSA. Zn translocation from roots to shoots and higher Fe concentration was observed in NH_4^+ supplemented plants. As Zn is a key player in enzyme activation, protein synthesis and carbohydrate metabolism, and Fe is required for the activation of several enzymes, especially those from redox system , it is possible that NH_4^+ treated plants tried to activate enzymatic and other defence pathways in order to restrain PSA colonization. (Broadley *et al*., 2012)

4.2.6 Protein concentration

Protein concentrations on plants tissues respond to environmental cues, including pathogenic attacks. The interactions between planta/pathogen creates several proteomic changes in both organisms and their study, during the systemic infection, enables a better understanding of the essential pathways and key effectors assisting pathogen colonization. These aspects are particularly important for kiwifruit production upon the current challenge posed by PSA (Petriccione *et al.* 2013a, 2013b).

Regarding Trial II, a significant difference in protein content between shoots of control and inoculated plants with NH_4^+ supplementation was observed (Fig.21). Under this treatment PSA-inoculated plants showed 22.8 ± 1.0 % protein, which represents a 1.1-fold increment compared with control plants. In addition, in most cases, protein content was significantly different between N treatments, both in roots and in shoots, with NH_4^+ plants showing the highest values (22.8 ± 1.0 and 29.4 ± 1.5 %), as already observed in Trial I due to the rapid assimilation of NH_4^+ into organic compounds (Atanasova, 2008; Horchani *et al*., 2010).

Increased protein content in inoculated NH_4^+ treated plants shoots, could fit the hypothesis made by the increased concentrations of P, Fe and Zn, where these plants were under biotic stress and, for that reason, were trying to activate their metabolic and defence signalling pathways. It also goes along with the high concentration of N on these plants. In fact, the studies of Petriccione *et al.* (2013a and 2013b) on kiwifruit, focused on the proteomic approach to investigate *Actinidia* and PSA pathosystem. They reported 117 differentially represented protein spots only on *A.chinensis* shoot before and after the systemic infection. Among this 117 differentially represented gene products, proteins related to plant protection, including PR polypeptides, or components involved in basal protection, oxidative stress, heat shock, and related transport and signaling processes, were characterized as the most represented category of induced species (Petriccione *et al.* 2013a and 2013b).

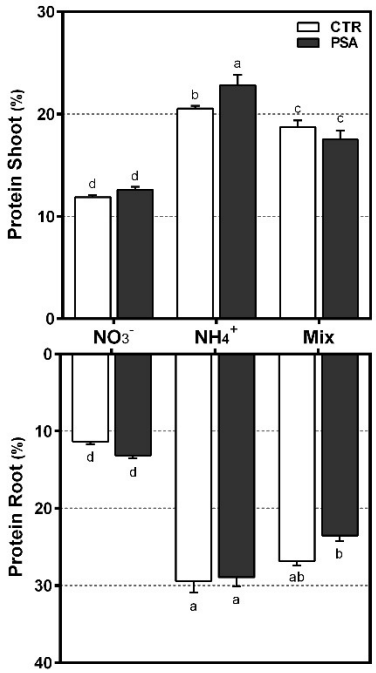


Figure 21: Percentage of protein on shoots and roots of *A. chinensis* var. *deliciosa* cv. 'Hayward' plants maintained in hydroponic solutions with 3 ppm NO_3^-, 3 ppm NH_4^+ or 1.5 ppm NO_3^- + 1.5 ppm NH_4^+ (Mix) with inoculation (PSA) and without inoculation (CTR). Each bar represents the mean of six biological replicates and vertical lines the standard error. Bars showing different letters are statistically different at $p < 0.05$.

4 Conclusions and future perspectives

The results from this study indicated that fertilization with NO_3^- seemed to present better effects on 'Hayward' plants both in general development, with higher values of biomass, height, root length and mineral concentrations, and on tolerance to PSA, displaying lower bacterial colonization compared to NH_4^+ treated plants.

The rapid NH_4^+ assimilation of NH_4^+ treated plants resulted on higher N values, which may be a key factor for stronger PSA colonization in these plants, referring to the possibility that PSA infection is reinforced in the presence of greater concentrations of this nutrient. Also, the lower concentrations of important nutrients for plants defence (Ca, Mg and K), as a possible result of environment acidification of NH_4^+ assimilation or from competitive uptake cues between NH_4^+ and these cations, could explain the greater disease severity showed by plants supplemented with NH_4^+.

Uptake of minerals related to plants metabolism, enzyme activation and defence like P, K, Zn and Fe were regulated on NH_4^+ inoculated plants along with protein concentration probably due to the stress induced by higher disease severity, in other words, these plants seemed to activate several metabolic pathways in response to the high bacterial colonization.

In the future, it would be important to explore the genetic regulation that leads to a lower susceptibility to PSA in NO_3^--treated plants, such as antioxidant defence related genes and genes involved in the hypersensitive response, and to test individually the altered minerals upon inoculation from the NH_4^+-treated plants to ascertain their role in the kiwifruit plant.

5 List of references

Abelleira A, Ares A, Aguin O, Peñalver J, Morente M, López M, Sainz M, Mansilla J (2015) Detection and characterization of *Pseudomonas syringae* pv. *actinidifoliorum* in kiwifruit in Spain. Journal of Applied Microbiology, **119**:1659-1671;

Abelleira A, López M, Peñalver J, Aguín O, Mansilla J, Picoaga A, García M (2011) First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Spain. Plant Disease, **95**(12): 1583-1583;

Adams F (1980) Interactions of Phosphorus with Other Elements in Soil and Plants. In FE Khasawneh, EC Sample, EJ Kamprath, eds, The Role of Phosphorus in Agriculture. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp 655-680;

Agrios NG (2005) Plant pathology, Ed 5. Elsevier Academic Press, New York, 922p.;

Alcoz MM, Hons FM, Haby VA (1993) Nitrogen Fertilization Timing Effect on Wheat Production, Nitrogen Uptake Efficiency, and Residual Soil Nitrogen. Agronomy Journal, **85**(6): 1198-1203;

Armengaud P, Breitling R, Amtmann A (2004) The potassium-dependent transcriptome of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. Plant Physiology, **136**: 2556–2576;

Arnon DI and Stout PR (1939) The Essentiality of Certain Elements in Minute Quantity for Plants with Special Reference to Copper. Plant Physiology, **14**:371-375;

