# Development of methods for the control of fusarium wilt and crown rot on tomato

Ali A. Almasrahi

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## Abstract

*Fusarium oxysporum* (*Fo*) is a major pathogen that causes economic losses of many important crops around the world and affects a very wide range of plants including tomato. *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) that causes fusarium wilt disease and *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*) which is the causal agent of crown and root rot disease are considered amongst the most devastating pathogens on tomato plants (*Solanum lycopersicum* L.). The use of UV-C seed treatments, silicon and biocontrol agents are considered as safe and potential options for controlling plant diseases.

This study focused on the evaluation of low doses (0, 2, 4 and 6 kJ/$m^2$) of Ultraviolet light (UV-C) on disease development of *Fol* and *Forl*. The results showed that there were significant reductions in disease severity for plants grown from UV-C-treated seeds when inoculated with *Fol* or *Forl*. For *Fol*, The percentage disease severity score was decreased from 87.5% (control) to 75.0% and 66.6% with 13.71% and 23.88% respectively, whilst a 6 kJ $m^{-2}$ dose significantly reduced *Forl* severity from 69.4% (control) to 50.0%. The results showed that there was a correlation between the reduction in the disease severity by UV-C seed treatments (4 and 6 $kjm^{-2}$) and the DNA quantity in the root, crown and stem samples for *Fol* and *Forl*.

The inhibitory potential of sodium silicate against *Fol* and *Forl* was investigated under *in vitro* and *in vivo* conditions. Under laboratory

conditions, the higher concentrations (30, 40 and 50 mM) showed significant reduction in the mycelial growth of both *Fol* and *Forl*. The 30, 40 and 50 mM concentrations recorded 57.23, 73.77 and 96.25% reductions for *Fol*, and 46.25, 81.99 and 97.73% for *Forl* respectively. In *in vivo* experiments, 30, 40, and 50 mM concentrations were highly significant compared to the other treatments. The disease severity was 56.0, 50.0, and 46.6% respectively for *Fol* whilst for *Forl*, the disease severity was 45.83, 41.66, and 29.16% respectively.

The expression of some pathogen related genes, *GluA* (acidic extracellular b-1,3-glucanase), *GluB* (basic intracellular b-1,3-glucanase), *Chi3* (acidic extracellular chitinase), *Chi9* (basic intracellular chitinase), *PR-1a* (PR-1 protein isoform PR-P6) and *PAL* (phenylalanine ammonia-lyase) induced in tomato by UV-C, sodium silicate, *Trichoderma* spp. and the combination of these treatments against *Fol* and *Forl* have been investigated. The results showed that the expression of all tested genes was varied in all treatments. However, they were significant compared to the control. The combination treatment recorded high accumulation of all tested genes compared to other treatments. LeChi3 and LeChi9 accumulation were higher in roots samples in plants inoculated with *Fol,* whilst in plants inoculated with *Forl*, LeChi3, LeChi9 and LePR1a accumulation were higher in root and crown samples.

In conclusion, UV-C seed treatments combined with sodium silicate have been shown to be an effective disease control option that could contribute to induced disease resistance to *Fol* and *Forl* pathogens of tomato.

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# 1. Introduction

## 1.1 Tomato

Tomato (*Solanum lycopersicum*) belongs to the family Solanaceae and originated from the Andes region of South America (Sims, 1980). It was first classified by Miller in 1754 as *Lycopersicon esculentum* and was then renamed as *Solanum lycopersicum* by Child (1990) and Peralta and Spooner (2006). The exact origin of the tomato plant is under debate and believed to be in either Mexico or Peru. Tomatoes were first introduced into Europe in the 16th century but it was not until the 18th century that tomato consumption became widespread across Europe (Peralta and Spooner, 2006).

Tomato is ranked as the world's second most important vegetable crop after potato with annual production of about 160 million tonnes on an area of about 4.8 million hectares in year 2011 (FAOSTAT 2013). The economic importance of tomato has risen dramatically from being an ornamental plant in Europe initially to becoming the $10^{th}$ most economically important non-meat commodity with a global gross production value of 12.7 billion US dollars in 1961 rising to 62.2 billion US dollars in 2013 (FAOSTAT, 2015). It is particularly valued as one of the most important vegetables and fruits because it is a rich source of minerals, phenolic antioxidants, organic acids, citric acid and vitamins A, B, and C (Pedro and Ferreira, 2007).

Tomato grows well in warm conditions ranging from 15 to 25 °C, and soils of pH 5-7 that are well drained and rich in organic matter are best for tomato cultivation (Hanson *et al.,* 2001). It is grown throughout the world and the major tomato producing countries include China, the USA, India, Turkey, Egypt, and Italy (FAOSTAT, 2017).

## 1.2 Tomato diseases and control

Tomato plants are subject to attack by over 200 fungal, oomycete, bacterial, viral and nematode disease-causing agents that can result in significant yield losses (Table 1.1) (Watterson, 1986). Tomato diseases can be spread through the soil, air, water, seed or vectors and can infect plant foliar tissues as well as the roots and fruits. Tomato cultivars can be defined as susceptible, tolerant or resistant to these biotic stresses based on their ability to prevent infection and the severity of the symptoms caused by the casual agents. In susceptible crops, the causal agent can colonize the plant tissues, which leads to the disease symptoms. On the contrary, if the crop is resistant, the plant is able to avoid the infection, often through induction of plant defence responses, resulting in fewer or no symptoms of disease. Tolerant crops are defined as those that are successfully infected and colonized by the pathogens, but the plant symptoms are reduced and there is only limited impact on the crop yield (Blancard, 1994; Hawks *et al*., 2007; Jones, 2008).

**Table 1.1 Commonly and economically important tomato diseases and their causal agents**

| Disease | Casual agents | Distribution | Disease severity |
|---|---|---|---|
| | **Fungi** | | |
| Late blight | *Phytophthora infestans* | Worldwide | Severe |
| Verticillium wilt | *Verticillium albo-atrum* | Worldwide | Severe |
| Anthracnose | *Colletotrichum gloeosporioides* | Worldwide | Severe |
| Fusarium wilt | *Fusarium oxysporum* f.sp. *lycopersici* | Worldwide | Severe |
| Fusarium crown and root rot | *Fusarium oxysporum* f.sp. *radicis lycopersici* | Worldwide | Considerable |
| Powdery mildew | *Oidiopsis sicula* | Worldwide | Considerable |
| Early blight | *Alternaria solani* | Worldwide | Severe |
| Gray leaf spot | *Stemphylium lycopersici* | Worldwide | - |
| Damping off | *Rhizoctonia solani* *Pythium aphanidermatum* | Worldwide | - |
| Septoria leaf spot | *Septoria lycopersici* | | - |
| | **Bacteria** | | |
| Bacterial speck | *Pseudomonas syringae* pv. *tomato* | Worldwide | Severe |
| Bacterial wilt | *Ralstonia solanacearum* | Worldwide | Severe |
| Bacterial spot | *Xanthomonas campestris* | | - |
| Bacterial canker | *Clavibacter michiganensis* | | - |
| | **Viruses** | | |
| Tomato Mosaic Virus | *Tomato mosaic virus* | Worldwide | Severe |
| Tomato Spotted Wilt Virus | *Tomato spotted wilt virus* | Worldwide | Severe |
| Curly top | *Curly top virus* | Worldwide | Severe |
| Tomato necrosis | *Alfalfa mosaic virus* | Worldwide | Severe |
| Tomato etch | *Tobacco etch virus* | Worldwide | Severe |
| | **Nematodes** | | |
| Root-knot | *Meloidogyne* spp. | Worldwide | Severe |
| Reniform | *Rotylenchus reniformis* | | - |
| Potato cyst nematodes | *Globodera* spp. | Worldwide | Severe |
| Stunt | *Tylenchorhynchus* spp. | | - |

(Jones *et al.,* 1991; Blancard, 2012).

### 1.2.1 Fusarium wilt disease

*Fusarium oxysporum* (*Fo*) is a major pathogen that causes economic losses of many important crops around the world and affects a very wide range of plants including tomato, onion, strawberry, watermelon and banana. Economically and scientifically, it is considered as the fifth most important plant pathogenic fungus (Summerell, 2006; Michielse and Rep, 2009; Leslie and Dean *et al*., 2012). *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*), which is a pathogenic form that causes vascular wilt disease, and *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*) (Section 1.2.2), which is the causal agent of crown and root rot disease are considered the most devastating pathogens on tomato plants (*S. lycopersicum* L.).

Fusarium wilt is a soilborne disease that is common in field and greenhouse grown tomatoes. It affects tomatoes grown in warm conditions and has become of worldwide importance as it has been reported in 32 countries all over the world and is considered to be the more devastating disease than *Forl*, with up to 80% yield losses on tomato crops (Wong, 2003; Sheu *et al*., 2006; Amini *et al*., 2010). *Fol* can survive extensively in soil as chlamydospores, and upon germination the infection hyphae penetrate the root surface and then invade the root cortical cells intercellularly and enter the vascular system through the xylem pits. Within the vessels, the fungus starts to produce microconidia, which are transported upwards

through the sap stream. Wilt symptoms appear due to vessel blockage triggered by a combination of host-pathogen interactions, such as the release of toxins, gums, gels, tyloses and fungal hyphae. Typical disease symptoms then start to appear such as wilting, brownish discoloration of the vascular system, yellowing of older leaves and eventually this can result in plant death (Fig. 1.1) (McGovern, 2015; Renu Joshi, 2018).

There are three known *Fol* races (Races 1, 2 and 3) known to be pathogens of tomato cultivars and these can be distinguished by their reactions on cultivars with the corresponding principle resistance genes. Some reports indicate that races 1 and 2 occur throughout the tomato growing regions all over the world, whilst race 3 has been reported in California, Georgia, Australia and Mexico. Most commercial tomato varieties grown throughout the world have some resistance to races 1 and 2, and a few are resistant to race 3 (Biju *et al.*, 2017).

### 1.2.2. Crown and root rot disease

Fusarium crown and root rot is a second damaging soil-borne disease of tomato and is becoming more common in greenhouse and field tomato production, causing significant losses in tomato production all over the world (Adams *et al*., 2001). The severity of crown and root rot of tomato varies widely by site and season and is favoured by cool temperatures and

can cause serious problems to commercial yields of tomato, which have been reported to be reduced by 15-65% (Benhamou and Theriault, 1992; Besri, 2000; Benhamou *et al.,* 2001). Fusarium crown and root rot is caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). The disease was initially reported in 1974 in Japan and has subsequently been reported in Canada, Mexico, Israel, Japan, and many countries in Europe (Benhamou and Theriault, 1992; Anonymous, 1996).

Symptoms appears on the root system as dry brown lesions that develop in the cortex of the tap or main lateral roots. A necrotic lesion may also develop on the surface of the stem from the soil line to 10 to 30 cm above it (Fig. 1.2). Foliar symptoms on plants include yellowing along the margin of the oldest leaves, followed by necrosis and older plants may die (Brammall and Higgens, 1987; Blancard and Lecoq, 1994).

**Figure 1.1** Fusarium wilt symptoms include wilt, yellowing, and stunting of foliage caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*).

**Figure 1.2** Necrosis symptoms on the crown and roots caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*).

## 1.3. Control of Fusarium wilt and Crown and root rot

Fusarium wilt disease can cause significant damage with 10-90% losses in yield of tomato in temperate regions. These losses can be minimized through management strategies, which include the use of effective soil fumigation treatments, resistant cultivars and biological control agents (Juliano and Bettiol, 2005; Hibar *et al*., 2006).

### 1.3.1. Chemical based pesticides

The most obvious solution to control plant diseases is to use chemical methods to prevent the disease complex from occurring. The use of chemical pesticides is widespread due to their low cost, effectiveness, availability and stability. They are also generally fast-acting, which helps limit the damage done to crops by the disease. The mode of action of pesticides is varied, but most work through blocking metabolic pathways or through the disturbance of the cell walls of pathogens. However, the number of chemicals that are available for controlling plant diseases is declining, and there is increasing concern over the development of new fungicide-resistant isolates of pathogens, and the potential hazards to the environments and human health of the chemicals (Dekker, 1981; Koenning *et al*., 2004).

Because of the perceived inappropriate use of certain fungicides, which were found to have hazardous effects on ecosystems, and the possible carcinogenic risk from some fungicides, there have been some recent moves to develop biopesticides as alternative management methods to reduce the dependence on synthetic pesticides. For example, plant extracts and essential oils are natural products that are starting to be more widely used as antifungal agents to control a wide range of fungal diseases (Islam *et al.,* 2004). In the case of *Fol*, Hadian *et al*. (2011) demonstrated the potential effectiveness of plant extracts through testing neem seed extracts to control fusarium wilt disease and root-knot nematodes on tomato. The results showed that neem seed powder significantly reduced the disease severity of fusarium and root-knot nematodes. It decreased the root knot index from 4.7 in control treatments (fungi+nematode) to 0.25, and also decreased disease severity from 85% in control treatments to 12% in neem treated. Moreover, the neem treatment contributed to increasing the growth characters such as plant weight and length compared to untreated plants.

### 1.3.2 Resistant cultivars

The use of fusarium resistant tomato cultivars can provide some degree of control. However, resistance genes can also be overcome by further genetic adaptations of the pathogen and development of new pathogenic races (Larkin and Fravel, 1998).

In tomato, most genes for disease resistance to *Fol* and *Forl* are dominant and have been transferred by the introgression of resistance genes or loci from wild relatives and closely related species, or generated via mutagenesis treatment (Causse *et al*., 2007). Resistance to fusarium wilt disease in tomato varieties is controlled by the *I* genes (*I* for immunity). The *I* and *I-1* genes refer to the genes that confer resistance to race 1, the *I-2* gene conveys resistance to race 2, the *I-3* gene to race 3, and the *Twf* gene confers tolerance to fusarium wilt (Bournival and Vallejos, 1991; Davis *et al*., 2013). Beckman (2000) reported that the *I* genes in resistant varieties that recognize Avr proteins of *Fol* were able to induce a defence response in the xylem which mainly involves callose deposition, the accumulation of phenolics and the formation of tyloses.

In the case of *Forl,* the *Frl* gene is a single dominant gene on chromosome 9 that induces resistance (Fazio *et al*., 1998). Tanyolaç and Akkale (2010) investigated the existence of resistance genes (*Frl* and *I-2*) in 115 tomato varieties using Randomly Amplified Polymorphic DNA (RAPD) and Cleaved Amplified Polymorphic Sequences (CAPS) markers. Their results showed that out of 115 plants, 42 were homozygous resistant, 38 were heterozygous resistant and 35 were homozygous susceptible.

### 1.3.3 Biological control

Biological control of fusarium wilt diseases of tomato has become increasingly popular as an alternative disease management strategy to the use of chemicals. Antagonistic organisms, including nonpathogenic fungi (*Trichoderma* spp.) and rhizobacteria have successfully reduced the incidence of fusarium wilt in tomato crops in greenhouses and fields.

*Trichoderma* spp. have been successfully used as biocontrol agent against soil-borne, seed-borne and storage rots diseases in many crops. The mechanisms used by *Trichoderma* species for controlling pant pathogens include competition, mycoparasitism, antibiosis and induced resistance in host plants (Schirmböck *et al*., 1994; Chet, 1987). Karkachi *et al*. (2010) tested the effect of the culture filtrate of *T. harzianum* on the mycelial growth of *F. oxysporum*. f. sp. *lycopersici* (*Fol*) under laboratory condition and the results showed that the growth inhibition of *Fol* was between 40-95%. Hibar *et al*. (2006) investigated the effect of *T. harzianum* to induce resistance in tomato plants against *F. oxysporum*. f. sp. *radicis lycopersici* (*Forl*) under greenhouse condition and the *T*. *harzianum* significantly reduced the level of disease incidence when applied a week before inoculation and tomato roots were found protected from penetration by the pathogen.

In further studies, Ojha and Chatterjee (2012) investigated the effect of *T. harzianum* (TH) with different concentrations of salicylic acid (SA) (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) as individual and combined treatments on the induction of phenolic accumulation content and defence enzymes in tomato plants infected with *Fol.* The results showed that the phenolic content was recorded to be higher in all the treatments compared to the healthy plants. The activities of both peroxidases and polyphenol oxidases reached their maximum on the 28th day, when the plants were treated with *F. oxysporum* and SA (1.5 mM). The combined application of SA (1.5 mM) and TH in fusarium infected tomato plants also enhanced the activities of both enzymes.

In the case of plant growth promoting rhizobacteria (PGPR), species such as *Bacillus*, *Enterobacter* and *Pseudomonas* strains can be a significant biocontrol agent to control plant diseases (Alabouvette *et al*., 1998; 1993; Datnoff *et al*., 1995; Schmidt *et al*., 2004).The increasing importance of application of PGPR to disease management and improved crop productivity in sustainable agriculture has been expanded these days because of their ability in inducing plant resistance and antagonistic actions to the pathogens (Ramamoorthy *et al.,* 2001).

Loganathan *et al*. (2014) investigated the role of two PGPR *Bacillus* spp: *B. amyloliquefaciens* and *B*. *subtilis* alongside chemical control (Carbendazim)

in reduction of disease progression caused by *Fol* under field conditions. Comparing the two isolates of *Bacillus* and Carbendazim, the result showed that *B*. *subtilis* resulted in 58.4% wilt reduction over the control, which was greater than the chemical treatment (48.97%) and *B. amyloliquefaciens* (45.39%).

Xu and Kim (2014) evaluated the efficacy of seven (PGPR) *Paenibacillus* strains (SC02-09, SC09-21, SG09-01, SG09-02, SR04-02, SR04-16 and SR07-23) in the reduction of crown and root rot disease caused by FORL under greenhouse conditions. The results showed all tested strains significantly reduced the severity of disease compared to the control. Strain SC09-21 reduced the disease severity by > 80.0%, whereas strains SC02-09 and SG09-02 resulted in disease reductions of > 60.0%.

## 1.4 Ultraviolet (UV) Light treatment

In terms of possible novel control methods for plant diseases, seed treatment with UV-C radiation has been shown to induce resistance in plants through the accumulation of phytoalexins, synthesis of phenylpropanoid compounds, and pathogeneses related proteins that play a major role in induced defence responses in plants. For example, Mercier *et al*. (2000) reported some evidence that UV-C acts as an inducer of disease resistance of carrot to *Botrytis cinerea* and the resistance was

associated with the accumulation of phytoalexins. Similarly, ultra-structural studies in tomato indicated that UV treatment induces accumulation of phenolic compounds and formation of lignin and suberin in layers of cells near the surface of plants (Charles, 1998).

Ultraviolet (UV) light is the genotoxic electromagnetic radiation with wavelengths that fall between 100 and 400 nm. UV radiation has been classified as UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm) and can induce significant biological effects in crops (Bintsis *et al*., 2000; Hollósy, 2002; Shama, 2007). UV-C radiation has direct affects in killing microbes but only penetrates around 5-30 microns into tissues. Therefore, it has mainly been used to date as a surface treatment for the disinfection of equipment, glassware and air by food and medical industries for many years (Kowalski, 2009).

### 1.4.1 UV-C hormesis

Hormesis is the application of potentially harmful agents at low doses to living organisms in order to induce stress responses. The positive effects of hormetic UV-C treatments have been reported in reducing and preventing postharvest storage rots including in onions, potatoes, sweet potatoes, carrots, tomato, peaches, mangoes and strawberries. While the application of hormesis using UV-C treatments have shown significant

effects on plant tissues, hormetic responses have also been shown in bacteria, fungi, animals and humans (Shama, 2007).