Ashraf M and Sultana R (2000) Combination Effect of NaCl Salinity and Nitrogen Form on Mineral Composition of Sunflower Plants. Biologia Plantarum, **43**(4): 615–619;

Atanasova E (2008) Effect of nitrogen sources on the nitrogenous forms and accumulation of amino acid in head cabbage. Plant, Soil and Environment, **54**(2): 66-71;

Atkinson MM, Koppler LD, Orlandi EW, Baker CJ, Mischke CF (1990) Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco. Plant Physiology, **92**: 215–221;

Amtmann A, Troufflard S, Armengaud P (2008) The effect of potassium nutrition on pest and disease resistance in plants. Physiologia Plantarum, **133**(4): 682–691;

Balestra GM, Mazzaglia A, Quattrucci A, Renzi M, Rossetti A (2009a) Current status of bacterial canker spread on kiwifruit in Italy. Australasian Plant Disease Notes, **4**:34–36;

Balestra GM, Mazzaglia A, Quattrucci A, Renzi M, Rossetti A (2009b) Occurrence of *Pseudomonas syringae* pv. *actinidiae* in Jin Tao kiwi plants in Italy. Phytopathologia Mediterranea, **48**(2): 299-301;

Balestra GM, Renzi M, Mazzaglia A (2010) First report of bacterial canker of *Actinidia* deliciosa caused by *Pseudomonas syringae* pv. *actinidiae* in Portugal. New Disease Reports **22**:10;

Bastas KK (2014) Effects on tomato bacterial canker of resistance inducers and copper compounds in greenhouse**.** Selcuk Journal of Agriculture and Food Sciences, **28:**1–10;

Bateman DF and Lumsden RD (1965) Relation between calcium content and nature of the peptic substances in bean hypocotyles of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology, **55**: 734–738;

Berry SZ, Madumadu GG, Uddin MR (1988) Effect of calcium and nitrogen nutrition on bacterial cancer disease of tomato. Plant Soil, **112**(1): 113–120;

Blachinski D, Shtienberg D, Dinoor A, Kafkafi U, Sujkowski LS, Zitter TA, Fry WE (1996) Influence of foliar application of nitrogen and potassium on Alternaria diseases in potato, tomato and cotton. Phytoparasitica, **24**:281–292;

Blair GJ, Miller MH, Mitchell WA (1970) Nitrate and ammonium as sources of nitrogen for corn and their influence on the intake of other ions. Agronomy Journal, **62**: 530-532;

Bolle-Jones EW and Hilton RN (1956) Zinc-deficiency of *Hevea braziliensis* as a predisposing factor to *Oidium* infection. Nature (London) **177**: 619–620;

Borgognone D, Colla G, Rouphael Y, Cardarelli M, Rea E, Schwarz D (2013) Effect of nitrogen form and nutrient solution pH on growth and mineral composition of self-grafted and grafted tomatoes. Scientia Horticulturae, **149**: 61–69;

Bourrain, L (2018) In vitro propagation of *Actinidia melanandra* Franch. and *Actinidia rubricaulis* Dunn. from shoot tip explants. New Zealand journal of crop and horticultural science, **46**: 162-173;

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F (2012) Function of Nutrients: Micronutrients. In P Marschner eds, Marschner's Mineral Nutrition of Higher Plants, Ed 3. Academic Press, pp 135–189;

Büschbell T, Hoffmann GM (1992) The effects of different nitrogen regimes on the epidemiologic development of pathogens on winterwheat and their control. Journal of Plant Diseases and Protection **99**: 381–403;

Cameron A, Sarojini V (2014) *Pseudomonas syringae* pv. *actinidiae*: chemical control, resistance mechanisms and possible alternatives. Plant pathology **63**(1): 1-11;

Cameron A, Zoysa GHD, Sarojini V (2014) Antimicrobial peptides against *Pseudomonas syringae* pv. *actinidiae* and *Erwinia amylovora*: Chemical synthesis, secondary structure, efficacy, and mechanistic investigations. Peptide Science **102**(1): 88-96;

Calonnec A, Burie J-B, Langlais M, Guyader S, Saint-Jean S, Sache I, Tivoli B (2012) Impacts of plant growth and architecture on pathogen processes and their consequences for epidemic behavior. European Journal of Plant Pathology, **135**(3), 479–497;

Cao P, Lu C, Yu Z (2018) Historical nitrogen fertilizer use in agricultural ecosystems of the contiguous United States during 1850–2015: application rate, timing, and fertilizer types. Earth System Science Data, **10**: 969-984;

Cazzonelli CI and Pogson BJ (2010) Source to sink: regulation of carotenoid biosynthesis in plants. Trends in Plant Science, **15**(5): 266–274;

Cellini A, Fiorentini L, Buriani G, Yu J, Donati I, Cornish D, Novak B, Costa G, Vanneste J, Spinelli F (2014) Elicitors of the salicylic acid pathway reduce incidence of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidae*. Annals of Applied Biology **165**(3): 441-453;

Chapman JR, Taylor RK, Weir BS, Romberg MK, Vanneste JL, Luck J, Alexander BJR (2012) Phylogenetic relationships among global populations of *Pseudomonas syringae* pv. *actinidiae*. Phytopathology **102**(11): 1034-1044;

Chase AR (1989) Effect of nitrogen and potassium fertilizer rates on severity of *Xanthomonas* blight of *Syngonium podophyllum*. Plant Disease, **73**:972–975**;**

Ciarroni S, Gallipoli L, Taratufolo MC, Butler MI, Poulter RT, Pourcel C, Vergnaud G, Balestra GM, Mazzaglia A (2015) Development of a multiple loci variable number of tandem repeats analysis (MLVA) to unravel the intra-pathovar structure of *Pseudomonas syringae* pv. *actinidiae* populations worldwide**.** PLOS ONE **10**(8): e0135310;

Colombi E, Straub C, Künzel S, Templeton MD, McCann HC, Rainey PB (2017) Evolution of copper resistance in the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* through acquisition of integrative conjugative elements and plasmids. Environmental Microbiology **19**(2), 819–832;

Conlin KC and McCarter SM (1983) Effectiveness of selected chemicals in inhibiting *Pseudomonas syringae* pathovar tomato in-vitro and in controlling bacterial speck. Plant Disease, **67**: 639-644;

Costa G, Donati I, Mauri S, Graziani S, Cellini A, Buriani G, Fiorentini L, Rocchi L, Giacomuzzi V, Spinelli F (2013) Influence of orchard management on kiwifruit bacterial disease. Proceedings of 1st International Symposium on Bacterial Canker of Kiwifruit (Psa). Mt Maunganui, New Zealand;

Costa G, Kukuriannis B, Monet R (1992) KIWIFRUIT PRODUCTION IN EUROPE. Acta Horticulturae, **297**: 141–150;

Costa G, Lain O, Vizzotto G, Johnson S (1997) Effect of nitrogen fertilization on fruiting and vegetative performance, fruit quality and post-harvest life of kiwifruit cv hayward. Acta Horticulturae, **444**: 279–284;

Cunnac S, Lindeberg M, Collmer A (2009) *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. Current Opinion in Microbiology**,** **12**(1): 53–60;

Cunty A, Poliakoff F, Rivoal C, Cesbron S, Fischer-Le Saux M, Lemaire C, Jacques MA, Manceau C, Vanneste JL (2014) Characterisation of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolated from France and assignment of Psa biovar 4 to a de novo pathovar: *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov. Plant Pathology, **64**(3): 582-596;

Datson P, Nardozza S, Manako K, Herrick J, Martinez-Sanchez M (2015) Monitoring the *Actinidia* germplasm for resistance to *Pseudomonas syringae* pv. *Actinidiae.* Acta Horticulturae **1095**: 181–184;

Demmig-Adams Band Adams WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends in Plant Science, **1**(1): 21–26;

DGAV (2013). Plano de ação nacional para o controlo da *Pseudomonas syringae* pv.*actinidiae* do kiwi (PSA). 24pp;

Di Lallo G, Evangelisti M, Mancuso F, Ferrante P, Marcelletti S, Tinari A, Superti F, Migliore L, D'Addabbo P, Frezza D (2014) Isolation and partial characterization of bacteriophages infecting *Pseudomonas syringae* pv. *actinidiae*, causal agent of kiwifruit bacterial canker. Journal of basic microbiology, **54**(11): 1210-1221;

Dietrich R, Ploss K, Heil M (2004). Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. Plant, Cell and Environment **27**(7): 896–906;

Dinev N and Stancheva I (1995) Response of wheat and maize to different nitrogen sources: I. Plant growth and biomass accumulation. Journal of Plant Nutrition, **18**(6): 1273–1280;

Ditommaso A and Watson AK (1995). Impact of a fungal pathogen, *Colletotrichum coccodes* on growth and competitive ability of *Abutilon theophrasti*. New Phytologist, **131**(1): 51–60;

Dodds WK and Whiles MR (2010) Nitrogen, Sulfur, Phosphorus, and Other Nutrients. In: WK Dodds and MR Whiles eds, Freshwater Ecology: Concepts and Environmental Applications of Limnology, Ed 2. Academic Press, pp 345-373;

Donati I, Buriani G, Cellini A, Mauri S, Costa G, Spinelli F (2014) New insights on the bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*). Journal of Berry Research **4**(2): 53-67;

Dordas C (2008) Role of nutrients in controlling plant diseases in sustainable agriculture. A review. Agronomy for Sustainable Development **28**: 33-46;

Durner J, Klessig DF (1999) Nitric oxide as a signal in plants. Current Opinion in Plant Biology **2**: 369–374;

Engelhard AW (1989) Soilborne Plant Pathogens: Management of Diseases with Macro-and Microelements St. Paul, MN: The American Phytopathological Society Press, 217 pp.;

EPPO (2018) European and Mediterranean Plant Protection Organization GlobalDatabase. Reporting Service Articles, https://gd.eppo.int/taxon/PSDMAK/reporting

Everett KR, Taylor RK, Romberg MK, Rees-George J, Fullerton RA, Vanneste JL, Manning MA (2011) First report of *Pseudomonas syringae* pv. *actinidiae* causing kiwifruit bacterial canker in New Zealand. Australasian Plant Disease Notes **6**: 67–71;

Fageria VD (2001) Nutrient interactions in crop plants. Journal of Plant Nutrition, **24**(8): 1269-1290;

FAOSTAT (2019) Food and Agriculture Organization of the United Nations, http://www.fao.org/faostat/en/#home (Accessed 2019-07-26);

Fawe A, Menzies JG, Chérif M, Bélanger RR (2001) Silicon and disease resistance in dicotyledons. In: Datnoff LE, Snyder GH, Korndörfer GH eds, Silicon in Agriculture, 1Ed Vol 8. Elsevier Science, New York, pp 159-169;

Félix A, Cavaco M (2004) Caracterização do ecossistema agrário da cultura da actinídea (*Actinidia deliciosa* A. CHEV) (*Actinidiaceae*). *VII-Encontro Nacional de Protecçao Integrada, 1-8*;

Ferguson AR (1984) Kiwifruit: A botanical review. Horticultural Reviews, **6**: 1–64;

Ferguson AR (2013) Kiwifruit: the wild and the cultivated plants. Advances in Food and Nutrition Research, **68**:15–32;

Ferguson AR, Huang H (2007) Genetic resources of kiwifruit: domestication and breeding. Horticulture Reviews, **33**:1–121;

Ferrante P, Scortichini M (2009) Identification of *Pseudomonas syringae* pv. *actinidiae* as causal agent of bacterial canker of yellow kiwifruit (*Actinidia chinensis Planchon*) in central Italy. Journal of Phytopathology **157**:768–770;

Ferrante P, Scortichini M (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. Plant Pathology **59**(5), 954–962;

Ferrante P, Scortichini M (2014). Redefining the global populations of *Pseudomonas syringae* pv. *actinidiae* based on pathogenic, molecular and phenotypic characteristics. Plant Pathology **64**: 51–62;

Forde BG (2002) Local and long-range signaling pathways regulating plant responses to nitrate. Annual Review of Plant Biology **53**: 203–224;

Frampton RA, Taylor C, Moreno AVH, Visnovsky SB, Petty NK, Pitman AR, Fineran PC (2014) Identification of bacteriophages for the biocontrol of the kiwifruit canker phytopathogen *Pseudomonas syringae* pv. *actinidiae*. Applied and environmental microbiology, **80**(7): 2216–2228;

Franco J (2008) História e Desenvolvimento Comercial. In Antunes D eds., Kiwi: da produção à comercialização. Universidade do Algarve, 13-19;

Freeman BC, Beattie GA (2008) An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor, 1-14;