### 1.4.2 UV-induced disease resistance

Plants are protected by two main physical barriers, the cuticle and the cell wall, which provide the first lines of plant defence against pathogen invasion (Lai and Mengiste, 2013). Initial activation of plant defence reactions is based on the ability of the plant to detect the presence of microorganisms through the recognition of highly conserved molecular patterns called Microbe-Associated Molecular Patterns (MAMPs). These pathogenic derived patterns are encoded by pathogenic microorganisms and include such things as peptidoglycan, fungal chitin and bacterial flagella, which are also commonly referred to as Pathogen Associated Molecular Patterns (PAMPs). All of these molecular patterns, also known as general elicitors, can be recognized by pattern recognition receptors (PRRs) present at the cell surface (Chinchilla, 2006; Gust *et al*., 2007; Henry *et al*., 2012; Newman *et al*., 2013; De Lorenzo *et al*., 2018).

As well as being exposed to biotic stressors (ie fungi, oomycetes, bacteria, viruses, nematodes and insects), plants are also exposed to abiotic stressors such as heat, cold, drought, salinity, and UV radiation. These abiotic stresses give rise to patterns that are referred to as Damage-

Associated Molecular Patterns (DAMPs). MAMPs/PAMPs/DAMPs, signaling compounds such as salicylic acid, jasmonic acid and ethylene, priming agents and plant defence activators (PDAs) work together to then trigger the activation of a non-host resistance known as Pathogen- or Pattern-Triggered Immunity (PTI).

Whilst PTI forms the first layer of induced defences against potentially pathogenic organisms, pathogens have often found ways to evade or overcome PTI. Effectors, that are the products of the pathogens' avirulence genes, are believed to be one of the key mechanisms through which pathogens overcome PTI. In turn, plants have therefore evolved a second line of induced resistance that is activated in response to recognition of extracellular or intracellular pathogen effectors through transmembrane receptors or resistance (R) proteins (Allen *et al*., 2004; Chisholm *et al.*, 2006; Dodds *et al*., 2006; Jones and Dangl, 2006). It is this second form of induced resistance that forms the basis of the breeding of resistant cultivars as discussed in section 1.3.2.

In terms of how hormesis relates to induced resistance mechanisms, it is known that the primary metabolites (carbohydrates, proteins, amino acids, lipids) are essential and vital for every living cell. Beyond this, the secondary metabolites which are organic compounds derived through methylation, glycosylation or hydroxylation from primary metabolites are

considered not essential but play important roles in plant defence systems to contribute to stress responses as signaling molecules, potent scavengers of Reactive Oxygen Species ROS, and to the protection of plants against pathogens (Korkina, 2007; Buer *et al*., 2010; Samanta *et al*., 2011).

Low UV-C doses may trigger acclimation responses in plants, including activation of enzymatic and non-enzymatic defence systems. However, high UV-C doses could activate repair mechanisms in order to overcome plant stresses. It has been reported that UV-C can be related to the induction of DNA repair mechanism, secondary metabolite production, and different signal transduction pathways (Loyall *et al*., 2000; Jansen, 2002; Brown *et al*., 2005; Katerova and Todorova, 2011; Rai *et al.,* 2011).

Duval *et al.* (2000) demonstrated that UV-C light exposure can stimulate phenolic-antioxidant production in cells of *Chlamydomonas nivalis* (called snow alga) which may provide a valuable source of pharmacological products targeted for anticancer, anticoagulant, antimicrobial, or anti-inflammatory treatments. Balouchi *et al*. (2009) investigated the changes in photosynthetic pigments of durum wheat leaves after being exposed to UV-C radiation. The results showed that flavonoids, anthocyanins, carotenoids, and proline content increased significantly through UV-C treatment as compared to the controls.

A group of three terpenoids; β-ionone, geranylacetone and terpinyl acetate, have been observed to be upregulated in melon and tomato after treatment of the plants with UV-C (Widiastuti *et al.*, 2011). β-ionone, geranylacetone, terpinyl acetate and rishitin have all been shown to act as phytoalexins, which are antimicrobial compounds, whilst β-ionone may also play a role in signalling during UV hormesis (Ramel *et al*., 2012).

Similarly, Casado-Diaz *et al*. (2006) have observed upregulation of genes coding for PR proteins such as chitinases, β-1,3-glucanases, osmotins, γ-thionins, and Fra a1, in strawberry leaves infected by *Colletotrichum acutatum* after being treated with UV-C radiation. In peach fruit a rapid induction of chitinase, b-1,3-glucanase, and phenylalanine ammonia lyase (PAL) activities have been observed after 6 h from UV-C treatment (El Ghaouth *et al*., 2003).

There are also a few studies that support the induction of defence responses when postharvest fruit have been inoculated and treated with UV-C radiation (Table 1.2).

**Table 1.2 Postharvest hormetic UV- C treatments on fruit induced disease resistance.**

| Fruit | Pathogen | Effective Dose (kJ/m2) | Intensity (µW/cm2) | Results | Reference |
|---|---|---|---|---|---|
| Tomato cv. Trust | *Botrytis cinerea* | 3.7 | 2055 | - 91% reduction in lesion size.<br>- Production of phytoalexin rishitin and hypersensitive-like response were induced | Charles et al., 2008 |
| Peach cv. Elberta and Loring | *Monilinia fructicola* | 7.5 | 1260 | - Incidence of disease was reduced by 44 and 78% and lesion size was reduced by 84 an d 89% on Elberta and Loring cultivars, respectively. | Stevens et al., 1997 |
| Melon | *Fusarium oxysporum* and *Alternaria alternata* | 4.0/6.0 | 555 | - The incidence and lesion diameter of both pathogens was reduced. | Huang et al., 2015 |
| Orange | *Penicillium digitatum* | 2.0 | 370 | - Reduced the disease progression by 70%.<br>- Inhibited 100% of spore germination. | Terao et al., 2017 |
| Carrot cv. Caropak | *Botrytis cinere*a | 0.88 | - | - Induced strong local accumulation of 6-methoxymellein and an 82% reduction in lesion size when inoculated 50 Day Post Treatment.<br>- A 24kDa chitinase was upregulated. | Mercier et al., 2000 |
| Lemon cv. Eureka | *Penicillium digitatum* | 5 | - | - 44% reduction in incidence<br>- Increased levels of scoparone were observed after UV treatment. | Ben-Yehoshua et al., 1992 |
| Tomato cv. Tuskege-80- 130 and Floradade | *Rhizopus stolonifer* | 3.6 | 1260 | Incidence of disease was reduced by 50 and 78% and lesion size was reduced by 33 and 51% on cultivars Tuskege 80, 130 and Floradade, respectively. | Stevens et al., 1997 |

### 1.4.3 UV-C induced resistance in tomato

Ultraviolet (UV)-C hormesis is one of the potential alternative approaches to the application of chemical fungicides for controlling pre and postharvest diseases of tomato and other crops. It contributes to the induction of natural host defences for protecting postharvest crops against pathogenic fungi, bacteria and other abiotic stresses, and has been shown to reduce disease susceptibility in stored tomato and other harvested crops (Liu *et al*., 1993; Charles *et al.,* 1996). Charles *et al.* (2008a, 2008d) investigated the impact of UV-C treatment to induce resistance against *Botrytis cinerea* in tomato fruit. The results showed increased biosynthesis and accumulation of a range of phenolic compounds, the phytoalexin rishitin and three PR proteins including a 33.1 kDa β-1,3-glucanase and two chitinases of 37.1 and 20.6 kDa in size. Moreover, physical modifications of the fruit surface and pericarp were observed after UV-C treatment which lead to the formation of a physical barrier that overcome *B. cinerea* ingress.

Liu *et al*. (2011) performed a transcriptional analysis assay using the Tomato GeneChip microarray (Affymetrix) for monitoring the regulation of 9254 genes of tomato treated with 4 kJ/m2 of UV-C irradiation. The results showed that a total of 677 genes were differentially expressed. Out of 677 genes, 274 were upregulated whose functions were mainly involved in

signal transduction, defence responses and metabolism. The remaining genes were downregulated and were responsible for photosynthesis, lipid metabolism and cell wall disassembly.

In our laboratory at the University of Nottingham, Scott *et al*. (2018) have monitored the expression of genes involved in defence, ripening and secondary metabolism following high-intensity, pulsed polychromatic light (HIPPL) and low-intensity UV-C (LIUV) treatments of tomato fruit. The result showed that *CHI9, OPR3*, *P4* and *GLUB* were significantly upregulated at 10 d post treatment (10 DPT) and 12 h post inoculation with *B. cinerea* (12 HPI). *FLS* and *PG* were significantly downregulated at 10 DPT and 12 HPI. *ACO1*, and *CRTR-B1* were only significantly upregulated at 24 HPT whereas *PAL* was significantly upregulated at 12 HPI.

## 1.5 Silicon

As well as UV, silicon (Si) can also have beneficial impacts on the growth and production of a wide range of plant species. Silicon plays a major role in enhancing plant resistance against both fungal and bacterial pathogens. Silicon is involving in activating defence-related enzymes, stimulating antimicrobial compound production, regulating the complex network of signal pathways, and activating the expression of defence-related genes

(Wang *et al.,* 2017). As a biochemical mechanism, Si enhances biochemical resistance that is associated with increasing the activity of defence-related enzymes, such as polyphenoloxidase, glucanase, peroxidase, and phenylalanine ammonia-lyase (PAL); inducing antimicrobial compounds production, such as phenolic, flavonoids, phytoalexins and pathogenesis-related (PR) proteins in plants; and regulating systemic signals, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET); (Fauteux *et al*., 2005; Datnoff *et al*., 2007; Fortunato *et al*., 2012b; Van *et al*., 2013).

Silicon (Si) has a great potential to be part of an effective integrated disease management strategy against plant diseases and has been identified as a potential inducer of plant defences against different fungal pathogens. Rahman *et al.* (2015) demonstrated the role of Si in reduction of disease incidence and severity of gray leaf spot disease on perennial ryegrass caused by *Magnaporthe oryzae* under field conditions. The soil was amended with three different levels of calcium silicate (1, 5, or 10 t/ha) in addition to the unamended soil as a control. The results showed that at 5 t/ha, calcium silicate was found to significantly suppress gray leaf spot in perennial ryegrass, including a significant reduction of disease incidence (39.5%) and disease severity (47.3%). Moreover, the study observed significant increases in chlorogenic acid, flavonoids, and relative expression levels of genes encoding phenylalanine ammonia lyase (PALa

and PALb) and lipoxygenase (LOXa) in Si-amended plants compared with that of nonamended control plants.

## 1.6 Aims and objectives

Management of soil-borne pathogens such as *Fol* and *Forl* is difficult due to the high survival rate of the spores. Therefore, management strategies for fusarium wilt epidemics have in the past often relied on applying soil fumigants to produce disease-free plants (UNEP, MBTOC 2002).

As well as *Fol* and *Forl*, other closely related species are also often found associated with tomato roots, including, for example, *Plectosphaerella lycopersici*, and some of these have in the past been found to be useful as biocontrol agents against the more severe strains. Other bacteria and fungi, such as *Bacillus* spp. and *Trichoderma* spp. have also been developed as potential biocontrol agents against *Fol* and *Forl* on tomato and other crop species, and more recently techniques such as application of silicates, or treatment of plants with UV-C, have also been examined as potential alternative means for disease control. How exactly these different treatments work, is poorly understood, but it is likely that some work by inducing systemic defence responses in the plant tissues.

Quantitative PCR assays have been developed for evaluation and monitoring disease development and pathogen biomass for plant pathogen forms and races. This is a very useful method to detect and quantify plant pathogens (Brouwer *et al*., 2003), and related reverse-transcriptase (RT) qPCR methods can also be developed to analyse and quantify the expression of defence related genes in plants to determine genes that are potentially up or down regulated.

The objectives of this project are therefore:

1) To develop robust quantitative DNA-based diagnostic techniques for pathogenic strains of the fungi *Fol* and *Forl,* and for the potential biocontrol agents to be used (*Trichoderma* spp.), that can be used to quantify levels of the microbes in roots, crown and stem.

2) To evaluate the efficacy of different control treatments (biocontrol, silicates, UV-C) in control of fusarium wilt and crown rot.

3) To investigate the mechanisms through which these controls work in plants through the analysis of induction of specific defence-related genes.

# Chapter 2. GENERAL MATERIALS AND METHODS

## 2.1 Culturing and storage of fungal isolates

*Fusarium oxysporum* (*Fo*) cultures were supplied from the University of Nottingham's fungal collection. The *Fol* culture was provided by Alison Jackson (University of Warwick, UK) (Table 2.1). All 19 *Fo* isolates were sub-cultured on potato dextrose agar (Sigma Aldrich) supplemented with penicillin G sodium salt (Sigma Aldrich) at 33 mg $L^{-1}$ and streptomycin sulphate salt (Sigma Aldrich) at 133 mg $L^{-1}$ and were incubated at 27 °C for 7 days. The fungal cultures were then preserved at 4 °C on PDA slops for future use. *Fusarium oxysporium* f. sp. *lycopersici* and *F. oxysporium* f. sp. *radices-lycopersici* spores were collected from 7 days old cultures following instructions in section (2.3), suspended in 20% glycerol and stored at -80 °C for long term storage.

## 2.2 DNA extraction of fungal isolates

Fungal DNA was extracted from approximately 100 mg of 7 day old mycelia of all 24 fusarium isolates placed in 2.0 ml Fastprep tubes containing 10 sterilized 2 mm glass beads (Sigma-Aldrich, Haverhill, UK). Mycelia were disrupted by vigorous shaking in a Fastprep (QBiogene, Cambridge, UK) for 2 cycles of 45 seconds at 6.5 $ms^{-1}$. The DNA was extracted using the

QIAGEN DNeasy plant mini kit (Qiagen, Crawley, UK), following the manufacturer protocols. DNA extracts were stored at -20 °C for PCR amplification.

**Table 2.1 List of *Fusarium oxysporum* (*Fo*) isolates**

| Fusarium isolates | location | isolate code |
|---|---|---|
| *Fusarium solani* 1 | Lab collection | F1 |
| *Fusarium solani* 2 | Lab collection | F2 |
| *Forl* A | Lab collection | F3 |
| *Forl* B | Lab collection | F4 |
| *Fusarium oxysporum* Bx15/4a | Lab collection | F5 |
| *Fusarium oxysporum* Bx15/4b | Lab collection | F6 |
| *Fusarium oxysporum* Bx15/4c | Lab collection | F7 |
| *Foc* 2A | Lab collection | F8 |
| *Foc* 2B | Lab collection | F9 |
| *Fusarium* sp. Bx14/153a | Lab collection | F10 |
| *Fusarium* sp. Bx14/153b | Lab collection | F11 |
| *Fusarium* sp. Bx14/153c | Lab collection | F12 |
| *Fusarium* sp. Bx14/152a | Lab collection | F13 |
| *Fusarium* Ex. Root Bx14/128a | Lab collection | F14 |

## Table 2.1 Continued

| | | |
|---|---|---|
| *Fusarium* sp. Bx14/168a | Lab collection | F15 |
| *Fusarium* sp. Bx14/168b | Lab collection | F16 |
| *Fusarium* sp. Bx14/168c | Lab collection | F17 |
| *Fusarium. oxysporum* f. sp . *lycopersici* Race1 | UK | F18 |
| *Fusarium. oxysporum* f. sp . *lycopersici* Race2 | UK | F19 |
| *Fusarium. oxysporum* f. sp. *radicis lycopersici* | UK | F20 |
| *Fusarium* (*Fol*) BX08/54 | UK | F21 |
| *Fusarium* (*Forl*) BX09/15 | UK | F22 |
| *Fusarium* (*Forl*) BX10/15 | UK | F23 |
| *Fusarium (Fol*??) BX13/39 | UK | F24 |

## 2.3 Identification of fungal isolates

PCR amplification was performed using the universal primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'- TCCTCCGCTTATTGATATG-3', which anneal to conserved regions of the 18S and 28S rRNA genes, respectively (White *et al*., 1990). The PCR mixture was comprised of 12.5 µl of MangoMixTM (Bioline), 10.5 µl of HyClone™ molecular grade water (GE Healthcare) and 0.5 µl of both forward and reverse universal fungal primers (from 10mM stocks) (Table 2.1). PCRs were performed on a BioRad® S1000TM thermal cycler programme using the following reaction cycle. Initial denaturation 94 °C for 2 minutes followed by 35 cycles of; 94 °C for 30 seconds, 53 °C for 1 minute and 72 °C for 1 minute; with a final extension of 72 °C for 5 minutes. The PCR products were then electrophoresed on a 1.5% agarose gel containing 1X TAE (Tris base Acetic acid- EDTA) buffer amended with 50 µl/l ethidium bromide run at 150 V for 30 minutes using a PowerPac BaisicTM (BioRad). The gel was viewed by UV camera InGenius3 on software platform GeneSys manufactured by Syngene, UK. PCR products were then purified using the GenElute TM kit (Sigma-Aldrich) following the manufacturer's protocol and sent to Eurofins (Germany) for sequencing.

## 2.4 Bioinformatics

Returned sequences were analysed and identified by the Basic Local Alignment Search Tool (BLAST) software produced by The National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1990).

## 2.5 inoculum preparation

Seven days old cultures on Petri dishes were flooded with 15 ml of sterile distilled water and 0.03% Tween 20. Spores were released via scrubbing the cultures and then filtered through a double layer of muslin cloth. Samples were vortexed vigorously, centrifuged at 1000 rpm for 10 minutes in a Centaur 2 (MSE). The supernatant was discarded, and the pellet was re-suspended in 30 ml of water, vortexed and centrifuged as stated before. The supernatant was discarded, and the pellet was re-suspended in 5ml of sterile distilled water. A haemocytometer was used to calculate the spore concentration and the inoculum concentration was adjusted accordingly.

## 2.6 Plant material

Seeds of the tomato Moneymaker was purchased from Johnsons Seeds. Seeds were stored at 6 °C at a relative humidity of <50%. Seeds were planted in 1 L pots of Levington M3 compost and grown in a heated and ventilated glasshouse at the University of Nottingham. Lighting by the

means of 400 W HPS SON-T lighting (Phillips) was used with a 16/8 h photoperiod. Day and night ventilation temperatures were 24/18 °C.

## 2.7 Low-intensity UV-C sources and treatments

Low-intensity UV-C (LIUV) treatments were carried out using a U-shaped amalgam UV-C source (UVI 12OU2G11 CP15/469) obtained from Dr Hőnle AG, Gräfelfing, Germany. Its peak emission was at 254 nm and the source was housed within an anodized aluminum parabolic reflector (Fig. 2.1 A, B and C). The LIUV source was switched on at least 30 minutes before treatment and not terminated until the end of the experiment to allow constant emission. Intensity was measured with a portable radiometer (Model UVX, UVP Instruments, Cambridge) fitted with a 254 nm sensor.

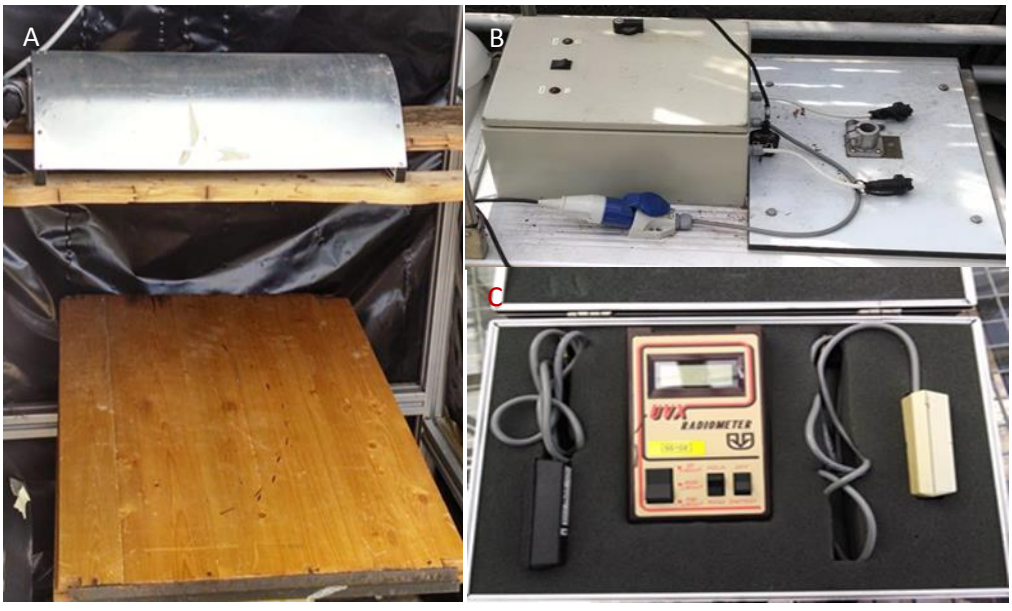


**Figure 2.1: A)** The enclosed counter in which treatments were performed, **B)** the low-intensity UV-C source, and **C)** portable radiometer (Model UVX, UVP Instruments, Cambridge).

# Chapter 3. PCR screening for the presence of SIX genes in *Fusarium oxysporum* isolates.

## 3.1 Introduction

Three physiological races (1, 2 and 3) of *Fusarium oxysporum* f. sp. *lycopersici* have been reported. Race 1 is the most widely distributed and has been reported from most geographical areas. Race 2 was first reported in Ohio in 1940, and after that has rapidly spread to and been reported in several state in the US and several other countries, including Australia, Brazil, the United Kingdom, Israel, Mexico, the Netherlands and Iraq. Race 3 was observed in Australia in 1978 (Grattidge and O'Brien, 1982) and was subsequently reported in several US states: Florida (Volin and Jones, 1982), California (Davis *et al.,* 1988), Georgia (Chellemi *et al.,* 1992), Arkansas and North Carolina (Marlatt *et al.,* 1996) and Tennessee (Bost, 2001).

Distinguishing between *Fol* races has previously relied on pathogenicity tests on different host plants, but the presence/absence of certain Secreted in Xylem (*SIX)* genes or sequence differences in these genes have been used more recently to identify and distinguish races of *Fol.* Race 2 and 3 isolates appear to lack the *SIX4* gene which is found in race 1, and the variation in *SIX3* sequences can be used to distinguish between races 2 and 3 (Lievens *et al.,* 2009).

The fungal genes that are required for pathogenesis and for disease development, but not essential for its life cycle, are defined as pathogenicity genes. These genes are also referred to as effectors, which are defined as small secreted proteins that function within the plant host to alter host cell structure, promote infections and suppress plant immunity responses. These proteins are generally cysteine rich and lack homologs in closely related species. Some of these effectors can act as avirulence genes that can be recognized by the plant host and contribute to plant resistance (Van de Wouw and Howlett, 2011).

A study conducted by Van Dam *et al.* (2016) on whole genome analysis of 59 isolates belonging to five *F. oxysporum* formae speciales (ff. spp. *cucumerinum, niveum, melonis, radicis-cucumerinum and lycopersici*) identified a total of 104 effectors and indicated that effector profiles of different formae speciales infecting the same hosts tend to be similar when compared to other formae speciales. Strains belonging to different lineages within the same forma specialis tend to share identical effector profiles.

Secreted in Xylem (*SIX*) genes were the first effectors identified in *Fusarium oxysporum* f.sp. *lycoperisci* (*Fol*) using a combination of proteomic and in silico approaches, along with gene knock-out and Agrobacterium-based assays (Rep *et al.,* 2004; Schmidt *et al.,* 2013).

The SIX1 protein was first identified by proteome analysis of xylem sap of a tomato plant infected by *F. oxysporum* f. sp. *lycopersici* (Rep *et al.,* 2004). So far, fourteen proteins have been identified in *Fol,* and the majority of SIX proteins are located on lineage specific chromosome 14 (Ma *et al.,* 2010; Schmidt *et al.*, 2013). At least 122 transposons and 227 predicted genes, including the *SIX* genes, are on chromosome 14 and are shared between different formae speciales and may play a more general role in pathogenicity (van der Does and Rep, 2007). SIX proteins are generally cysteine rich with 300 amino acids, an N-terminal signal and non-autonomous transposable elements (MITEs) present at the promoter sites for the majority of these SIX genes (Rep *et al.,* 2004; Houterman *et al.,* 2007; Lievens *et al.,* 2009; Ma *et al.,* 2010; Schmidt *et al.,* 2013). Homologs of *SIX* genes are also present in other formae speciales, including *Fusarium oxysporum* ff. spp. *cepae, canariensis, cubense*, *pisi* and *vasinfectum*, with *SIX* gene profiles distinct from one another (Lievens *et al.,* 2009; Chakrabarti *et al.,* 2011; Meldrum *et al.,* 2012; Fraser-Smith *et al.,* 2014; Laurence *et al.,* 2015; Taylor *et al.,* 2016).

In F*ol,* the lineage-specific (LS) genomic regions that include four entire chromosomes (chromosomes 3, 6, 14 and 15) are rich in repetitive elements and contain genes that encode known or putative effector proteins (Ma *et al., 2*010). Fourteen genes were identified to encode small, cysteine-rich SIX proteins, secreted into the xylem during host infection

(Houterman *et al.,* 2007; Schmidt *et al.,* 2013). Four of these SIX proteins (SIX1, SIX3, SIX5 and SIX6) have been associated with host specificity (Rep *et al.,* 2005; Lievens *et al.,* 2009; Gawehns *et al.,* 2014; Ma *et al.,* 2015), and three (SIX1, SIX3 and SIX4) behave as avirulence determinants when corresponding resistance genes are present in tomato (Rep *et al.,* 2004; Houterman *et al.,* 2008, 2009). For example, *Fol* SIX1 is recognized by the I-3 protein, which confers resistance to *Fol* races 2 and 3 (Rep *et al.,* 2004, 2005; Catanzariti *et al.,* 2015).

Loop-mediated isothermal amplification (LAMP) is a robust diagnostic technique, which is highly sensitive and less time consuming than PCR-based methods, and less subject to inhibition from DNA preparations. LAMP is characterized as using four different primers (two internal and two external) specifically designed to recognize six distinct regions of the target gene. Amplification products contain loop regions to which further primers can bind making the amplification continue isothermally. An additional pair of "loop primers" can bind to the loop regions to accelerate the reaction (Notomi *et al.,* 2000; Tomlinson *et al.,* 2012).

## 3.2 Objectives

The aim of this chapter was: 1) using PCR assays to validate the classifications of the *Fo* isolates using the universal primers ITS1 and ITS4.

In addition, specific primers targeting specific S*IX* genes in *Fol* DNA were used to distinguish between *Fol* races. Determination of the severity of infection of the *Fol* isolates in causing fusarium wilt in tomato have also been determined and the most aggressive isolates have been selected for further use in this study. 2) screening for fourteen Secreted in Xylem (*SIX*) genes in 21 *Fo* isolates using specific SIX primers and develop a Loop-Mediated Isothermal Amplification (LAMP) method for detection of some of the *SIX* genes that have been found in the *Fo* isolates to provide a specific and rapid approach for detecting pathogenic isolates of the fungus as an alternative to PCR-based diagnostics.

## 3.3 Materials and methods

### 3.3.1 Molecular identification of Fusarium isolates using Polymerase Chain Reaction (PCR) Analysis

All DNA of the *Fo* isolates listed in table 2.1 were subjected to PCR using the universal primers ITS1 and ITS4 and were performed as stated in section 2.3.

### 3.3.2 PCR Purification

All *Fo* amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocols and quantified on a Nanodrop spectrophotometer.

### 3.3.3 Sequencing

The purified PCR products were sequenced by Eurofins Genomics (https://ecom.mwgdna.com/services/home.tcl) and then identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were queried against GenBank using BLAST to confirm their identity.

### 3.3.4. Discrimination between *Fol* races and *Forl*

The cultural features of *Fol* races seem to be similar making this an unreliable method to be used for race discrimination. In general, the mycelium textures of all the isolates were fluffy, but colours of the aerial mycelium were varied (Fig. 3.1). Recently, molecular PCR based methods have been used to distinguish between FOL races using specific primers. Four primer sets (*SIX3*a, b, c, and *SIX4*) were used to target three SIX protein genes and distinguish between *Fol* races, and two primer sets (*SIX1* and *sprl*) to distinguish between *Fol* and *Forl* (Table 3.1). All DNA of *Fol* and

*Forl* isolates were subjected to PCR analysis using specific primers that amplified the *SIX1, SIX 3, SIX4* and *pgx4* protein genes. PCR reactions were performed as stated in section 2.2 using 59° and 62° C as the annealing temperatures for *Fol* and *Forl* respectfully.

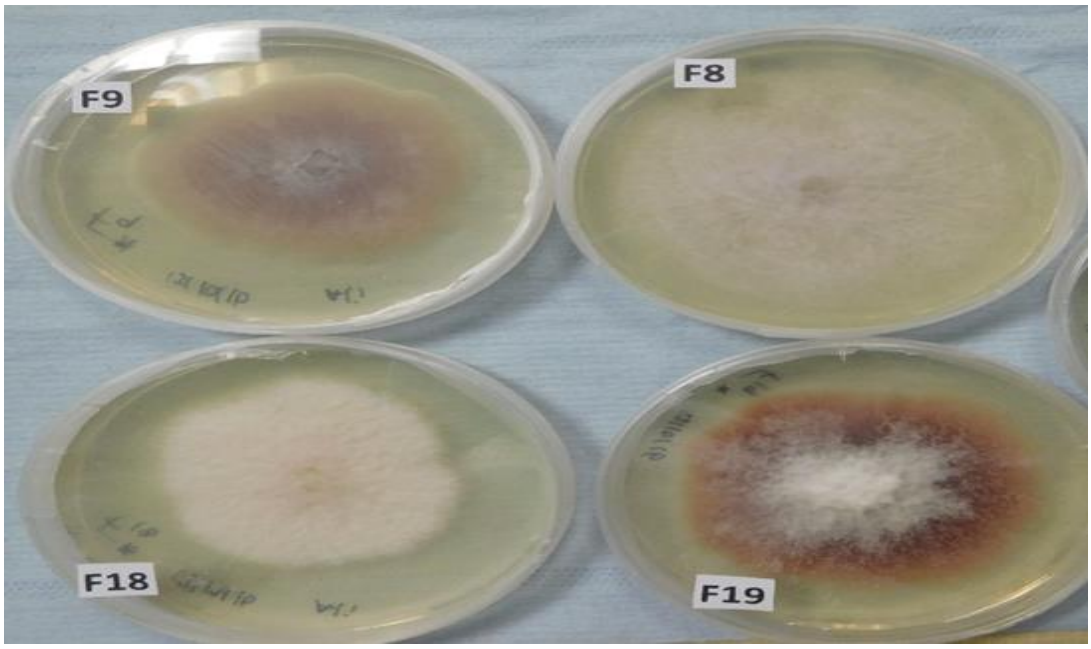


**Figure 3.1**. Cultural and morphological features of four *Fusarium oxysporum*. f. sp. *lycopersici* (F8, F9, F18, and F19) isolates on PDA agar.

**Table 3.1 Primers used in this study with their size and references.**

| Primers | Sequence 5'- 3' | Product size | Reference |
|---|---|---|---|
| ITS1 | TCCGTAGGTGAACCTGCGG | 600-650bp | White et al., 1990 |
| ITS4 | TCCTCCGCTTATTGATATG | | White et al., 1990 |
| SIX1F | GTACCCTCCGGATTTTGAGC | 992bp | Van Der Does et al., 2008 |
| SIX1R | AATAGAGCCTGCAAAGCATG | | Van Der Does et al., 2008 |
| SIX4F | TCAGGCTTCACTTAGCATAC | 967bp | Lievens et al., 2009a |
| SIX4R | GCCGACCGAAAAACCCTAA | | Lievens et al., 2009a |
| Six3aF | ACGGGGTAACCCATATTGCA | 608bp | Lievens et al., 2009a |
| Six3bF | TTGCGTGTTTCCCGGCCA | | Lievens et al., 2009a |
| Six3cF | GCGTGTTTCCCGGCCGCCC | | Lievens et al., 2009a |
| Six3 R | GGCAATTAACCACTCTGCC | | Van Der Does et al., 2008 |
| sprlF | GAGTTCACTTCAAGGACTCC | 947bp | Hirano and Arie, 2006 |
| sprlR | AGCAATCATCACCAATGTCA | | Hirano and Arie, 2006 |

### 3.3.5. Pathogenicity testing

Pathogenicity tests were carried out using the Moneymaker tomato variety, which is susceptible to Fusarium wilt disease, to determine the virulence of the four *Fol* strains (F8, F9, F18, and F19) that were identified by ITS analysis. *Fol* cultures were grown on potato dextrose agar (PDA) as stated in section 2.1. The inoculation of *Fol* was performed on cv. Moneymaker seedlings 22 days after planting. Spore suspensions of $10^6$ $ml^{-1}$ were made from 7-day-old cultures. The compost was moistened, and

seedlings were carefully removed. Roots were gently rubbed with Celite (Sigma Aldrich) and incubated in 30 ml of spore solution for 30 min. Plants were repotted and not watered until the following day.

### 3.3.6. Disease severity

Disease severity was scored at 30 days post inoculation (dpi) from 1-5 as follows: (1) Plant free from symptoms; (2) Plant showing: (a) vascular browning, (b) without wilting; (3) Plant showing: (a) vascular browning, (b) wilting symptoms, (c) without leaf yellowing; (4) Plant showing (a) severe wilting, (b) foliar chlorosis, (c) foliar necrosis; (5) Dead plant (Reis and Boiteux, 2007). Two independent experimental repeats were performed with a total of 12 biological replicates per treatments. In addition, inoculation of six seedlings with sterilized water served as the control.

Disease scores were converted to disease severity by using the following formula (Weitang *et al*., 2004):

$$\frac{\sum (\text{scale} \times \text{number of plants infected})}{\text{highest scale} \times \text{total number of plants}} \times 100$$

### 3.3.7 Detection of *SIX* Genes by PCR

All *Fo* isolates listed in Table 2.1 were screened for the presence or absence of the *SIX1* through to the *SIX14* genes using published primers. Table 3.2 indicates the primers, their annealing temperatures and amplicon size, along with their reference sources. The PCR was performed as stated in section 2.3.

### 3.3.8 Loop-Mediated Isothermal Amplification for Detection of SIX genes in *Fusarium oxysporum* isolates.

#### 3.3.8.1 Loop-mediated isothermal amplification assay

Six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers F-Loop and B-Loop) were designed from the sequences of the *SIX1, SiX2, SIX6, SIX7* and *SIX13* genes that were obtained from PCR products using the LAMP Designer software from OptiGene http://www.optigene.co.uk/lamp-designer/. (Table 3.3). LAMP assays were carried out on a Genie II instrument (OptiGene, Horsham, United Kingdom) in 20 µl reaction mixtures containing 14.3 µl of Isothermal MasterMix (ISO-001, OptiGene), 2.8 µl of PrimerMix (4 µl F3 and B3, 20 µl FIP, BIP, LoopF and LoopB (all at 10 mM) and 46 µl water), 5.7 µl water and 1 µl of the DNA sample. The reaction was held at 66°C for 35 min.

**Table 3.2 Primers used for molecular characterisation of *Fusarium oxysporum* isolates with product size, annealing temperature and relevant publications.**

| Primers | Sequence 5'- 3' forward primer reverse primer | Gene | Product size | Annealing temp. | References |
|---|---|---|---|---|---|
| SIX1 | GTATCCCTCCGGATTTTGAGC AATAGAGCCTGCAAAGCATG | SIX1 | 992 | 59 | Lievens et al., 2009 |
| SIX2 | CAACGCCGTTTGAATAAGCA TCTATCCGCTTTCTTCTCTC | SIX2 | 749 | 59 | Lievens et al., 2009 |
| SIX3 | CCAGCCAGAAGGCCAGTTT GGCAATTAACCACTCTGCC | SIX3 | 608 | 59 | Lievens et al., 2009 |
| SIX4 | TCAGGCTTCACTTAGCATAC GCCGACCGAAAAACCCTAA | SIX4 | 967 | 59 | Lievens et al., 2009 |
| SIX5 | ACACGCTCTACTACTCTTCA GAAAACCTCAACGCGGCAAA | SIX5 | 667 | 59 | Lievens et al., 2009 |
| SIX6 | CTCTCCTGAACCATCAACTT CAAGACCAGGTGTAGGCATT | SIX6 | 793 | 59 | Lievens et al., 2009 |
| SIX7 | CATCTTTTCGCCGACTTGGT CTTAGCACCCTTGAGTAACT | SIX7 | 862 | 59 | Lievens et al., 2009 |
| SIX8 | TCGCCTGCATAACAGGTGCCG TTGTGTAGAAACTGGACAGTCGATG | SIX8 | 250 | 59 | Meldrum et al., 2012 |
| SIX9 | GGGTGGACCATATCACGATGTTCG GAATACCTGAGTGGAGTTGTGTCTTG | SIX9 | 458 | 69 | Taylor et al., 2016 |
| SIX10 | GTTAGCAACTGCGAGACACTAGAA AGCAACTTCCTTCCTCTTACTAGC | SIX10 | 636 | 65 | Taylor et al., 2016 |
| SIX11 | ATTCCGGCTTCGGGTCTCGTTTAC GAGAGCCTTTTTGGTTGATTGTAT | SIX11 | 559 | 61 | Taylor et al., 2016 |
| SIX12 | CTAACGAAGTGAAAAGAAGTCCTC GCCTCGCTGGCAAGTATTTGT | SIX12 | 449 | 61 | Taylor et al., 2016 |
| SIX13 | CCTTCATCATCGACAGTACAACG ATCAAACCCGTAACTCAGCTCC | SIX13 | 1027 | 61 | Taylor et al., 2016 |
| SIX14 | ATAAAGTGCGACTGGACTTCTGCC ACCCCCATCCACATTCCTAAGCGA | SIX14 | 422 | 67 | Taylor et al., 2016 |

**Table 3.3** LAMP Primers used for detection *SIX* genes in Fusarium isolates.

| | |
|---|---|
| **Six 2** | |
| F3 | CGAGAGGTATCACGATAACG |
| B3 | GACAGTTGACGTTCGTGA |
| FIP(F1c+F2) | CAGCCATTGTAGCCGTCGTATCTATGAGAAGCAATGATCCG |
| BIP(B1c+B2) | CCAACTCCGACACGCAGATTCGGACATGGTCAACATAGG |
| LoopF | CAGAGTTGAGTGAAAGATTGGC |
| LoopB | ACACCAACCACCGTCTTC |
| **Six 6** | |
| F3 | CGCTGTACACAAATCTCCC |
| B3 | TCGCATCAATCTTGGCTG |
| FIP(F1c+F2) | TTCCCGTCTCGACCTGTCCAAGCTCCGAATCTTGTCG |
| BIP(B1c+B2) | AATTCGCCTTCGGTGGTTCAGCTGAGCACACCATCAAT |
| LoopF | ACCGCTCCGTCTGCTATA |
| LoopB | CTCTTCTGGAGCGATGTCAATA |
| **Six 7** | |
| F3 | GTTGCCTGGTACTGTAGTATG |
| B3 | AACCAGAGTAAGTTATGTGGC |
| FIP(F1c+F2) | TCAACACCACCACGACCACCGCTAGTGAACGTGTTGA |
| BIP(B1c+B2) | AAGCATTGCCCTTGTCTTGGATTTCCATTTCGCCCTGTTT |
| LoopF | GCTAGAGAGGTGACATTTGACA |
| LoopB | TAGGTCGAGCATAGGTATAGCA |
| **Six13** | |
| F3 | CGTTGCATGAACTATCTTCATC |
| B3 | CTGGTTCTCCTATTGCTTAGG |
| FIP(F1c+F2) | GTAGAGCGAACAGAGCATACGGGGTGTTCGTCGTCTTGAC |
| BIP(B1c+B2) | GCTCGTCCACGACTTTGAGTAGTCTGATCAGCCTCCTAGC |
| LoopF | CTAGTCTCGGAAGCAGCAG |
| LoopB | TTCGTTGAAGGCCTGATCC |

### 3.3.9 Experimental Design and Data Analysis

A randomized experimental design was used. Disease severity indexes were compared using one-way ANOVA. A Tukey's post-hoc test was performed to compare the means. Statistical significance is defined as $P < 0.05$ for all experiments.