Fujikawa T, Sawada H (2016). Genome analysis of the kiwifruit canker pathogen *Pseudomonas syringae* pv. *actinidiae* biovar 5. Scientific Reports **6**(1);

Garbin LM; Dillenburg RL (2008) Effects of different nitrogen sources on growth, chlorophyll concentration, nitrate reductase activity and carbon and nitrogen distribution in *Araucaria angustifoli.* Brazilian Journal of Plant Physiology, **20**(4): 1677-9452;

Gardan L, Shafik H, Belouin S, Brosch R,Grimont F, Grimont PAD (1999) DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). International Journal of Systematic Bacteriology, **49**: 469–78;

Gerendás J, Zhu ZJ, Bendixen R, Ratcliffe RG, Sattelmacher B (1997) Physiological and biochemical processes related to ammonium toxicity in higher plants, Zeitschrift für Pflanzenernährung und Bodenkunde, **160**(2): 239–251;

Gowariker V, Krishnamurthy VN, Gowariker S, Dhanorkar M, Paranjape K (2009) The Fertilizer Encyclopedia, Ed 1. Wiley, pp 880;

Graham DR (1983) Effects of nutrients stress on susceptibility of plants to disease with particular reference to the trace elements. Advances in Botanical Research**, 10**:221–276;

Greenshields DL, Liu G, Wei Y (2007) Roles of Iron in Plant Defence and Fungal Virulence. Plant Signal Behaviour, **2**(4): 300–302;

Gupta KJ, Brotman Y, Segu S, Zeier T, Zeier J, Persijn S, **Cristescu SM, Harren FJ, Bauwe H, Fernie AR, Kaiser WM, Mur LA** (2013) The form of nitrogen nutrition affects resistance against *Pseudomonas syringae* pv. *phaseolicola* in tobacco. Journal of Experimental Botany **64**(2): 553-68;

Gupta N, Debnath S, Sharma S, Sharma P, Purohit J (2017) Role of Nutrients in Controlling the Plant Diseases in Sustainable Agriculture. In: Meena V., Mishra P., Bisht J., Pattanayak A. eds, Agriculturally Important Microbes for Sustainable Agriculture, 1Ed Vol.28. Springer, Singapore 33–46;

Hackett C (1972) A method of applying nutrients locally to roots under controlled conditions and some morphological effects of locally applied nitrate on the branching of wheat roots. Australian Journal of Biological Science, **25**: 1169-1180;

Hakulinen J, Julkunen-Tiitto R, Tahvanainen J (1995) Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? Trees, **9**: 235-240;

Han HS, Nam HY, Koh YJ, Hur J-S, Jung JS (2003) Molecular bases of high-level streptomycin resistance in Pseudomonas marginalis and *Pseudomonas syringae* pv. *actinidiae*. The Journal of Microbiology **41**(1), 16-21;

Hanley Z (2018) Kiwifruit (*Actinidia* spp.) Breeding. In J Al-Khayri, S Jain, D Johnson eds, Advances in Plant Breeding Strategies: Fruits, Vol. 3. Springer, Cham, pp 377-401;

Hawkesford M, Horst W, Kichey T, Lambers H, Schjoerring J, Møller IS, White P (2012) Functions of Macronutrients. In P Marschner eds, Marschner's Mineral Nutrition of Higher Plants, Ed 3. Academic Press, pp 135–189;

Haynes RJ and Goh KM (1978) Ammonium and nitrate nutrition of plants. Biological Reviews, **53**: 465-510;

Heckman JR, Clarke BB, Murphy JA (2003) Optimizing Manganese Fertilization for the Suppression of Take-All Patch Disease on Creeping Bentgrass. Crop Science, **43**(4): 1395-1398;

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Current Opinion in Plant Biology, **4**:210–218;

Hoffland E, Jeger MJ, van Beusichem ML (2000) Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. Plant and Soil, **218** (1/2): 239–247;

Hoffmann C, Ladewig E, Claassen N, Jungk A (1994) Phosphorus uptake of maize as affected by ammonium and nitrate nitrogen - Measurements and model calculations -. Journal of Plant Nutrition and Soil Science, **157**(3): 225–232;

Holmes A (2012) Effect of Soil Nutrition and Composition on the Susceptibility of Hayward and Hort16A to *Pseudomonas syringae* pv. *Actinidiae*. ZESPRI Group, Tauranga, New Zeland, 81pp;

Hoopen FT, Cuin TA, Pedas P, Hegelund JN, Shabala S, Schjoerring JK, Jahn TP (2010). Competition between uptake of ammonium and potassium in barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences. Journal of Experimental Botany, **61**(9): 2303–2315;

Hopping ME (1990) Floral biology, pollination, and fruit set. In IJ Warrington, GC Weston eds., Kiwifruit: Science and management. Ray Richards Publisher and New Zealand Society for Horticultural Science, Auckland, pp 71–96;

Horchani F, Hajri R, Aschi-Smiti S (2010) Effect of ammonium or nitrate nutrition on photosynthesis, growth, and nitrogen assimilation in tomato plants. Journal of Plant Nutrition and Soil Science, **173**(4): 610–617;

Howard DD, Chambers AY, Logan J (1994) Nitrogen and fungicide effects on yield components and disease severity in wheat. Journal of Production Agriculture, **7**: 448–454.

Huang H (2016) Kiwifruit: The Genus *ACTINIDIA*. Academic Press, 350p;

Huber DM and Jones JB (2012). The role of magnesium in plant disease. Plant and Soil, **368**(1-2), 73–85;

Huber D, Römheld V, Weinmann M (2012) Relationship between Nutrition, Plant Diseases and Pests. In P Marschner eds, Marschner's Mineral Nutrition of Higher Plants, Ed 3. Academic Press, pp 283-298;

Huber DM and Thompson IA (2007) Nitrogen and plant disease. In LE Datnoff, WH Elmer, DM Huber eds, Mineral Nutrition and Plant Disease Vol 55. APS Press, St. Paul, Minnesota, USA, pp 31–44.;

Huber DM and Graham RD (1999) The role of nutrition in crop resistance and tolerance to disease, In: Rengel Z eds, Mineral nutrition of crops fundamental mechanisms and implications. Food Product Press, 205–226;

Huber DM and Jones JB (2012). The role of magnesium in plant disease. Plant and Soil, **368**(1-2): 73–85;

Huber DM and Watson RD (1974) Nitrogen form and plant disease. Annual Review of Phytopathology, **12**:139–165;