### 3.3.10 Results and discussion

#### 3.3.10.1 ITS sequence analysis

The ITS region was successfully amplified from most of the *Fo* isolate DNAs using the fungal specific universal primer pairs ITS1 and ITS4. The lengths of sequences as determined by capillary electrophoresis ranged from 600 bp to 650 bp (Figs 3.2 and 3.3). The BLAST analysis of the ITS rDNA sequence data showed the closest matches (98–100% similarity) in the NCBI GenBank database were found to be with *F. oxysporum*, *Fol* and *Forl,* and supported the morphological identifications (Table 3.4).

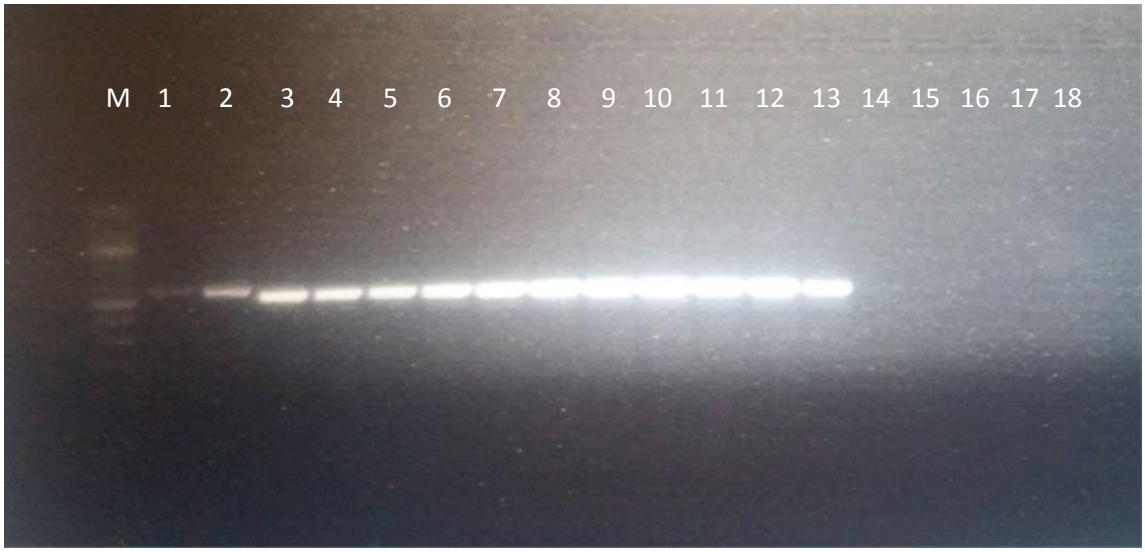


**Figure 3.2** ITS PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100-bp marker. Lanes 1 through 12 show ITS amplicons from F1, F2, F3, F4, F5, F6, F7, F8, F9, F9, F10,F11 and F12 respectively. Lane 13 represents positive control. Lanes 14 to 19 represent negative controls.


**Figure 3.3** ITS PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100-bp marker. Lanes 1 through 12 show ITS amplicons from F13, F14, F15, F16, F17, F18, F19, F20, F21, F22, F23 and F24 respectively. Lane 13 represents positive control. Lanes 14 to 19 represent negative controls.

**Table 3.4 Identification of *Fusarium oxysporum* isolates using PCR method with gene targeted, identity (%) and accession number.**

| Isolate code | Isolate name | gene | Identity% | Accession No. |
|---|---|---|---|---|
| F2 | *Fusarium solani* | ITS | 100 | KT716021.2 |
| F3 | *Fusarium oxysporum* f.sp. *radices- lycopersici* | pgx4 | 99 | AB256798.1 |
| F8 | *Fusarium oxysporum* f.sp. *lycopersici* | SIX1 | 99 | AJ608703.3 |
| F9 | *Fusarium oxysporum* f.sp. *lycopersici* | SIX1 | 97 | GQ268948.1 |
| F18 | *Fusarium oxysporum* f.sp. *lycopersici* | SIX4 | 99 | AB674509.1 |
| F19 | *Fusarium oxysporum* f.sp. *lycopersici* | SIX1 | 99 | AJ664546.1 |
| F21 | *Fusarium oxysporum* | ITS | 100 | KU382653.1 |
| F22 | *Fusarium oxysporum* | ITS | 100 | KT828536.1 |
| F23 | *Fusarium oxysporum* | ITS | 99 | KT358875.1 |
| F24 | *Fusarium oxysporum* | ITS | 100 | KR708632.1 |

### 3.3.10.2 *Fol* races and *Forl*

The result of the PCR based assay showed that a 992 bp fragments was amplified with the SIX1 primer set from *Fol* isolates and that isolates F8, F9, F18, and F19 were *Fol*, whereas the 947 bp fragment that was amplified with the sprl primers indicated that the F3 isolate was *Forl.* A 608 bp fragment that was amplified with the *SIX3a*, b, and c primers indicated that F8, F9 and F19 were *Fol* race 3, whilst the 967 bp fragment that was

amplified with the *SIX4* primers indicated that F18 was *Fol* race 1 (Figs 3.4 A and B and Table 3.5).

**Table 3.5 Molecular identification of *Fol* races and *Forl***

| | ITS | SIX1 | SIX4 | SIX3a | SIX3b | SIX3c | sprl | |
|---|---|---|---|---|---|---|---|---|
| F3 | + | - | - | - | - | - | + | *Forl* |
| F8 | + | + | - | + | + | + | - | *Fol* race3 |
| F9 | + | + | - | + | + | + | - | *Fol* race3 |
| F18 | + | + | + | - | - | - | - | *Fol* race1 |
| F19 | + | + | - | + | + | + | - | *Fol* race3 |

[+, amplicon was present; -, amplicon was absent]

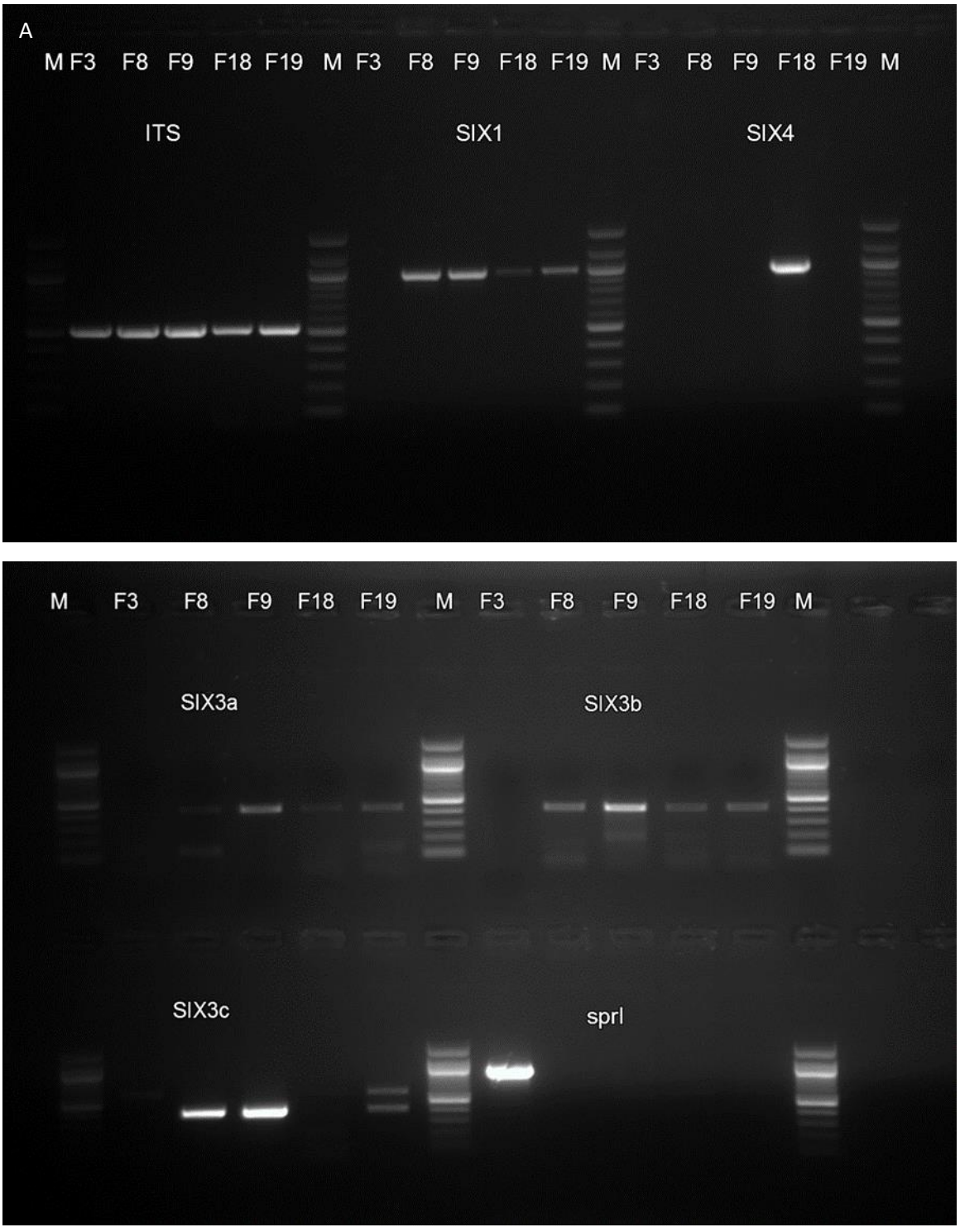


**Figure 3.4** PCR products amplified from isolates F3, F8, F9, F18 and F19 on 1.5% agarose gel. **A)** PCR products amplified with ITS, SIX1 and SIX4 primers. **B)** PCR products amplified with SIX3a, SIX3b, SIX3c and sprl primers.

### 3.3.10.3 Evaluation of four *Fol* isolates in causing Fusarium wilt disease on Moneymaker tomato variety.

Symptoms on infected plants appeared two weeks after inoculation. All four *Fol* isolates caused typical symptoms of Fusarium wilt including wilting, stunting, yellowing leaves and browning vascular tissue. The aggressiveness of the isolates was variable but generally high (Fig. 3.5A, B and C). Isolate F18 showed the strongest virulence with a score of 3.66 and disease severity greater than 91%. Isolates F8, F9, and F19 had virulence scores of 3.08, 2.16, 2.25 and disease severities of 77.08, 72.22 and 75.00% respectively (Table 3.6).

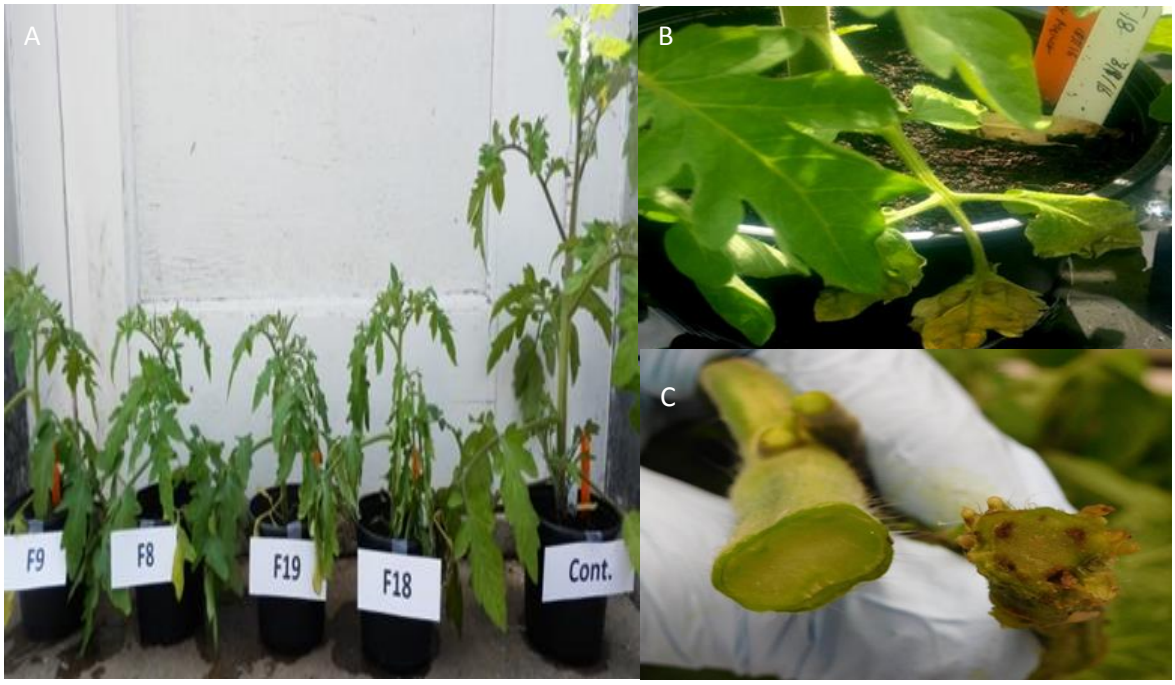


**Figure 3.5 A)** Comparison between *Fol* isolates (F8, F9, F18, f18) and the control 30 PDI. **B)** Wilting symptoms. **C)** Browning vascular symptoms.

**Table 3.6** Disease severity of *Fol* isolates on Moneymaker tomato variety.

| Treatments | Disease Severity Index (1-5) | Disease Severity % |
|---|---|---|
| Cont. | 1.00[a]* | 00.00 |
| F8 | 3.08[c] | 77.08 |
| F9 | 2.16[b] | 72.22 |
| F18 | 3.66[d] | 91.66 |
| F19 | 2.25[b] | 75.00 |
| Cont. | 1 | 0.00 |

*Values represents the mean of twelve replicates.

-Mean values followed by the same letters are not significantly different at $P<0.05$.

In this study, molecular techniques (PCR based assays) and pathogenicity tests have been shown to be efficient for identifying *Fo* formae speciales and races. The results showed that all isolates of *Fol* that were identified by PCR as race 1 and 3 were pathogenic to the tomato variety tested. However, the virulence of these races was varied. *Fol* F18 (race1) showed the greatest disease severity compared to the others and was therefore selected to be use in all this study's experiments.

### 3.3.10.4 Molecular identification of Fusarium isolates using SIX1 to SIX14 primers.

All *Fo* isolates listed in Table 2.1 were tested for the presence of 14 *SIX* genes by PCR assays. All PCR products of *Fo* were electrophoresed on 1.5% agarose gel to determine the size of the PCR products (Figs 3.6 to 3.10). Resulting amplicons were sequenced to verify gene identity. A BLAST of *Fo* predicted proteomes, queried with all available SIX proteins from NCBI GenBank and their alignments using a cutoff of 90% similarity and a minimum e-value of 0.001, predicted the presence of all 14 SIX proteins. Most *SIX* genes were found to be present in four formae specialis *lycopersici* (F8, F9, F18 and F19) but not in other formae speciales or nonpathogenic isolates (Table 3.7). The nucleotide sequence matches of *Fol six1*, *six2*, *six6*, *six7* and *six13* is shown in Figs 3.11 to 3.15.


**Figure 3.6** SIX1 PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100 bp marker. Lanes 1 through 19 show SIX1 amplicons from F1, F2, F3, F6, F7, F8, F9, F10, F11, F12, F13, F14, F16, F18, F19, F21, F22, F23 and F24.

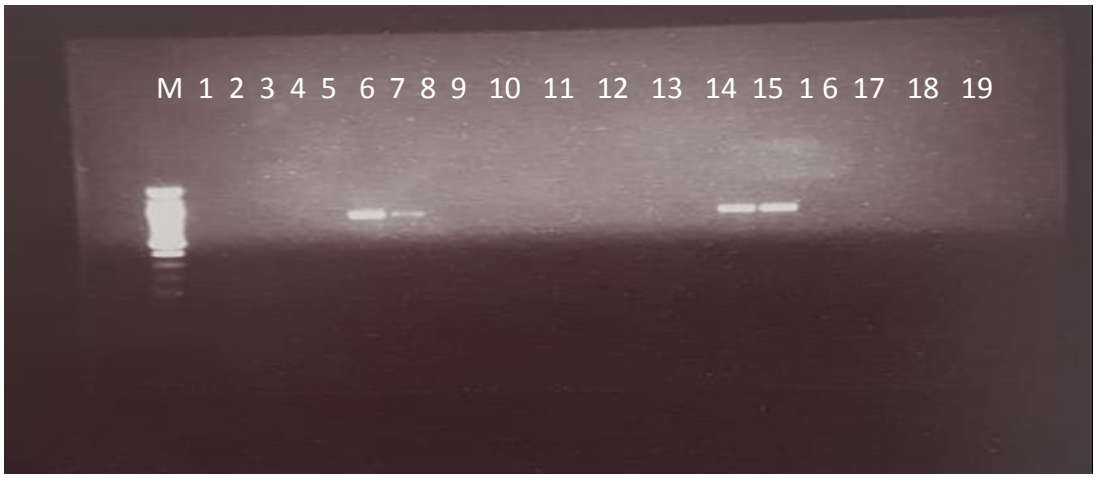


**Figure 3.7** SIX2 PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100 bp marker. Lanes 1 through 19 show SIX2 amplicons from F1, F2, F3, F6, F7, F8, F9, F10, F11, F12, F13, F14, F16, F18, F19, F21, F22, F23 and F24.

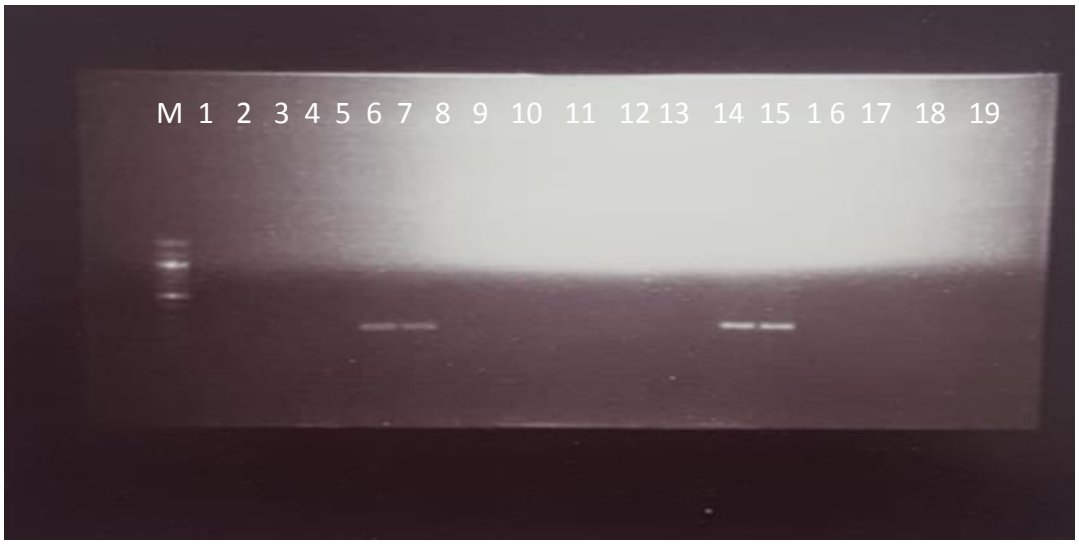


**Figure 3.8** SIX6 PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100 bp marker. Lanes 1 through 19 show SIX6 amplicons from F1, F2, F3, F6, F7, F8, F9, F10, F11, F12, F13, F14, F16, F18, F19, F21, F22, F23 and F24.


**Figure 3.9** SIX7 PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100 bp marker. Lanes 1 through 19 show SIX7 amplicons from F1, F2, F3, F6, F7, F8, F9, F10, F11, F12, F13, F14, F16, F18, F19, F21, F22, F23 and F24.