Ibrahim MH, Jaafar HZE, Rahmat A, Rahman ZA (2011) Effects of Nitrogen Fertilization on Synthesis of Primary and Secondary Metabolites in Three Varieties of Kacip Fatimah (*Labisia Pumila* Blume). International Journal of Molecular Sciences, **12**(8): 5238–5254;

INE (2019) Estatísticas Agrícolas 2018. Instituto Nacional de Estatística, https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main (2019-07-26);

Jiang C-D, Gao H.-Y, Zou Q, Jiang G-M, Li L-H (2006) Leaf orientation, photorespiration and xanthophyll cycle protect young soybean leaves against high irradiance in field. Environmental and Experimental Botany, **55**(1-2): 87–96;

Jones C and Jacobsen J (2009) Module 11: Fertilizer Placement and Timing. Modules of Nutrient Management Self-Study Course. Montana State University, pp 1-16;

Jones CG and Hartley SE (1999) A protein competition model of phenolic allocation. Oikos, **86**: 27–44;

Kawasaki T (1995) Metabolism and Physiology of Calcium and Magnesium. In T Matsuo, K Kumazawa, R Ishii, K Ishihara, H Hirata eds, Science of the Rice Plant, Vol 2. Food and Agricultural Policy Research Center, Tokyo, Japan, pp 412-419;

Kiiski H, Dittmar H, Drach M, Vosskamp R, Trenkel ME, Gutser R, Steffens G (2016b) Fertilizers, 2. Types. In Wiley VCH eds, Ullmann's Encyclopedia of Industrial Chemistry, pp 1–53;

Kiiski H, Scherer HW, Mengel K, Kluge G, Severin K (2016a) Fertilizers, 1. General. In Wiley VCH eds, Ullmann's Encyclopedia of Industrial Chemistry, pp 1–30;

Kiraly Z (1976) Plant disease resistance as influenced by biochemical effects of nutrients and fertilizers. In: Proceedings of colloquium 12, fertilizer use and plant health. International Potash Institute, Atlanta, pp 33–46;

Kirkby E (2012). Introduction, Definition and Classification of Nutrients. In P Marschner eds, Marschner's Mineral Nutrition of Higher Plants Ed 3. Academic Press, pp 3–5;

Kisaki G, Tanaka S, Ishihara A, Igarashi C, Morimoto T, Hamano K, Endo A, Sugita-Konishi S, Tabuchi M, Gomi K, *et al.* (2018) Evaluation of various cultivars of *Actinidia* species and breeding source *Actinidia rufa* for resistance to *Pseudomonas syringae* pv. *actinidiae* biovar 3. Journal of General Plant Pathology **84**(6): 399-406;

Koh HS, Kim GH, Lee YS, Koh YJ, Jung JS (2014) Molecular characteristics of *Pseudomonas syringae* pv. *actinidiae* strains isolated in Korea and a multiplex PCR assay for haplotype differentiation. Plant Pathology Journal, **30**: 96-101;

Koh YJ, Kim GH, Jung JS, Lee YS, Hur JS (2010) Outbreak of bacterial canker on Hort16A (*Actinidia chinensis* Planchon) caused by *Pseudomonas syringae* pv. *actinidiae* in Korea. New Zealand Journal of Crop and Horticultural Science **38**:275-282;

Koh YJ, Park S, Lee D (1996) Characteristics of bacterial canker of kiwifruit occurring in Korea and its control by trunk injection. Korean Journal of Plant Pathology **12**: 324–30;

Koricheva J, Larsson S, Haukioja E, Keinanen M (1998) Regulation of woody plant secondary metabolism by resource availability: Hypothesis means by meta-analysis. Oikos, **83**: 212–226;

Kulbat K (2016) The role of phenolic compounds in plant resistance Ed 2 Vol 8. Institute of General Food Chemistry, Lodz University of Technology, Wolczanska, pp 97-108;

Lastdrager J, Hanson J, Smeekens S (2014) Sugar signals and the control of plant growth and development. Journal of Experimental Botany, **65**(3), 799–807;

Lattanzio V (2013) Phenolic compounds – Introduction, In KG Ramawat, JM Merillon eds, Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes. Springer, Berlin, Heidelberg, pp 1543-1580;

Lee JH, Kim JH, Kim GH, Jung JS, Hur J-S, Koh YJ (2005) Comparative analysis of Korean and Japanese strains of *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker of kiwifruit. Plant Pathology Journal **21**: 119–26;

Lemon C (1993). *Anatomy and Histochemistry of the Root System of the Kiwifruit Vine, Actinidia deliciosa var. deliciosa.* Annals of Botany, **71**(2): 117–129;

Ling Q, Huang W, Jarvis P (2010) Use of a SPAD-502 meter to measure leaf chlorophyll concentration in Arabidopsis thaliana. Photosynthesis Research, **107**(2): 209–214;

Lionakis SM, Schwabe WW (1985) Some effects of mineral nutrient deficiencies on the growth of *Actinidia chinensis* Planch. Journal of horticultural Sciences, **60**: 411–422;

Liu Y, Lai N, Gao K, Chen F, Yuan L, Mi G (2013) Ammonium Inhibits Primary Root Growth by Reducing the Length of Meristem and Elongation Zone and Decreasing Elemental Expansion Rate in the Root Apex in Arabidopsis thaliana. PLoS ONE, **8(**4), e61031;

Lu YX, Li CJ, Zhang FS (2005) Transpiration, potassium uptake and flow in Tobacco as affected by nitrogen forms and nutrient levels. Annals of Botany, **95**(6), 991–998;

Magalhaes JR and Wilcox GE (1983). Tomato growth and mineral composition as influenced by nitrogen form and light intensity. Journal of Plant Nutrition, **6**(10): 847–862;

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London, p. 889;

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany, **105**(7): 1141–1157;

Mauri S, Cellini A, Buriani G, Donati I, Costa G, Spinelli F (2016) Optimization of cultural practices to reduce the development of *Pseudomonas syringae* pv. *actinidiae*, causal agent of the bacterial canker of kiwifruit. Journal of Berry Research, **6**(4): 355–371;

McCrimmon JN, Karnok KJ, Mills HA (1993) Effect of nitrogen-form on plant growth and nutrient composition of creeping bentgrass, In: MAC Fragoso, ML Van Beusichem, A Houwers eds, Optimization of Plant Nutrition, Vol 53. Developments in Plant and Soil Sciences, Springer, Dordrecht, pp 291-297;

McCrimmon JN, Mills HA, Karnok KJ (1992) Effect of N-form on growth and nutrient content of creeping bentgrass1. Journal of Plant Nutrition, **15**(8): 1235–1252;

Melakeberhan H, Jones AL, Bird GW (2000) Effects of soil pH and *Pratylenchus penetranson* the mortality of "Mazzard" cherry seedlings and their susceptibility to *Pseudomonas syringaepv.syringae*. Canadian Journal of Plant Pathology, **22**(2): 131–137.