**Figure 3.10** SIX13 PCR products amplified from different *Fusarium oxysporum* isolates on 1.5% agarose gel. M, 100 bp marker. Lanes 1 through 19 show SIX13 amplicons from F1, F2, F3, F6, F7, F8, F9, F10, F11, F12, F13, F14, F16, F18, F19, F21, F22, F23 and F24.

**Fusarium oxysporum f. sp. lycopersici mRNA for secreted in xylem 1 protein (six1 gene)**

**Sequence ID:** AJ608702.1 **Length:** 1190 **Number of Matches:** 1

**Range 1: 181 to 1064** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1622 bits(878) | 0.0 | 883/885(99%) | 1/885(0%) | Plus/Minus |

```
Query  2     GGAGAGGCTTTGGATCGCAACTTAGTGTGGGCTGGTATATCCAAACGCCCAAGTTGCGC
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1064  GGAG-GGCTTTGGATCGCAACTTAGTGTGGGCTGGTATATCCAAACGCCCAAGTTGCGC

Query  62    ATATGTGTTTGTGATAGTCACCTTGCCTCTCGGGCAATTAATGCATGACCATGAATGTC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1005  ATATGTGTTTGTGATAGTCACCTTGCCTCTCGGGCAATTAATGCATGACCATGAATGTC

Query  122   ATATCCGTGAGAGCTCGTCTTATATCCATCCGAATAAACACCGTCATACCCGAAGTACC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  945   ATATCCGTGAGAGCTCGTCTTATATCCATCCGAATAAACACCGTCATACCCGAAGTACC

Query  182   ATTGAGAGTGCCCGCCATCTGGGCGAAATAATCGTAGGTCCCAGGCGATTTAGGCGATT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  885   ATTGAGAGTGCCCGCCATCTGGGCGAAATAATCGTAGGTCCCAGGCGATTTAGGCGATT

Query  242   GGGGTACCAGGTTCCCTGCCACTCCGTGTGTATCCCTATATCATGTCTGTCCTTCACCT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  825   GGGGTACCAGGTTCCCTGCCACTCCGTGTGTATCCCTATATCATGTCTGTCCTTCACCT

Query  302   AATTCGGGGCCCACAGACAGGAAAAGTAACCTGATGGTGACGGTTACGAAAGTTGTACG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  765   AATTCGGGGCCCACAGACAGGAAAAGTAACCTGATGGTGACGGTTACGAAAGTTGTACG

Query  362   ATTGAAAGTGGTGCACTCCTGGCCCTTTGGGCAAGCTCTGGTCACTGATCTGTCTCTCT
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  705   ATTGAAAGTGGTGCACTCCTGGCCCTCTGGGCAAGCTCTGGTCACTGATCTGTCTCTCT

Query  422   CCTGACAGACGGTTTATTACATTCGATTTTCCTAGTCGCCCGACGATCAGTGTAACATA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  645   CCTGACAGACGGTTTATTACATTCGATTTTCCTAGTCGCCCGACGATCAGTGTAACATA

Query  482   CCCACTAACCGCTCGATAGCCTGTGCCAAATCTTTCAACGCACAGATCATGAAGCGCCT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  585   CCCACTAACCGCTCGATAGCCTGTGCCAAATCTTTCAACGCACAGATCATGAAGCGCCT

Query  542   GAGCATGTCTTGTGTGACACGATCGTTGCGAGACTCCTCCCCGAAAGGCTCACGCTTTC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  525   GAGCATGTCTTGTGTGACACGATCGTTGCGAGACTCCTCCCCGAAAGGCTCACGCTTTC

Query  602   AAGGTTGACAATGTGACCACGCCTCGATAGGCTGCTGCCCGTCTCGGTCTGGACACCAT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465   AAGGTTGACAATGTGACCACGCCTCGATAGGCTGCTGCCCGTCTCGGTCTGGACACCAT

Query  662   GTCCGTCTCGTTTCCAGGAAAGCTGCCCATAGTGTCATTCCAGGGCAAATCACTCTTGT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405   GTCCGTCTCGTTTCCAGGAAAGCTGCCCATAGTGTCATTCCAGGGCAAATCACTCTTGT

Query  722   TGGGGGACTCCCGTGGGATGCTGCCACCTTATCCAGATATTCCGTGTAGGTCAAGTTGA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345   TGGGGGACTCCCGTGGGATGCTGCCACCTTATCCAGATATTCCGTGTAGGTCAAGTTGA

Query  782   AAATATCTGGGGCTCCCGAACCGCAGCCTCTTGAGCATAAGCCCCAAACCCAAGGATTG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285   AAATATCTGGGGCTCCCGAACCGCAGCCTCTTGAGCATAAGCCCCAAACCCAAGGATTG

Query  842   GAGGGCGCCAAGGAGTACCATGCTATAGGGCGCCATTTTGTCGAA  886
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225   GAGGGCGCCAAGGAGTACCATGCTATAGGGCGCCATTTTGTCGAA  181
```

**Fig. 3.11** Alignment of *six1* from *Fol* (F18, Query) with *six1* nucleotide sequence from Fol (accession number AJ608702.1, Subject).

**Fusarium oxysporum f. sp. lycopersici isolate Fol59 secreted in the xylem 2 (SIX2) gene, partial cds**

**Sequence ID:** MN745205.1 **Length:** 641 **Number of Matches:** 1

**Range 1: 1 to 641** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1173 bits(635) | 0.0 | 640/642(99%) | 1/642(0%) | Plus/Plus |

```
Query  22   TATATCCATAAAAGAAAATATGCTCTTCAAAATCGCGTGGGTCTCACTATTTACGACCTG
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TATATCCATAAAAG-AAATATGCTCTTCAAAATCGCGTGGGTCTCACTATTTACGACCTG

Query  82   GGCCATCTCGGTAGCTGCAAACCCGGCTGGGGATTCTCTGCCCGACGATGCGCATCTCCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   GGCCATCTCGGTAGCTGCAAACCCGGCTGGGGATTCTCTGCCCGACGATGCGCATCTCCC

Query  142  GGATCGTCGCCTATCGCCCTCCGAGGTTCAAGCTTTGAAAAAAGCCCAAATCTATCCTCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120  GGATCGTCGCCTATCGCCCTCCGAGGTTCAAGCTTTGAAAAAAGCCCAAATCTATCCTCC

Query  202  AGGTTACATTCATAAGAGGGTCACATTTGGAGAGGGCAAAGACGCCGTGGAAGTTCCCAT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  AGGTTACATTCATAAGAGGGTCACATTTGGAGAGGGCAAAGACGCCGTGGAAGTTCCCAT

Query  262  CGTTGAAGCTGATGTCGAGATGCTTCTAAACAACGAGAAGGGCGTCAAGGCACGTTCCCT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  CGTTGAAGCTGATGTCGAGATGCTTCTAAACAACGAGAAGGGCGTCAAGGCACGTTCCCT

Query  322  AGCACCACGAGGCTCCTGCTTTAGTTTTCCTACGCCAGCAAGAGGAAGCTGCATGATCGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  AGCACCACGAGGCTCCTGCTTTAGTTTTCCTACGCCAGCAAGAGGAAGCTGCATGATCGA

Query  382  TTACTGCTGGCGTGACGACAACGGGGTTATCTACTCGAGAGGTATCACGATAACGGGCAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360  TTACTGCTGGCGTGACGACAACGGGGTTATCTACTCGAGAGGTATCACGATAACGGGCAG

Query  442  TAACGGGGCGTCTAATCCTACATCTATGAGAAGCAATGATCCGGCCAATCTTTCACTCAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  TAACGGGGCGTCTAATCCTACATCTATGAGAAGCAATGATCCGGCCAATCTTTCACTCAA

Query  502  CTCTGTATTCAACGACGGCTACAATGGCTGGTTCCCGCACGGACACGCATGCTCCAACTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480  CTCTGTATTCAACGACGGCTACAATGGCTGGTTCCCGCACGGACACGCATGCTCCAACTC

Query  562  CGACACGCAGATTTACACCAACCACCGTCTTCTACAGGGCGTCAATGGTGTGGCCTATGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540  CGACACGCAGATTTACACCAACCACCGTCTTCTACAGGGCGTCAATGGTGTGGCCTATGT

Query  622  TGACCATGTCCGATGTGAAAACTGCAACTTCACGAACGTCAA  663
            |||||||||||||||||||||||||||||| |||||||||
Sbjct  600  TGACCATGTCCGATGTGAAAACTGCAACTTCAGGAACGTCAA  641
```

**Fig. 3.12** Alignment of *six2* from *Fol* (F18, Query) with *six2* nucleotide sequence from *Fol* (accession number MN745205.1, Subject).

**Fusarium oxysporum f. sp. lycopersici secreted in xylem Six6 (SIX6) mRNA, complete cds**

**Sequence ID:** FJ755835.2 **Length:** 678 **Number of Matches:** 1

**Range 1: 1 to 619** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1144 bits(619) | 0.0 | 619/619(100%) | 0/619(0%) | Plus/Minus |

```
Query  70   TTTTCATATATCGGCTTTTCGAGAACTGGAAATTGTCGCTGCTGAAGTACGATGTCGATC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  619  TTTTCATATATCGGCTTTTCGAGAACTGGAAATTGTCGCTGCTGAAGTACGATGTCGATC

Query  130  CACCAATACCTTCATTCACGTTGCCAGGCTCGCCGAAGTAGCTGATCTTATCAACTTCAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  559  CACCAATACCTTCATTCACGTTGCCAGGCTCGCCGAAGTAGCTGATCTTATCAACTTCAA

Query  190  TAGGATTCTTGTTAATGTCATACACAATGGTACCCAGATGATGATAACCTGCAGGGTTGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499  TAGGATTCTTGTTAATGTCATACACAATGGTACCCAGATGATGATAACCTGCAGGGTTGA

Query  250  CTGAGGTCGTGGTGCAGCCTTCCTTGTTCCCATTTGCGCTCGTCTTCCACTCTACAAGGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  CTGAGGTCGTGGTGCAGCCTTCCTTGTTCCCATTTGCGCTCGTCTTCCACTCTACAAGGT

Query  310  CAACGATAGGAATGCACTTGGCAAAGCTTTTCCCGTTGCTGAGATTGCGCTGTACACAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  CAACGATAGGAATGCACTTGGCAAAGCTTTTCCCGTTGCTGAGATTGCGCTGTACACAAA

Query  370  TCTCCCGCGGCTGGCAAGGGGTGTCGGTAATCTTTTCCCGGTTGCTGCGAGGGTTCGCCA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  TCTCCCGCGGCTGGCAAGGGGTGTCGGTAATCTTTTCCCGGTTGCTGCGAGGGTTCGCCA

Query  430  CGCAAAAGCTCCGAATCTTGTCGGCTTTATAGCAGACGGAGCGGTCATATTTCTGTCCGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  CGCAAAAGCTCCGAATCTTGTCGGCTTTATAGCAGACGGAGCGGTCATATTTCTGTCCGG

Query  490  CTGGACAGGTCGAGACGGGAAGCGTGTCTCGAGAGACCAAAGATGATAAATTCGCCTTCG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  CTGGACAGGTCGAGACGGGAAGCGTGTCTCGAGAGACCAAAGATGATAAATTCGCCTTCG

Query  550  GTGGTTCAAACTCTTCTGGAGCGATGTCAATATAATTGATGGTGTGCTCAGCCACATCAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  GTGGTTCAAACTCTTCTGGAGCGATGTCAATATAATTGATGGTGTGCTCAGCCACATCAG

Query  610  CAGATTCGGATTCGGTTTGAGCAAGGGGACCAGCTACGCAGCCGGCAGCCAAGATTGATG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   CAGATTCGGATTCGGTTTGAGCAAGGGGACCAGCTACGCAGCCGGCAGCCAAGATTGATG

Query  670  CGATAAGAGCGAGCTTCAT  688
            ||||||||||||||||||
Sbjct  19   CGATAAGAGCGAGCTTCAT  1
```

**Fig. 3.13** Alignment of *six6* from *Fol* (F18, Query) with *six6* nucleotide sequence from *Fol* (accession number FJ755835.2, Subject).

**Fusarium oxysporum f. sp. lycopersici isolate PLM905 secreted in xylem 7 (SIX7) gene, complete cds**

**Sequence ID:** MH764405.1 **Length:** 672 **Number of Matches:** 1

**Range 1: 1 to 667** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1232 bits(667) | 0.0 | 667/667(100%) | 0/667(0%) | Plus/Minus |

```
Query  70   TTGACTACAGATTTACTACTGACTGTGTTAACCTATGAAAACGAAAGTCAGCAAGGCCCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667  TTGACTACAGATTTACTACTGACTGTGTTAACCTATGAAAACGAAAGTCAGCAAGGCCCC

Query  130  TGGGCTTATAAATCATCTGATGGGTTTTGGCGTAGCAACGGGCATTCATTAAGCGGTTGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  TGGGCTTATAAATCATCTGATGGGTTTTGGCGTAGCAACGGGCATTCATTAAGCGGTTGG

Query  190  CGTATTGCCTCTCAGGAATGACTTCAAGGGGGTAGGAGTGCTTGGGCTTACTTTGAATCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  CGTATTGCCTCTCAGGAATGACTTCAAGGGGGTAGGAGTGCTTGGGCTTACTTTGAATCC

Query  250  GGAGCCGATAGGTAATATCTCCTCGCCGACCCCAAGTTTGAAGGCAGACCGTAGTCACCT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  GGAGCCGATAGGTAATATCTCCTCGCCGACCCCAAGTTTGAAGGCAGACCGTAGTCACCT

Query  310  GACCCCAGCCCGCTAAACCACCGCCTGTGACCTCTTCGTTGGGCCTAAAGACGTTGCTAT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  GACCCCAGCCCGCTAAACCACCGCCTGTGACCTCTTCGTTGGGCCTAAAGACGTTGCTAT

Query  370  CGATAGCGCGATATCGACTTGTTCCGGGAGCATCCGGAAGCAAGACTACTTGCGCCTCGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  CGATAGCGCGATATCGACTTGTTCCGGGAGCATCCGGAAGCAAGACTACTTGCGCCTCGG

Query  430  CAGTACCGAAAACCCGAACCTCAACGAACGTAGTTCGTTGCCTGGTACTGTAGTATGCGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  CAGTACCGAAAACCCGAACCTCAACGAACGTAGTTCGTTGCCTGGTACTGTAGTATGCGC

Query  490  GCCATTGGTATCTAATATTCGAGAGTCCTACACCCTCAGTCCATGGTGTGGACGCGGCGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  GCCATTGGTATCTAATATTCGAGAGTCCTACACCCTCAGTCCATGGTGTGGACGCGGCGC

Query  550  TAGTGAACGTGTTGACATTTTGGGTGATGTCAAATGTCACCTCTCTAGCCGCGGGTGGTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  TAGTGAACGTGTTGACATTTTGGGTGATGTCAAATGTCACCTCTCTAGCCGCGGGTGGTG

Query  610  GTCGTGGTGGTGTTGAGCCGGTGGTGCTAAAGCATTGCCCTTGTCTTGGAAATAGGTCGA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  GTCGTGGTGGTGTTGAGCCGGTGGTGCTAAAGCATTGCCCTTGTCTTGGAAATAGGTCGA

Query  670  GCATAGGTATAGCACTCGCAAACAGGGCGAAATGGAAAAGGAGGTAAAGGTACTTCATAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   GCATAGGTATAGCACTCGCAAACAGGGCGAAATGGAAAAGGAGGTAAAGGTACTTCATAA

Query  730  CCTGCAT  736
            |||||||
Sbjct  7    CCTGCAT  1
```

**Fig. 3.14** Alignment of *six7* from *Fol* (F18, Query) with *six7* nucleotide sequence from *Fol* (accession number MH764405.1Subject).

**Fusarium oxysporum f. sp. lycopersici strain Fol007 Six13 (SIX13) mRNA, complete cds**

**Sequence ID:** KC701451.1 **Length:** 882 **Number of Matches:** 2

**Range 1: 1 to 443** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 819 bits(443) | 0.0 | 443/443(100%) | 0/443(0%) | Plus/Minus |

```
Query  490  ACATTATTATCCGTGATGGGGTGCATTGTATAGCAGCAAGCGCAAGTGATCCGCCTGAAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  443  ACATTATTATCCGTGATGGGGTGCATTGTATAGCAGCAAGCGCAAGTGATCCGCCTGAAC

Query  550  GTGCCTGCGCAAACCGAACCAGTTGAGCCAAACGTTTTAACATCGTTGTGCGTTTGAGTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  383  GTGCCTGCGCAAACCGAACCAGTTGAGCCAAACGTTTTAACATCGTTGTGCGTTTGAGTT

Query  610  CCCCATTTTGCGTTGCATGAACTATCTTCATCTGAATCAACATAAAGACGTCCTCCCCTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  323  CCCCATTTTGCGTTGCATGAACTATCTTCATCTGAATCAACATAAAGACGTCCTCCCCTC

Query  670  GGGCAAGGTCCGTTAGGGTGTTCGTCGTCTTGACGCTTCTGTACTGCTGCTTCCGAGACT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  263  GGGCAAGGTCCGTTAGGGTGTTCGTCGTCTTGACGCTTCTGTACTGCTGCTTCCGAGACT

Query  730  AGGGCCCGTTCCGTATGCTCTGTTCGCTCTACCGAGAGCTCGTCCACGACTTTGAGTAGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  203  AGGGCCCGTTCCGTATGCTCTGTTCGCTCTACCGAGAGCTCGTCCACGACTTTGAGTAGC

Query  790  TCCTCTTCGTTGAAGGCCTGATCCCGATAAGTATTTTCGACGCTAGGAGGCTGATCAGAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  143  TCCTCTTCGTTGAAGGCCTGATCCCGATAAGTATTTTCGACGCTAGGAGGCTGATCAGAC

Query  850  AAATCAGACACCTCTAACTCCCCTAAGCAATAGGAGAACCAGGAGAAAAGTAGTGGTAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  83   AAATCAGACACCTCTAACTCCCCTAAGCAATAGGAGAACCAGGAGAAAAGTAGTGGTAAA

Query  910  AGGATAAGATGAAACCGAGTCAT  932
            |||||||||||||||||||||||
Sbjct  23   AGGATAAGATGAAACCGAGTCAT  1
```

**Fig. 3.15** Alignment of *six13* from *Fol* (F18, Query) with *six13* nucleotide sequence from *Fol* (accession number KC701451.1, Subject).

**Table 3.7 Presence / absence of *SIX* genes in *Fusarium oxysporum* (*Fo*) isolates.**

| | | ***Secreted in Xylem (SIX)* genes** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fusarium Isolates** | **code** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| Fusarium solani 1 | **F1** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium solani 2 | **F2** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| FORL A | **F3** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| FORL B | **F4** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium Bx15/4a | **F5** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium Bx15/4b | **F6** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium Bx15/4c | **F7** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| FOC 2A | **F8** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| FOC 2B | **F9** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Fusarium* sp Bx14/153a | **F10** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium* sp Bx14/153b | **F11** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium* sp Bx14/153c | **F12** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium* sp Bx14/152a | **F13** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium* Ex.Root Bx14/128a | **F14** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium* sp Bx14/168a | **F16** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Fusarium oxysporum* f. sp. *lycopersici* Race1 | **F18** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Fusarium oxysporum* f. sp. *lycopersici* Race2 | **F19** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Fusarium oxysporum* f. sp. *radicis lycopersici* | **F21** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium (FOL) BX08/54 | **F22** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium (FORL) BX10/15 | **F23** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fusarium (FOL??) BX13/39 | **F24** | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Plus sign, present; minus sign, absent.

## 3.3.10.5 LAMP assay

The amplification plots visible in Figures 3.11 to 3.13 represent the results of LAMP sensitivity assays. Positive amplification plots were observed for all *Fol* isolates (F8, F9, F18 and F19) tested with SIX2, SIX6, SIX7 and SIX13 LAMP primers. No amplification was observed in *Fo* isolates (F22 and F23) and non-template controls. Table 3.8 and 3.9 represent the time needed and annealing temperatures for each isolate to be amplified.

**Table 3.8 Amplification time and annealing temperature for *Fol* and *Fo* isolates with SIX2 and SIX6 LAMP primers.**

| Well Number | Well Name | Amplification time mm.ss | Annealing °C |
|---|---|---|---|
| 1 | SIX2 F8 | 11:45 | 88.2 |
| 2 | SIX2 F9 | 13:30 | 88.2 |
| 3 | SIX2 F18 | 12:00 | 88.0 |
| 4 | SIX2 F19 | 16:15 | 88.3 |
| 5 | SIX2 F22 | - | - |
| 6 | SIX2 F23 | - | - |
| 7 | + | 08:45 | 88.2 |
| 8 | - | - | - |
| 9 | SIX6 F8 | 15:00 | 88.5 |
| 10 | SIX6 F9 | 14:45 | 88.4 |
| 11 | SIX6 F18 | 13:00 | 88.5 |
| 12 | SIX6 F19 | 17:15 | 88.5 |
| 13 | SIX6 F22 | - | - |
| 14 | SIX6 F23 | - | - |
| 15 | + | 09:15 | 88.3 |
| 16 | - | - | - |

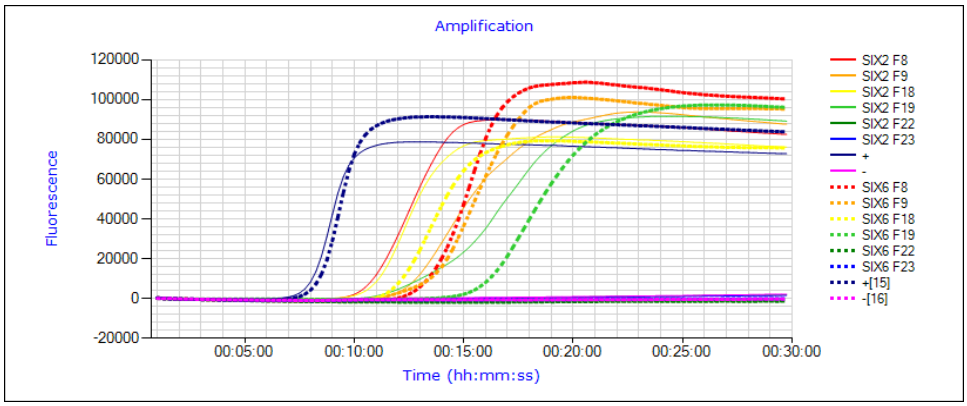


**Figure 3.16** The specificity of *Fol* LAMP detection method. The amplification plots of *Fol* isolates F8, F9, F18 and F19 detected with SIX2 and SIX6 primers. F22 and F23 showed no amplification. + and - stand for positive and non-template control respectively.

**Table 3.9 Amplification time and annealing temperature for *Fol* and *Fo* isolates with SIX7 and SIX13 LAMP primers.**

| Well Number | Well Name | Amplification time mm.ss | Annealing c° |
|---|---|---|---|
| 1 | SIX7 F8 | 12:45 | 88.1 |
| 2 | SIX7 F9 | 13:15 | 88.1 |
| 3 | SIX7 F18 | 11:45 | 88.0 |
| 4 | SIX7 F19 | 14:15 | 88.1 |
| 5 | SIX7 F22 | - | - |
| 6 | SIX7 F23 | - | - |
| 7 | + | 08:45 | 88.0 |
| 8 | - | - | - |
| 9 | SIX13 F8 | 12:15 | 88.0 |
| 10 | SIX13 F9 | 12:00 | 88.2 |
| 11 | SIX13 F18 | 11:15 | 88.2 |
| 12 | SIX13 F19 | 15:30 | 88.3 |
| 13 | SIX13 F22 | - | - |
| 14 | SIX13 F23 | - | - |
| 15 | + | 09:00 | 88.2 |
| 16 | - | - | - |

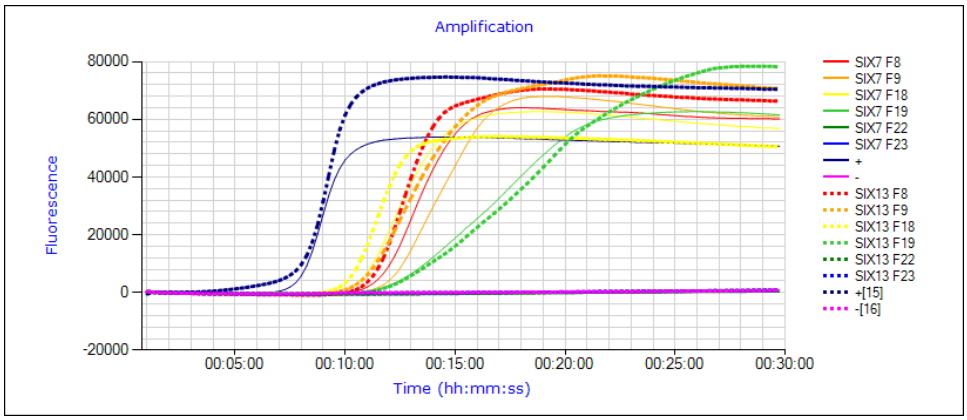


**Figure 3.17** The specificity of *Fol* LAMP detection method. The amplification plots of *Fol* isolates F8, F9, F18 and F19 detected with SIX7 and SIX13 primers. F22 and F23 showed no amplification. + and - stand for positive and non-template control respectfully.

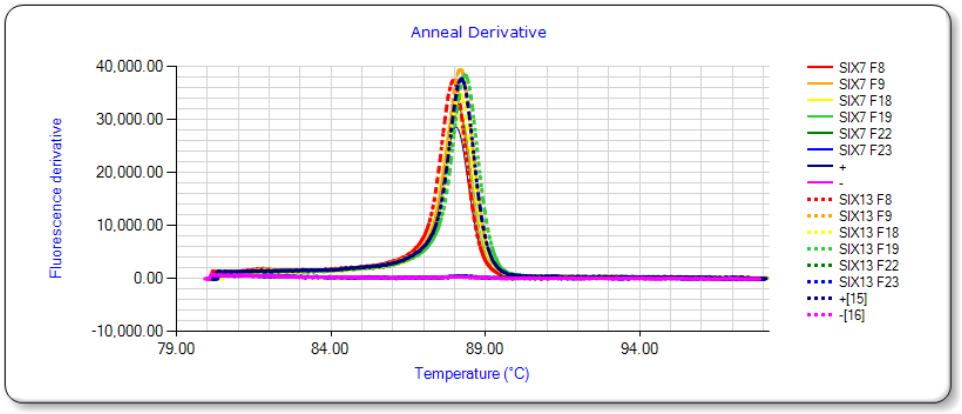


**Figure 3.18** Melting curve analysis of obtained amplification products with SIX7 and SIX13 primers.

The LAMP assays developed for *FoI* detection were specific and sensitive, with the SIX7 set being the slightly quicker in terms of time to amplification, and therefore the optimal set based on these preliminary studies. However, the other sets of primers were also effective and only slightly slower in their amplification times. Designed method and primers in this study were confirmed to be specific and fast to discriminate *FoI* from other *Fo* species. These results indicate that the developed LAMP method can accurately and effectively detect pathogens in DNA samples.

Detection of *SIX* genes in this study was based on PCR and LAMP assays and they demonstrated the importance of these molecular techniques to detect specific DNA in *Fo* isolates and discriminate between them.

## 3.4 Discussion

Six primer sets (*SIX3*a, b, c, *SIX4, SIX1 and sprl*) were used to target four *SIX* and pgx4 protein genes to distinguish between *Fol* races and *Forl.* This study showed that the primers tested can be an efficient tool to be used in discrimination of *Fol* races and *Forl* and can be also successful in order to discriminate *Fo* infecting tomato plants. The present findings are in agreement with Lievens *et al* (2009b) who used several six proteins for the identification of *Fo* forma specialis and races based on host- specific virulence genes.

21 *Fo* isolates were tested for the presence of 14 *SIX* genes by PCR assays. This study showed that only four *Fo* isolates (F8, F9, F18 and F19) seem to have all 14 SIX proteins and which have identified as *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). Based on the results presented here, this supports the idea that these genes are secreted into the xylem sap of infected tomato plants and responsible for the ability to cause tomato wilt. These findings are supported the results of Rep *et al.*, (2005a) who investigated the ability of *SIX1, SIX2* and *SIX3* to contribute the development of vascular wilt disease in tomato.

This study developed a loop-mediated isothermal amplification (LAMP) assay for detection and differentiation of *Fusarium oxysporum* (*Fo*). Five LAMP primers have used in this study to detect *SIX1, SiX2, SIX6, SIX7* and

*SIX13* genes in some of *Fo* isolates. The results showed that all *Fol* (F8, F9, F18 and F19) were positive with LAMP primers. These findings are in agreement with Ayukawa *et al*., (2016) who used *SIX4, SIX5* and *SIX1* LAMP primers as a rapid and simple tool for detection and discrimination of *Fol* races.

# Chapter 4. Investigating UV-C seed treatments for the control of fusarium wilt and crown and root rot disease on tomato.

## 4.1 Introduction

The application of fungicides to control fungal diseases is the most used and effective strategy in management of plant diseases. However, their use can cause negative impacts on the environment and frequent use enhances the risk of fungicide resistance developing. Therefore, there is a need to develop alternative methods to control plant diseases. Alternative techniques, such as biological control, physical treatments, and use of natural products have already been tested with success in controlling plant diseases (Marquenie, 2002; Tripathy and Dubey, 2004; Pal *et al.,* 2006).

UV is genotoxic electromagnetic radiation with wavelengths that fall between 100 and 400 nm. The portion of the electromagnetic light between 100 and 280 nm is referred to as the UV-C region. Hormesis is the application of low doses of UV-C to living organisms in order to induce stress responses. The use of UV-C has shown some benefits such as induced disease resistance, delayed chlorophyll degradation, and improved nutritional content (Shama and Alderson, 2005; Ribeiro *et al.,* 2012). Hormetic UV-C seed treatments have now been shown to have

positive impacts including increased germination rate, improved crop colour, delayed maturity, increased dry mass, increased seedling vigour index and an increased number of root nodules (Brown *et al.,* 2001; Hamid and Javvaid, 2011; Siddiqui *et al.,* 2011; Neelamegam and Sutha, 2015).

As examples of UV-C being used for enhancing disease resistance, Brown *et al.* (2001) reported reduced *Xanthomonas campestris* pv. *campestris* disease levels in cabbage plants when seeds were treated with UV-C. The results showed seeds treated with UV-C at 3.6 kJ $m^{-2}$ resulted in reduced disease incidence of black rot of cabbage by 90% compared to the control plants. Also, Sid-diqui *et al.* (2011) demonstrated reductions of up to 88% in the incidence of disease caused by *Fusarium* spp., *Rhizoctonia solani* and *Macrophomina phaseolina* on mung bean (*Vigna radiata*) and groundnut (*Arachis hypogaea*).

## 4.2 Aims and objectives

The aim of this study was to investigate the effect of a range of UV-C seed treatments on disease severity of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) on tomato. In addition, experiments were undertaken to confirm the presence of DNA of the pathogens in treated plants using LAMP assays. Quantification of the

levels of pathogen DNA in treated plants were also analysed using qPCR assays.

## 4.3 Materials and Methods

### 4.3.1 Seeds and UV-C treatment

Seeds of the tomato cv. Moneymaker were purchased from Johnsons' Seeds. Seeds were stored at 6 °C at a relative humidity of <50%. UV-C treatments were performed with a U-shaped amalgam UV-C source (UVI 120 U 2G11 C P 15/469) with peak emission at 254 nm, obtained from Dr Gilbert Shama, University of Loughborough, UK. The source was housed within an anodized aluminium parabolic reflector. Seeds were treated at an intensity of 20 W $m^{-2}$ at 0, 2, 4, and 6 kJ $m^{-2}$ and immediately stored in the dark at 21 °C for 5 days following the procedures of Brown *et al.* (2001). UV-C intensity was measured using a radiometer (Model UVX; UVP Instruments) fitted with a 254 nm sensor.

### 4.3.2 Plant material

Seeds of the tomato Moneymaker were planted in 1 L pots of Levington M3 compost and grown in a heated and ventilated glasshouse. The lighting and ventilation were as stated in section 2.5.

### 4.3.3 inoculum preparation and inoculation

*Fol* and *Forl* inoculations were performed on cv. Moneymaker seedlings 22 days after planting. Plants were inoculated with spore solutions of $10^6$ $ml^{-1}$ as was stated in section 2.4. The compost was moistened, and seedlings were carefully removed. Roots were gently rubbed with Celite (Sigma Aldrich) and incubated in 30 ml of spore solution for 30 min. Plants were repotted and not watered until the following day. Disease severity was scored at 30 days post-inoculation following the methods of Reis and Boiteux (2007) and Parke and Grau (1992) for *Fol* and *Forl,* respectively. Two independent experimental replicates were performed with a total of twelve biological repeats per treatments. In addition, six seedlings were inoculated with sterile distilled water and used as a mock inoculated plants (control) for the inoculation procedures.

### 4.3.4 PCR and LAMP analysis

DNA was extracted from approximately 100 mg of tissue from roots, crown and stems of all treated plants using the DNeasy Plant Mini kit protocol (QIAGEN) as was stated in section 2.2. PCR amplification was performed using SIX1 primers to amplify the *SIX1* gene in *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and sprl primers to amplify exo-polygalacturonase (*Pgx4*) genes in *Fusarium oxysporum* f. sp. radicis *lycopersici* (*Forl*) (Table

4.1). Thermal cycling conditions consisted of 2 min at 94 °C, followed by 35 cycles of 45s at 94 °C, (annealing temperature is listed in Table 1) and 45s at 72 °C, with a final elongation step at 72 °C for 10 min. Amplified products were resolved electrophoretically in a 1.5% agarose gel and then visualized by UV.

LAMP assays were performed using SIX1 and sprl LAMP primers to amplify *FoI* and *ForI* DNA respectively, as stated in Table 4.2.

**Table 4.1.** Primers used for molecular characterisation of *FoI* and *ForI* isolates with product size, annealing temperature and relevant publications.

| **Primers** | **Sequence 5'- 3' forward primer reverse primer** | **Gene** | **Product size** | **Annealing temp.** | **References** |
|---|---|---|---|---|---|
| SIX1 | GTATCCCTCCGGATTTTGAGC<br>AATAGAGCCTGCAAAGCATG | *SIX1* | 992 | 59 | Lievens *et al.,* 2009 |
| sprl | GATGGTGGAACGGTATGACC<br>CCATCACACAAGAACACAGGA | *Pgx4* | 947 | 62 | Hirano &Arie 2006 |

**Table 4.2** LAMP Primers used for detection *SIX1* and *pgx4* genes in *Fol* and *Forl.*

| | |
|---|---|
| **Six 1** | |
| F3 | CCTGGAGCATGTCTTGTG |
| B3 | CTCAATCCTTGGGTTTGGG |
| FIP(F1c+F2) | CCTGGAAACGAGACGGACGAGGTTGACAATGTGACCAC |
| BIP(B1c+B2) | CCAGGGCAAATCACTCTTGTCTAACTTGACCTACACGGAATATC |
| LoopF | ATGGTGTCCAGACCGAGA |
| LoopB | GATGCTGCCACCTTATCCA |
| **Sprl1** | |
| F3 | GAGTTCACTTCAAGGACTCC |
| B3 | AGCAATCATCACCAATGTCA |
| FIP (F1c+F2) | CTTGAACGAGATGTCCTTGGCTGTCGTCACCTGTAAGGAAC |
| BIP(B1c+B2) | CACCAACGCCACTGCTCTCGCTCAACATTGAGGTTCT |
| LoopF | GATCATCAGGACTTGGCTAGG |
| LoopB | CTGACTTCTTTGACTCCCTGAA |

## 4.3.5 Quantification of *Fol* and *Forl* DNA in treated plants

Absolute Quantification was used to quantify the concentration of *Fol* and *Forl* DNA in treated plants using a standard curve generated with samples of a known quantity. A 10 µl reaction contained 5µl of 2 x Fast SYBR® Green master mix (Applied Biosystems), 2 µl of template DNA (1ng/ul) and 0.5 µl of each forward and reverse Primer (Table 4.3) Reactions were run on a

Mic PCR (Bio Molecular Systems) with 40 amplification cycles. The cycle was as follows; a pre-incubation of 5 min at 95 °C followed by 40 cycles of 95 °C for 5 s and the anneal for (FOL) DNA samples 60 °C and (FORL) samples 62 °C at 20 s, extension 72°C for 20 s, melting curves 65 to 95 °C.

DNA standard curves were obtained from 5-fold dilutions of *Fol* and *Forl* culture DNA, and were serially diluted (1:1, 1:10, $1:10^2$, $1:10^3$ and $1:10^4$). The Ct values obtained for each dilution series were used to generate a standard curve for absolute quantification of *Fol* and *Forl* DNA. The baseline threshold value of the real-time PCR was set automatically to determine the Ct value of DNA extracted from treated plants.

**Table 4.3.** Primers used for qPCR of *Fol* and *Forl* isolates with target gene and annealing temperature.

| **Primers** | **Sequence 5'- 3'**<br>**forward primer**<br>**reverse primer** | **Target Gene** | **Annealing temp.** |
|---|---|---|---|
| SIX1 | CCTGGAGCATGTCTTGTG<br>CTCAATCCTTGGGTTTGGG | *SIX1* | 59 |
| sprl | GAGTTCACTTCAAGGACTCC<br>AGCAATCATCACCAATGTCA | *Pgx4* | 62 |

### 4.3.6 Experimental Design and Data Analysis

A completely randomised experimental design within the glasshouse was applied to study the effect of four UV-C seed treatments, 0, 2, 4 and 6 $kJ/m^2$ on disease development for *Fol* and *Forl*. Two independent experimental replicates were performed with a total of twelve biological replicates per treatment group. Data was analysed in SPSS (IBM) by Kruskal Wallis (non-parametric ANOVA) with post-hoc testing by X utilising the adjusted p-values for pairwise comparisons. Statistical significance is defined as p=0.05 in all experiments

## 4.4 Results

### 4.4.1 UV-C seed treatments

The germination percentages of all treated seeds were approximately 90%, which suggests that the low doses of UV-C did not affect the seeds' germination rates (Table 4.4). All plants derived from tomato seeds that were treated with different UV-C doses and inoculated with *Fol* and *Forl* were scored for their symptoms after 30 days from inoculation. Stunting, wilting, necrosis, vascular browning, and yellowing leaves were observed in some treatments (Figs 4.1, 4.2, 4.3 and 4.4). The results showed that there were significant reductions in disease severity for plants grown from

UV-C-treated seeds when inoculated with *Fol* or *Forl*. For *Fol*, both 4 and 6 kJ $m^{-2}$ doses significantly decreased disease severity, with median scores dropping from 3.30 (control) to 2.25 and 2.00, respectively (Fig. 4.5). The percentage disease severity score was decreased from 87.5% (control) to 75.0% and 66.6% with 13.71% and 23.88% reductions in disease progression respectively when disease scores were converted to the disease severity (Table 4.5). A 6 kJ $m^{-2}$ dose significantly reduced *Forl* severity, with a median score of 1.5 compared to the control median of 2.0 (Fig. 4.6). The percentage of disease severtiy was decreased from 69.4% (control) to 50.0% with 27.95% when disease scores were converted to the disease severity (Table 4.6).

**Table 4.4** Germination % after 15 days from planting.

| Treatments | Tot | No. | % |
|---|---|---|---|
| 0 | 40 | 37 | **92.5** |
| 2 | 40 | 38 | **95.0** |
| 4 | 40 | 37 | **92.5** |
| 6 | 40 | 36 | **90.0** |


**Figure 4.1** Stunting and wilting symptoms caused by *Fol* on plants derived from treated seeds (0, 2, 4 and 6 kjm$^{-2}$ ) compared to the mock inoculated plant.