Miao L, Genjia T, Yao L, Kejian D, Zhulong C, Yun L (2009) Relationships between the contents of phenolics, soluble proteins in plants of kiwifruit cultivars and their resistance to kiwifruit bacterial canker by *Pseudomonas syringae* pv. *Actinidiae*. Plant Protection, 35(1): 37-41;

Moershbacher B, Noll U, Gorrichon L, Reisener HJ (1990) Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. Plant Physiology, **93**: 465–470;

Moreira WR, Bispo WM, Rios JA, Debona D, Nascimento CWA, Rodrigues FÁ (2015) Magnesium-induced alterations in the photosynthetic performance and resistance of rice plants infected with *Bipolaris oryzae*. Scientia Agricola, **72**(4): 328-333;

Mur LAJ, Simpson C, Kumari A, Gupta AK, Gupta KJ (2016) Moving nitrogen to the centre of plant defence against pathogens. Annals of Botany, **119**(5): 703–709;

Na L, Li Z, Xiangxiang M, Ara N, Jinghua Y, Mingfang Z (2014) Effect of Nitrate/Ammonium Ratios on Growth, Root Morphology and Nutrient Elements Uptake of Watermelon (*Citrullus Lanatus*) Seedlings. Journal of Plant Nutrition, **37**(11): 1859–1872;

Nakajima M, Goto M, Hibi T (2002) Similarity between copper resistance genes from *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *tomato*. Journal of General Plant Pathology **68**: 68–74;

Nardozza S, Boldingh H, Richardson A, Walter M, Kashuba P, Seelye R, Clearwater M, Gould N (2015) Kiwifruit Xylem Sap: Composition And In Vitro Growth Of A Virulent Strain Of *Pseudomonas Syringae* Pv. *Actinidiae*. Acta Horticulturae, **1095**: 123–128;

Nauš J, Prokopová J, Řebíček J, Špundová M (2010) SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynthesis Research, **105**(3), 265–271;

Nemli G, Kırcı H, Serdar B, Ay N (2003) Suitability of kiwi (*Actinidia chinensis* Planch.) prunings for particleboard manufacturing. Industrial Crops and Products, **17**(1): 39–46;

Neves N (2008a) Morfologia e fisiologia. In Antunes D eds., Kiwi: da produção à comercialização. Universidade do Algarve, 24-28;

Neves N (2008b) Informação Botânica. In Antunes D eds., Kiwi: da produção à comercialização. Universidade do Algarve, 29-39;

Nunes da Silva M, Machado J, Balestra GM, Mazzagalia A, Vasconcelos MV, Carvalho SMP (2019) Unravelling Actinidia molecular mechanisms against Pseudomonas syringae pv. actinidiae and P. syringae pv. actinidifoliorum – first steps. European Journal of Horticultural Science, **84**(4): 206-212;

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, **95**:351-358;

Oliveira JC, Albuquerque GMR, Mariano RLR, Gondim DMF, Oliveira JTA, Souza EB (2012) Reduction of the severity of angular leaf spot of cotton mediated by silicon. Journal of Plant Pathology, **94**:297–300;

Osaki M, Shirai J, Shinano T, Tadano T (1995) ^{15}N-Allocation of $^{15}NH_4$-N and $^{15}NO_3$-N to Nitrogenous Compounds at the Vegetative Growth Stage of Potato Plants. Soil Science and Plant Nutrition, **41**(4): 699–708;

Pacheco C, Calouro F, Vieira S, Santos F, Neves N, Curado F, Franco J, Rodrigues S, Antunes D (2008) Influence of nitrogen and potassium on yield, fruit quality and mineral composition of kiwifruit. International Journal of Energy and Environment, **2**(1): 9-15;

Pandey N (2018) Role of Plant Nutrients in Plant Growth and Physiology. In: M Hasanuzzaman, M Fujita, H Oku, K Nahar, B Hawrylak-Nowak (eds) Plant Nutrients and Abiotic Stress Tolerance. Springer, Singapore, pp 51-93;

Parangan-Smith A and Lindow S (2012) Contribution of Nitrate Assimilation to the Fitness of *Pseudomonas syringae pv. syringae* B728a on Plants. Applied and Environmental Microbiology, **79**(2): 678–687;

Peticila A, Scaeteanu GV, Madjar R, Stanica F, Asanica A (2015) Fertilization Effect on Mineral Nutrition of *Actinidia Deliciosa* (kiwi) Cultivated on Different Substrates. Agriculture and Agricultural Science Procedia, **6**: 132–138;

Petriccione M, Di Cecco I, Arena S, Scaloni A, Scortichini M (2013a) Proteomic changes in *Actinidia chinensis* shoot during systemic infection with a pandemic *Pseudomonas syringae* pv. *actinidiae* strain. Journal of Proteomics, **78**:461-476;

Petriccione M, Di Cecco I, Salzano A, Arena S, Scaloni A, Scortichini M (2013b) A proteomic approach to investigate the *Actinidia* spp./*Pseudomonas syringae* pv. *actinidiae* pathosystem. I International Symposium on Bacterial Canker of Kiwifruit 1095. pp. 95-101;

Petriccione M, Mastrobuoni F, Zampella L, Scortichini M (2015) Reference gene selection for normalization of RT-qPCR gene expression data from *Actinidia deliciosa* leaves infected with *Pseudomonas syringae* pv. *actinidiae*. Scientific Reports **5:**16961;

Pinto VA (2018) Avaliação do potencial do ácido salicílico e do metil jasmonato contra o cancro bacteriano do kiwi causado pela *Pseudomonas syringae* pv. *actinidiae* (Psa). Science Faculty, Oporto;

Prabhu AS, Fageria ND, Berni RF, Rodrigues FA (2007) Phosphorous and plant disease. In LE Datnoff, WH Elmer, DM Huber eds, Mineral nutrition and plant disease. APS Press, St Paul, pp 45–55;