**Figure 4.2** No clear foliar symptoms caused be *Forl* on plants derived from treated seeds (0, 2, 4 and 6 kjm$^{-2}$ ) compared to the mock inoculated plant.

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**Figure 4.3** Different levels of vascular browning in the stems of plants treated with (0, 2, 4 and 6 $kjm^{-2}$ ) and inoculated with *Fol*.


**Figure 4.4** Different levels of necrosis on the crown and roots of plants treated with (0, 2, 4 and 6 $kjm^{-2}$ ) and inoculated with *Forl*.

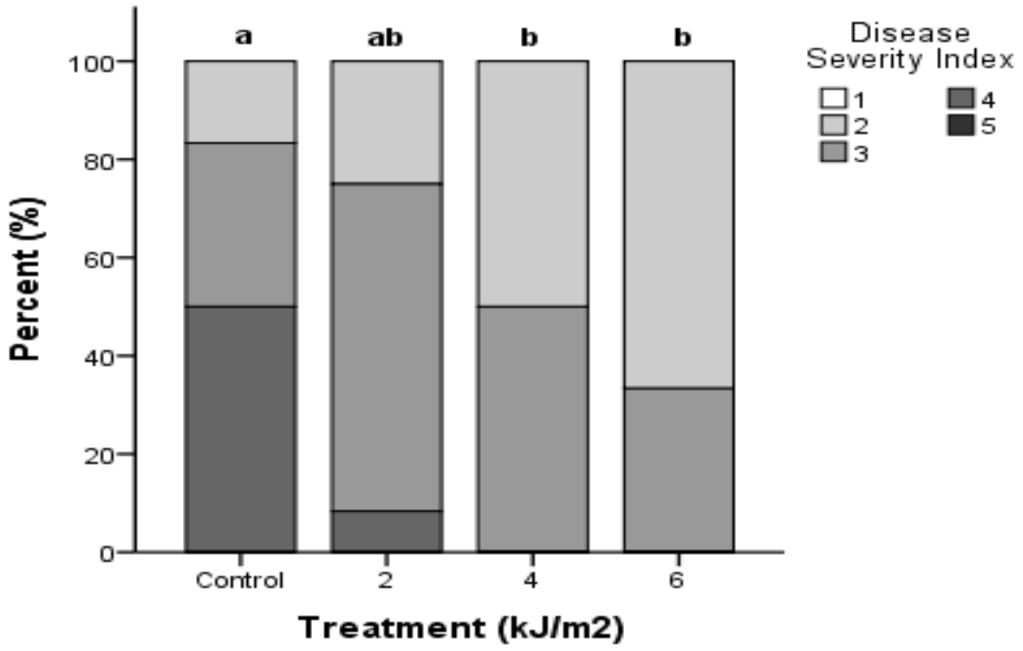


**Figure 4.5** The effect of UV-C seed treatments on the disease severity caused by *Fol*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=12.

**Table 4.5** Disease severity for *Fol* on Moneymaker tomato variety.

| UV-C doses | Disease Severity Index (1-5) | Disease Severity % |
|---|---|---|
| 0 | 3.33[a]* | 87.5 |
| 2 | 2.83[a] | 80.5 |
| 4 | 2.50[b] | 75.5 |
| 6 | 2.33[b] | 66.6 |

*Values represent the mean of twelve replicates.

-Mean values followed by the same letters are not significantly different at P<0.05.

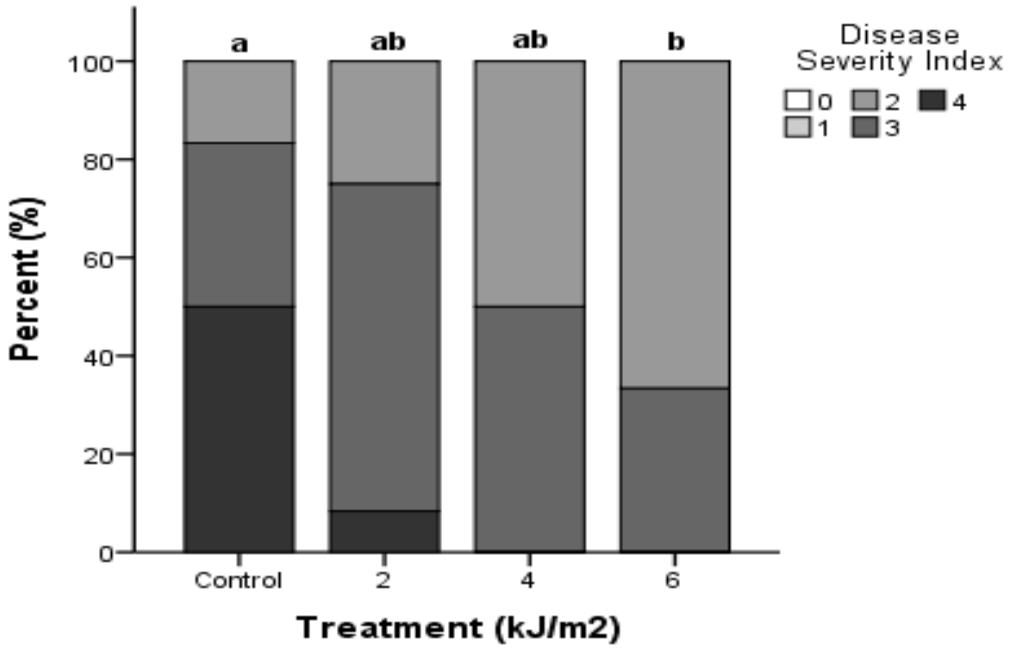


**Figure 4.6** The effect of UV-C seed treatments on the disease severity of *Forl*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=12.

**Table 4.6** Disease severity for *Forl* on Moneymaker tomato variety.

| UV-C doses | Disease Severity Index (0-4) | Disease Severity % |
|---|---|---|
| 0 | 2.08[a]* | 69.4 |
| 2 | 2.00[a] | 66.6 |
| 4 | 1.83[a] | 61.1 |
| 6 | 1.50[b] | 50.0 |

*Values represent the mean of twelve replicates.

-Mean values followed by the same letters are not significantly different at P<0.05.

## 4.4.2 PCR and LAMP analysis

PCR amplification of DNA extracted from all treated plants was carried out using SIX1 and Sprl primers. The PCR products were electrophoresed on 1.5% agarose gel to determine the size of PCR products for *Fol* and *Forl*. Amplicons of 992 bp were observed from extracted DNA for all root and stem samples inoculated with *Fol* using SIX1 primers and root and crown samples for *Forl* using sprl primers. The DNA of mock samples showed no amplicons (Figs 4.7 and 4.8). The results of LAMP assays showed that DNA from roots, crown, and stems of all treated plants were detected using SIX1 and Sprl LAMP primers (Figs 4.9 and 4.10). Tables 4.7 and 4.8 are showing times of amplification and annealing temperature for the detection assays.

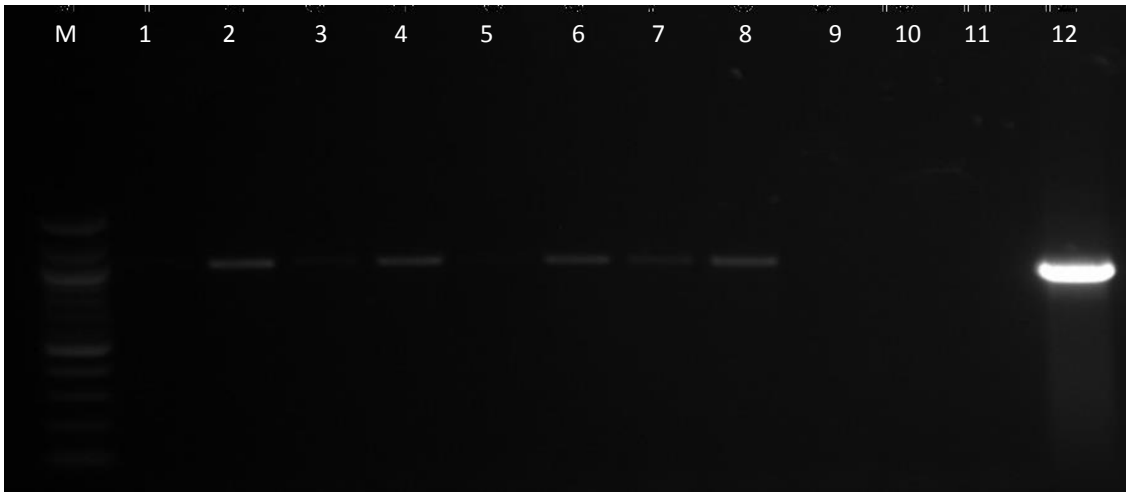


**Figure 4.7** PCR results showing a 992 bp PCR product with the SIX1 primers from the extracted DNA of all plants treated with *Fol*. **M:** 100bp marker. 1) 0 Roots. 2) 0 Stems. 3) 2 Roots. 4) 2 Stems. 5) 4 Roots. 6) 4 Stems. 7) 6 Roots. 8) 6 Stems. 9) -Cont. Roots. 10) Cont. Stems. 11) Negative control. 12) Positive control. The numbers 0, 2, 4 and 6 refer to the UV-C dosage.


**Figure 4.8** The 947 bp PCR products from the extracted DNA of all treated plants for *Forl* with the sprl primers. M: 100bp marker. 1) 0 Roots. 2) 0 Crowns. 3) 2 Roots. 4) 2 Crowns. 5) 4 Roots. 6) 4 Crowns. 7) 6 Roots. 8) 6 Crowns. 9) -Control Roots. 10) - Control Crowns 11) Negative control. 12) Positive control. The numbers 0, 2, 4 and 6 refer to the UV-C dosage.

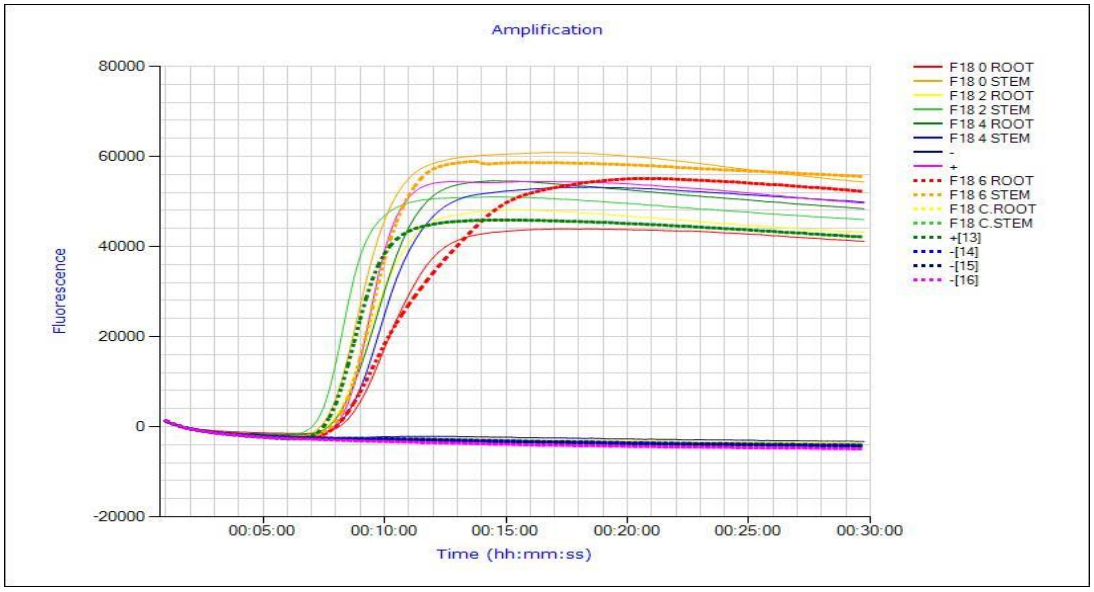


**Figure 4.9** The amplification plots for *Fol* DNA from roots and stem of 0, 2, 4 and 6 kjm$^{-2}$ treated plants detected with SIX1 LAMP primers. The control roots and stems showed no plots. + and - stand for positive and non-template controls respectively.

**Table 4.7** Amplification time and annealing temperature for *Fol* DNA from treated plants with SIX1 LAMP primers.

| Well N0. | Well Name | Amplification time Mm:ss | Anneal Derivative ˚C |
|---|---|---|---|
| 1 | F18 0 ROOT | 09:45 | 85.7 |
| 2 | F18 0 STEM | 08:45 | 85.9 |
| 3 | F18 2 ROOT | 09:00 | 85.9 |
| 4 | F18 2 STEM | 08:15 | 85.7 |
| 5 | F18 4 ROOT | 09:30 | 85.8 |
| 6 | F18 4 STEM | 09:30 | 85.8 |
| 7 | - | - - | |
| 8 | + | 09:15 | 85.6 |
| 9 | F18 6 ROOT | 09:15 | 85.7 |
| 10 | F18 6 STEM | 09:15 | 85.8 |
| 11 | F18 C.ROOT | - - | |
| 12 | F18 C.STEM | - - | |
| 13 | + | 08:45 | 85.6 |
| 14 | - | - - | |
| 15 | - | - - | |
| 16 | - | - - | |

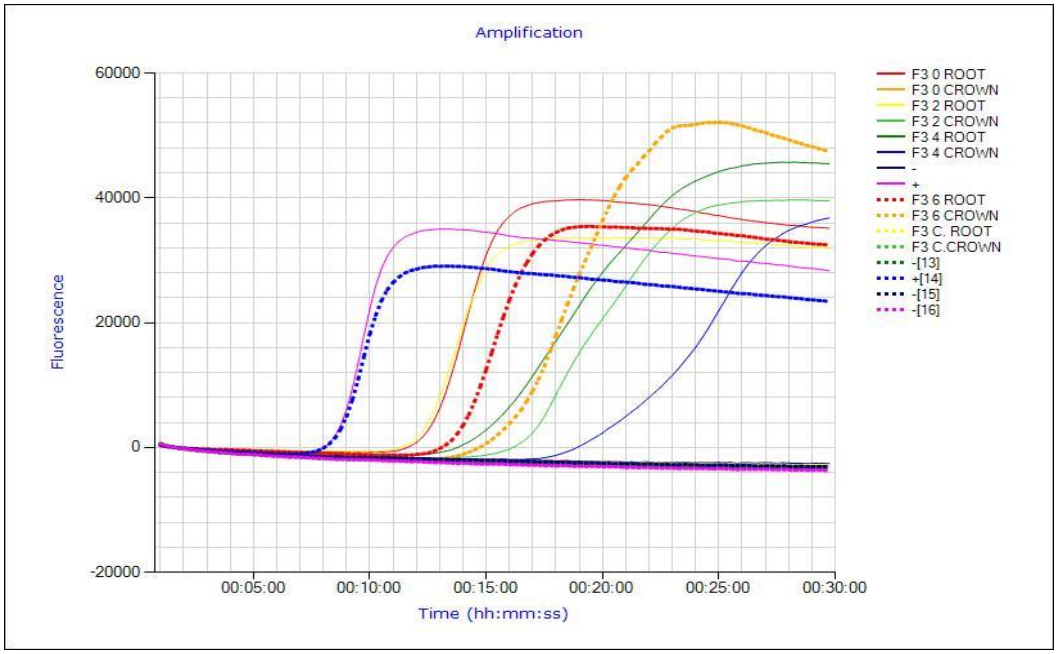


**Figure 4.10** The amplification plots for *Forl* DNA from roots and crowns of 0, 2, 4 and 6 kjm$^{-2}$ treated plants detected with Sprl LAMP primers. The control roots and crowns showed no plots. + and - stand for positive and non-template control respectively.

**Table 4.8** Amplification time and annealing temperature for *Forl* DNA from treated plants with sprl LAMP primers.

| Well N0. | Well Name | Amplification time Mm:ss | Anneal Derivative ˚C |
|---|---|---|---|
| 1 | F3 0 ROOT | 14:00 | 91.6 |
| 2 | F3 0 CROWN | | |
| 3 | F3 2 ROOT | 13:30 | 91.7 |
| 4 | F3 2 CROWN | 18:00 | 91.6 |
| 5 | F3 4 ROOT | | 91.6 |
| 6 | F3 4 CROWN | 25:00 | 91.6 |
| 7 | - | - - | |
| 8 | + | 09:45 | 91.7 |
| 9 | F3 6 ROOT | 15:15 | 91.6 |
| 10 | F3 6 CROWN | 18:00 | 91.6 |
| 11 | F3 C.ROOT | - - | |
| 12 | F3 C.CROWN | - - | |
| 13 | - | - - | |
| 14 | + | 09:45 | 91.7 |
| 15 | - | - - | |
| 16 | - | - - | |

### 4.4.3 qPCR analysis

Real-time qPCR amplification was used to quantify the *Fol* and *Forl* DNA present in samples from roots, crowns and stems collected from different treatments. DNA fragments of *Fol* and *Forl* from treated plants were successfully amplified using specific primers SIX1 and Sprl respectively (Figs 4.11 and 4.13). Standard regression lines were generated for *Fol* and *Forl* using genomic DNA ranging from 1ng to 0.1 pg/µl (Figs 4.12 and 4.16). The regression equation for the DNA standard curve for *Fol* was y= -2.67x+ 18.36 and ($R^2$=0.9960), and for *Forl* was y=-1.50x+18.91 and ($R^2$=0.8948). The results indicated that, in tested root samples, the quantity of *Fol* DNA extracted from 0, 2, 4 and 6 $kjm^{-2}$ treatments were 1.2892, 0.5456, 0.5332 and 0.3611 ng/µl, whereas in tested stem samples the DNA quantity were 1.0174, 0.9985, 0.5133 and 0.0935 ng/µl respectively (Table 4.9, Figs 4.13 and 4.14). For *Forl*, the DNA quantities from the 0, 2, 4 and 6 $kjm^{-2}$ treatments in tested root samples were 3.5322, 1.6222, 0.1836 and 0.0657 ng/µl, whilst in tested crown samples the DNA quantities were 1.4711, 2.1393, 0.1788 and 0.0377 ng/µl respectively (Table 4.10, Figs 4.17 and 4.18). The results showed that there was a correlation between the reduction in the disease severity by UV-C seed treatments (4 and 6 $kjm^{-2}$) and the DNA quantity in the root, crown and stem samples for *Fol* and *Forl*.