Ramani S, Kannan S (1974) Effects of Certain Cations on Manganese Absorption by Excised Rice Roots. Communications in Soil Science and Plant Analysis, **5**: 435-439;

Rayar AJ and van Hai T (1977) Effect of ammonium on uptake of phosphorus, potassium, calcium and magnesium by intact soybean plants. Plant and Soil, **48**(1): 81-87;

Reglinski T, Vanneste J, Wurms K, Gould E, Spinelli F, Rikkerink E (2013) Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae*. Frontiers in plant science, 4: 24;

Reuveni R, Dor G, Raviv M, Reuveni M, Tuzun S (200) Systemic resistance against *Sphaerotheca fuliginea* in cucumber plants exposed to phosphate in hydroponics system, and its control by foliar spray of mono-potassium phosphate. Crop Protection, **19**(5): 355-361;

Ribeiro MG (2018) Resposta metabólica e molecular de diferentes cultivares de *Actinidia* spp. à *Pseudomonas syringae* pv. *actinidiae* (PSA) e pv. *actinidifoliorum* (PFM). Science Faculty, Oporto;

Rinallo C and Modi G (2002) Content of oxalate in *Actinidia deliciosa* plants grown in nutrient solutions with different nitrogen forms. Biologia Plantarum, **45**(1): 137–139;

Robson AD and Pitman MG (1983) Interactions Between Nutrients in Higher Plants. In A Läuchli, RL Bieleski, Inorganic Plant Nutrition Vol 15. Springer, Berlin, Heidelberg, pp 147–180;

Roosta HR, Schjoerring JK (2007) Effects of ammonium toxicity on nitrogen metabolism and elemental profile of cucumber plants, Journal of Plant Nutrition, **30**: 1933-1951;

Roy RN, Flinck A, Blair GJ, Tandon HLS (2006) Guidelines for the management of plant nutrients and their sources. In RN Roy, A Flinck, GJ Blair, HLS Tandon eds, Plant nutrition for food security: A guide for integrated nutrient management. Food and agriculture organization of the united nations, Rome, pp 193-232;

Ruan J, Gerendás J, Härdter R, Sattelmacher B (2007) Effect of Nitrogen Form and Root-zone pH on Growth and Nitrogen Uptake of Tea (*Camellia sinensis*) Plants. Annals of Botany, **99**(2): 301–310;

Sawada H, Kondo K, Nakaune R (2016) Novel biovar (biovar 6) of *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker of kiwifruit (*Actinidia deliciosa*) in Japan. Japanese Journal of Phytopathology, **82**:101–115;

Sawada H, Miyoshi T, Ide Y (2014) Novel MLSA group (Psa5) of *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker of kiwifruit (*Actinidia chinensis*) in Japan. Japanese Journal of Phytopathology **80**: 171–184;

Sawada H, Shimizu S, Miyoshi T, Shinozaki T, Kusumoto S, Noguchi M, Naridomi T, Kikuhara K, Kansako M, Fujikawa T, *et al*. (2015) Characterization of biovar 3 strains of *Pseudomonas syringae* pv. *actinidiae* isolated in Japan. Japanese Journal of Phytopathology **81**:111–126;

Schindler C, Reith P, Lichtenthaler HK (1994) Differential Levels of Carotenoids and Decrease of Zeaxanthin Cycle Performance during Leaf Development in a Green and an Aurea Variety of Tobacco. Journal of Plant Physiology, **143**(4-5): 500–507;

Scortichini M (1994) Occurrence of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Italy. Plant Pathology **43**: 1035- 1038;

Scortichini M (2014) Field efficacy of chitosan to control *Pseudomonas syringae* pv. *actinidiae*, the causal agent of kiwifruit bacterial canker. European journal of plant pathology **140**(4): 887-892;

Scortichini M, Marcelletti S, Ferrante P, Petriccione M, Firrao G (2012) *Pseudomonas syringae* pv. *actinidiae*: a reemerging, multi-faceted, pandemic pathogen. Molecular Plant Pathology, **13**:631–640;

Scortichini M, Marchesi U, Di Prospero P (2002) Genetic relatedness among *Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P. s*. pv. *actinidiae*, and their identification. European Journal of Plant Pathology, **108**: 269–78;

Serizawa S and Ichikawa T (1993a) Epidemiology of bacterial canker of kiwifruit, 3: The seasonal changes of bacterial population in lesions and of its exudation from lesion. Annals of the Phytopathological Society of Japan, **59**(4): 469-476;

Serizawa S, Takikawa Y, Ichikawa T, Tsuyumu S, Goto M (1989) Occurrence of bacterial canker of kiwifruit in Japan: description of symptoms, isolation of the pathogen and screening of bactericides. Annals of the Phytopathological Society of Japan, **55**:427–436;

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of botany, 1–26;

Song YR, Choi MS, Choi GW, Park IK, Oh CS (2016) Antibacterial activity of cinnamaldehyde and estragole extracted from plant essential oils against *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker disease in kiwifruit. The plant pathology journal, **32**(4), 363;

Song Y, Sun L, Lin M, Chen J, Qi X, Hu C, Fang J (2019) Comparative transcriptome analysis of resistant and susceptible kiwifruits in response to *Pseudomonas syringae* pv. *Actinidiae* during early infection. PLoS ONE **14**(2): e0211913;

SOPI (2014) Situation and Outlook for Primary Industries. Ministry for Primary Industries, https://www.mpi.govt.nz/about-mpi/corporate-publications/ (Accessed 2019-04-08);

Srihuttagum M and Sivasithamparam K (1991). The influence of fertilizers on root rot of field peas caused by Fusarium oxysporum, Pythium vexans and Rhizoctonia solani inoculated singly or in combination. Plant and Soil, **132**(1): 21–27;

Strik B (2005) Growing Kiwifruit. Pacific Northwest Extension, pp 28;

Tagliavini M, Toselli M, Marangoni B, Stampi G, Pelliconi F (1995) NUTRITIONAL STATUS OF KIWIFRUIT AFFECTS YIELD AND FRUIT STORAGE. Acta Horticulture, **383**: 227-238;

Takashi Fujikawa and Hiroyuki (2016) Sawada Genome analysis of the kiwifruit canker pathogen *Pseudomonas syringae* pv. *actinidiae* biovar 5 Scientific Reports **6**:21399;

Takashi Fujikawa and Hiroyuki Sawada (2019) Genome analysis of *Pseudomonas syringae* pv. *actinidiae* biovar 6, which produces the phytotoxins, phaseolotoxin and coronatine; Scientific Reports **9**: 3836;

Takikawa Y, Serizawa S, Ichikawa T, Tsuyumu S, Goto M (1989) *Pseudomonas syringae* pv. *actinidiae* pv. nov.: the causal bacterium of kiwifruitfruit canker in Japan. Annals of the Phytopathological Society of Japan **55**:437–444;

Therios IN and Sakellariadis SD (1988) Effects of nitrogen form on growth and mineral composition of olive plants (*Olea europaea* L.). Scientia Horticulturae, **35**(3-4), 167–177;

Tsabarducas V, Chatzistathis T, Therios I, Patakas A (2017) How nitrogen form and concentration affect growth, nutrient accumulation and photosynthetic performance of *Olea europaea* L. (cv. “Kalamon”). Scientia Horticulturae, **218**: 23–29;

Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H (2007) Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynthesis Research, **91**(1): 37-46;

Valente LMP, Custódio M, Batista S, Fernandes H,Kiron V (2019) Defatted microalgae (*Nannochloropsis* sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. Fish Physiology and Biochemistry, **45**(3): 1067-1081;

Vanneste JL (2012) Pseudomonas syringae pv. actinidiae (Psa): a threat to the New Zealand and global kiwifruit industry. New Zealand Journal of Crop and Horticultural Science **40**:265–267;

Vanneste JL (2017) The scientific, economic, and social impacts of the New Zealand outbreak of bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*). Annual Review of Phytopathology, **55**:377–399;

Vanneste JL, Cornish DA, Yu J, Audusseau C, Paillard S, Rivoal C, Poliakoff F (2011a) Presence of the effector gene hopA1 in strains of *Pseudomonas syringae* pv. *actinidiae* isolated from France and Italy. New Zealand Plant Protection, **64**:252-258;

Vanneste JL, Cornish DA, Yu J, Stokes CA (2014) First Report of *Pseudomonas syringae* pv. *actinidiae* the Causal Agent of Bacterial Canker of Kiwifruit on *Actinidia arguta* Vines in New Zealand. Plant Disease, **98**:418;

Vanneste JL, Kay C, Onorato R, Spinelli F, Yu J, Cornish D.A, Max S (2011b) Recent Advances in the Characterisation and Control of *Pseudomonas syringae* pv. *actinidiae*, the Causal Agent of Bacterial Canker on Kiwifruit. Acta Horticulturae **913**:443-455;

Vanneste JL, Yu J, Cornish DA, Tanner DJ, Windner R, Chapman JR, Taylor RK, Mackay JF, Dowlut S (2013) Identification, virulence, and distribution of two biovars of *Pseudomonas syringae* pv. *actinidiae* in New Zealand. Plant Disease, **97**(6):708-719;

Vargas P, Farias GA, Nogales J, Prada H, Carvajal V, Baron M, Rivilla R, Martin M, Olmedilla A, Gallegos MT (2013) Plant flavonoids target Pseudomonas syringae pv. tomato DC3000 flagella and type III secretion system. Environmental Microbiology, **5**:841-850;

Vavala E, Passariello C, Pepi F Colone M, Garzoli S, Ragno R, Pirolli A, Stringaro A, Angiolella L (2016) Antibacterial activity of essential oils mixture against PSA. Natural product research **30**(4): 412-418;

Wang MY, Siddiqi MY, Glass DM (1996) Interaction between K+ and NH4 + : Effects on ion uptake by rice roots. Plant, Cell and Environment, **19**:1037–1046;

Wang T, Wang G, Jia ZH, Pan DL, Zhang JY, Guo ZR (2018) Transcriptome analysis of kiwifruit in response to *Pseudomonas syringae* pv. *Actinidiae* infection. International Journal of Molecular Sciences **19**(2): 373;

Wang X and Below F (1998) Accumulation and partitioning of mineral nutrients in wheat as influenced by nitrogen from. Journal of Plant Nutrition, **21**: 49–61;

White PJ and Brown PH (2010) Plant nutrition for sustainable development and global health. Ann Bot.; **105**(7): 1073–1080;

Xiao Y, Lu Y, Heu S, Hutcheson SW (1992) Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp cluster. Journal of Bacteriology, **174**(6): 1734–1741;

Xu L, Zhu L, Tu L, Liu LL, Yuan DJ, Jin L, Long L, Zhang X (2011) Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. Journal of Experimental Botany, **62**(15):5607–5621;

Yamazaki H and Hoshina T (1995) Calcium Nutrition Affects Resistance of Tomato Seedlings to Bacterial Wilt. HortScience **30**(1):91–93;

Yang X, Wang B, Liu Y, Duan C, Dai C 2002) Biological effects of *Actinidia chinensis* callus on mechanical vibration. Colloids and Surfaces B: Biointerfaces, **25**(3): 197–203;

Yoo SD, Greer DH, Laing WA, McManus MT (2003) Changes in photosynthetic efficiency and carotenoid composition in leaves of white clover at different developmental stages. Plant Physiology and Biochemistry, **41**(10): 887–893;

Young JM (2010) Taxonomy of Pseudomonas syringae. Journal of Plant Pathology **92**(1): S1.5-S1.14;

Yu JG, Lim JA, Song YR, Heu S, Kim GH, Koh YJ, Oh CS (2016) Isolation and characterization of bacteriophages against *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker disease in kiwifruit. Journal of Microbiology and Biotechnology, **26**: 385-393;

Zhang F, Niu J, Zhang W, Chen X, Li C, Yuan L, Xie J (2010). Potassium nutrition of crops under varied regimes of nitrogen supply. Plant and Soil, **335**(1-2): 21–34;

Zhang H, Jennings A, Barlow PW, Forde BG (1999) Dual pathways for regulation of root branching by nitrate. Proceedings of the National Academy of Sciences, **96**(11): 6529–6534;

Zhang HM, Rong HL Pilbeam D (2007) Signalling mechanisms underlying the morphological responses of the root system to nitrogen in Arabidopsis thaliana. Journal of Experimental Botany, **58**: 2329–2338;

Zhu Z, Yu M, Chen Y, Guo Q, Zhang L, Shi H, Liu L (2014) Effects of ammonium to nitrate ratio on growth, nitrogen metabolism, photosynthetic efficiency and bioactive phytochemical production of *Prunella vulgaris*. Pharmaceutical Biology, **52**(12): 1518–1525;