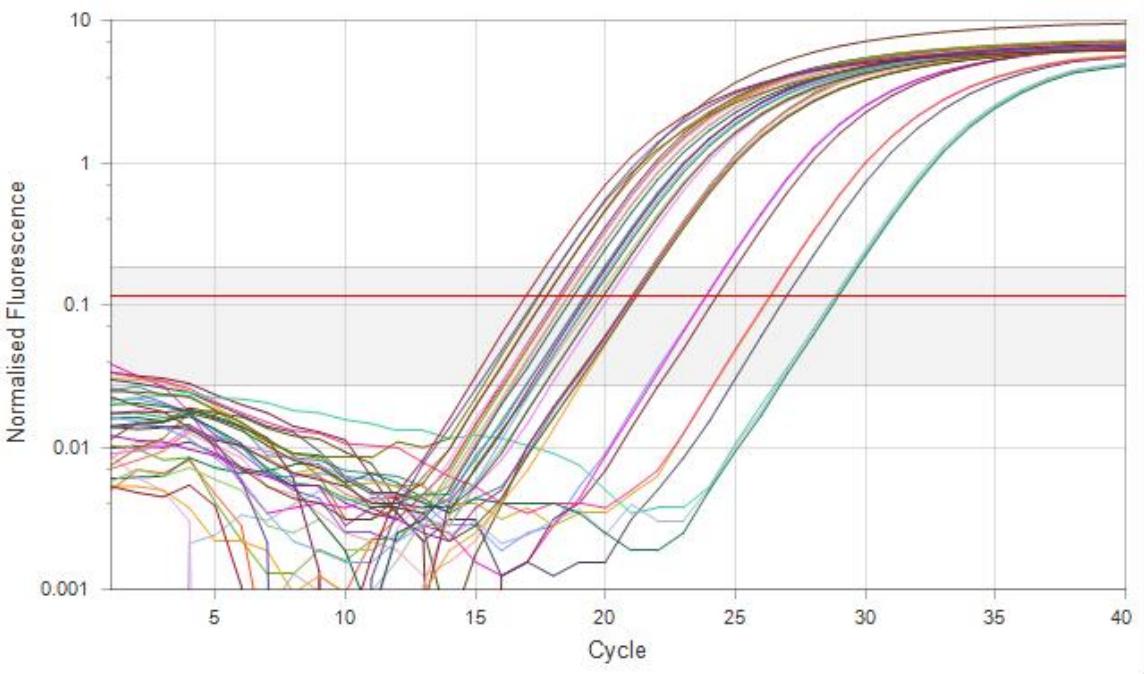


**Figure 4.11** Amplification curves of roots and stem DNA samples of *Fol*.

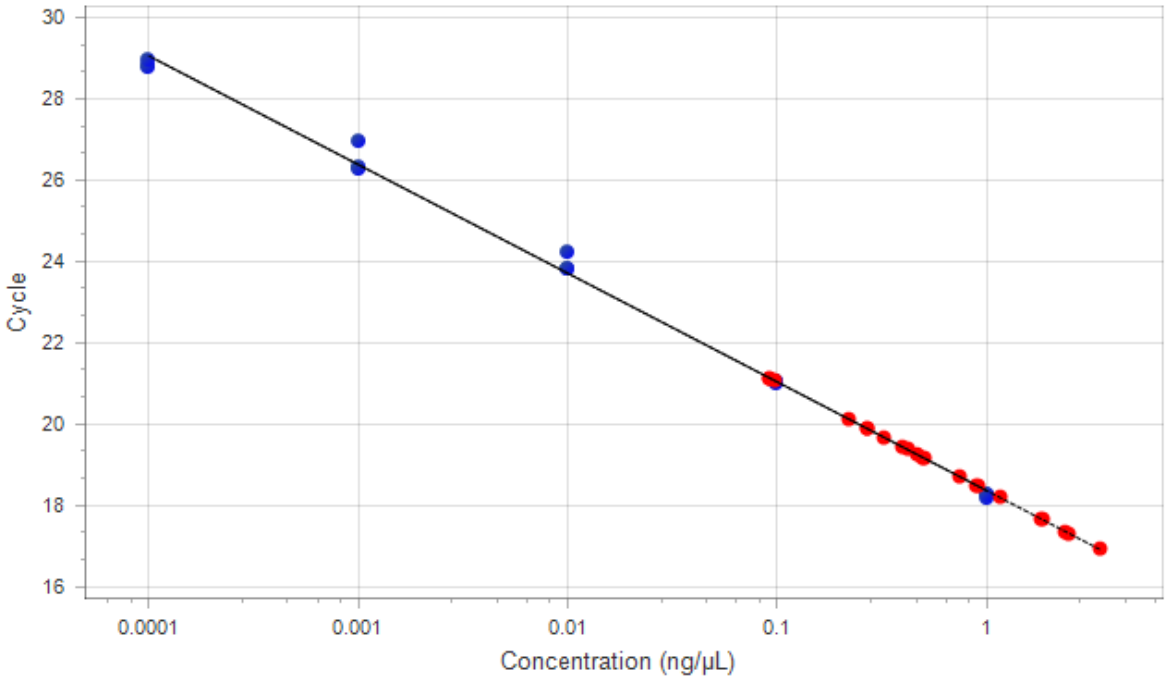


**Figure 4.12** Standard regression lines of 10-fold serial dilution of *Fol* culture DNA (blue dots) and DNA quantity of root and stem samples (red dots).

**Table 4.9** Ct values and DNA quantity of root and stem samples of *Fol*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µL)** |
| 16 | 0R | 18.20 | 1.15 |
| 17 | 0R | 17.66 | 1.833 |
| 18 | 0R | 18.50 | 0.8858 |
| 19 | 2R | 19.15 | 0.5077 |
| 20 | 2R | 20.12 | 0.2202 |
| 21 | 2R | 18.47 | 0.9093 |
| 22 | 4R | 18.71 | 0.7402 |
| 23 | 4R | 19.25 | 0.4671 |
| 24 | 4R | 19.45 | 0.3933 |
| 25 | 6R | 19.19 | 0.491 |
| 26 | 6R | 19.89 | 0.2685 |
| 27 | 6R | 19.67 | 0.3247 |
| 28 | 0S | 19.89 | 0.2673 |
| 29 | 0S | 17.36 | 2.369 |
| 30 | 0S | 19.38 | 0.4168 |
| 31 | 2S | 18.52 | 0.9652 |
| 32 | 2S | 18.71 | 1.236 |
| 33 | 2S | 19.38 | 0.792 |
| 34 | 4S | 19.22 | 0.523 |
| 35 | 4S | 19.31 | 0.386 |
| 36 | 4S | 20.15 | 0.6321 |
| 37 | 6S | 21.06 | 0.09813 |
| 38 | 6S | 21.13 | 0.09191 |
| 39 | 6S | 21.15 | 0.09091 |
| 40 | + | 16.93 | 3.447 |
| 41 | + | 17.67 | 1.813 |
| 42 | + | 17.33 | 2.444 |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | N | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

R=Roots, S=Stems, +=Positive and N=Negative.

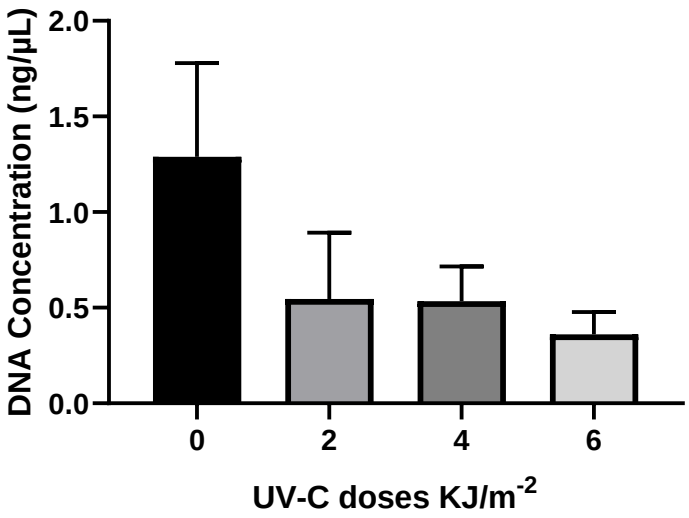


**Figure 4.13** The effect of UV-C seed treatments on DNA quantity of root samples caused by *Fol*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

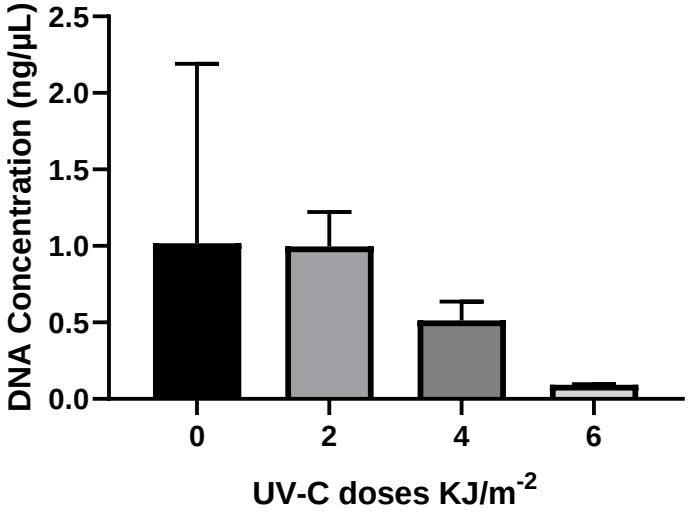


**Figure 4.14** The effect of UV-C seed treatments on DNA quantity of stem samples caused by *Fol*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

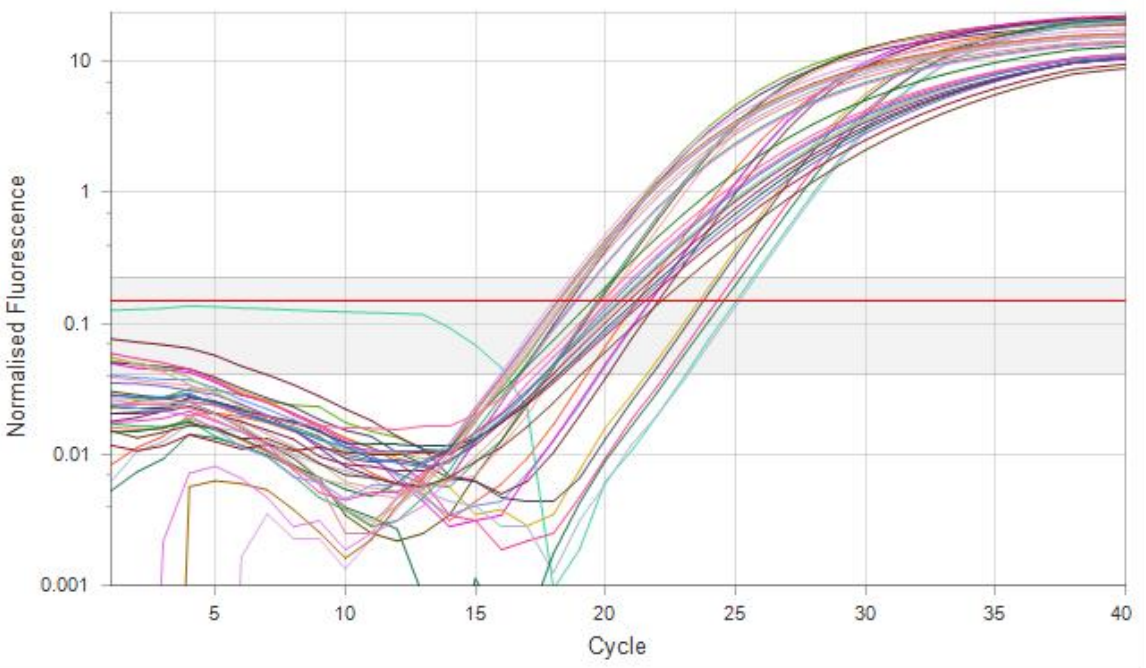


**Figure 4.15** Amplification curves of roots and stem DNA samples of *Forl*.

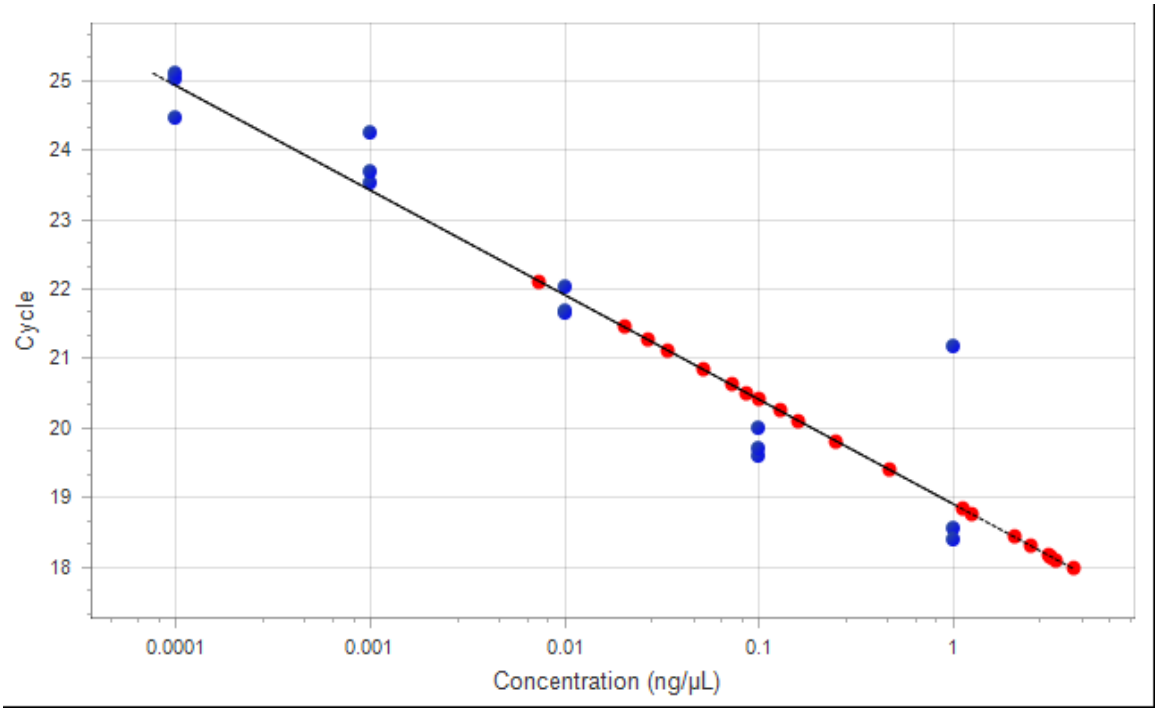


**Figure 4.16** Standard regression lines of 10-fold serial dilution of *Forl* culture DNA (blue dots) and DNA quantity of roots and crown samples (red dots).

**Table 4.10** Ct values and DNA quantity of roots and crown samples of *Forl*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µL)** |
| 16 | 0R | 17.99 | 4.129 |
| 17 | 0R | 18.12 | 3.389 |
| 18 | 0R | 18.18 | 3.08 |
| 19 | 2R | 18.77 | 1.248 |
| 20 | 2R | 18.85 | 1.108 |
| 21 | 2R | 18.31 | 2.512 |
| 22 | 4R | 21.13 | 0.03347 |
| 23 | 4R | 20.85 | 0.05146 |
| 24 | 4R | 19.41 | 0.466 |
| 25 | 6R | 21.28 | 0.02653 |
| 26 | 6R | 20.42 | 0.09901 |
| 27 | 6R | 20.63 | 0.07193 |
| 28 | 0C | 18.22 | 1.389 |
| 29 | 0C | 18.32 | 1.554 |
| 30 | 0C | - | - |
| 31 | 2C | 18.35 | 2.853 |
| 32 | 2C | - | - |
| 33 | 2C | 18.88 | 1.425 |
| 34 | 4C | 20.25 | 0.1281 |
| 35 | 4C | 19.82 | 0.2501 |
| 36 | 4C | 20.11 | 0.1588 |
| 37 | 6C | 21.46 | 0.02032 |
| 38 | 6C | 22.12 | 0.007345 |
| 39 | 6C | 20.52 | 0.08567 |
| 40 | + | 18.44 | 2.065 |
| 41 | + | 18.16 | 3.186 |
| 42 | + | 18.17 | 3.13 |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | N | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

R=Roots, C=Crowns, +=Positive and N=Negative.

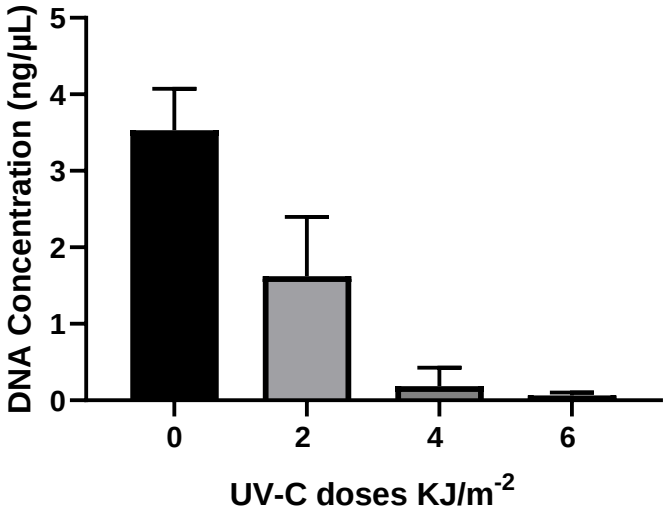


**Figure 4.17** The effect of UV-C seed treatments on DNA quantity of root samples caused by *Forl*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

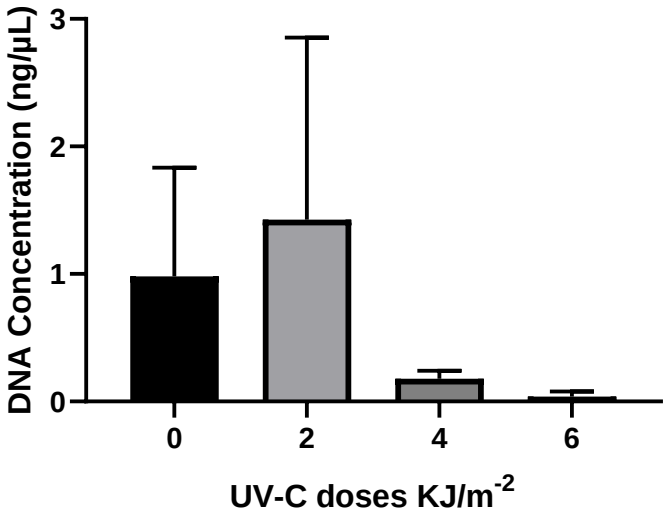


**Figure 4.18** The effect of UV-C seed treatments on DNA quantity of crown samples caused by *Forl*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

## 4.5 Discussion

Low doses of hormetic ultraviolet light-C (UV-C) seed treatments were used to elicit host resistance to Fusarium wilt and crown and root rot diseases of tomato in greenhouse studies. Different UV-C doses (0, 2, 4 and 6 kJ $m^{-2}$) were tested to determine their ability to induce resistance to fusarium wilt and crown and root rot diseases. The 4 and 6 $kJm^{-2}$ treatment showed significant reductions of 13.71% and 23.88% respectively in disease severity of *Fol*, whilst, the 6 $kJm^{-2}$ treatment showed significant reductions of 27.95% in disease severity of *Forl*.

It was noted that there was a reduction in disease severity and DNA quantity as UV-C doses increased for *Fol* and *Forl*. The present findings are in agreement with the findings of Brown *et al.* (2001) and Siddiqui *et al.* (2011) who reported reduced incidence of *X. campestris* pv*. campestris* on cabbage and *Fusarium* spp., *R. solani* and *M. phaseolina* on mung bean and groundnut following UV-C seed treatment. Further investigation is required to determine how UV-C seed treatments reduce disease progression by focusing on the molecular changes that occur in treated plants and determining what genes and physiological changes are induced by the UV-C treatment. The mechanisms supporting disease control following UV-C seed treatments are currently poorly understood. However, induced resistance by regulating resistance-related genes is a likely player and has been shown to occur against *Botrytis cinerea*

following UV-C treatment of tomato fruit (Charles *et al.,* 2008a; Scott *et al.,* 2018).

These findings are the first observations of UV-C seed treatment inducing disease control of *Fol* and *Forl* in tomato. This study has identified that UV-C seed treatments are an effective disease control method against *For* and *Forl* with 4 and 6 $kJ/m^{-2}$ doses being shown to significantly affect the reduction in disease progression. However, it is possible that cultivars may be varied in their response to UV-C doses and the biostimulation against different pathogens, so further studies are required to test additional cultivars and determine whether the hormetic UV-C treatments have protective effects against other pathogens.

# Chapter 5. Effect of Sodium Silicate for the control of fusarium wilt and crown and root rot disease on tomato.

## 5.1 Introduction

Silicon (Si) comprises about 28% of the Earth's crust and represents the second most abundant mineral element in soil (Ye *et al.,* 2013). The beneficial effects of silicon have been documented in many plant species for plant growth and resistance to both abiotic and biotic stresses. For example, sodium silicate, calcium silicate, and potassium silicate can play major roles in increasing plant resistance to diseases such as powdery mildew and Fusarium wilt in cucumber (*Cucumis sativus* L.) (Safari *et al.,* 2012), sheath blight and blast in rice (*Oryza sativa* L.) (Zhang *et al.,* 2013), and powdery mildew in wheat (*Triticum aestivum* L.) (Guével *et al.,* 2007).

The mechanisms of exogenous silicates in enhancing resistance in plants includes: first, Si increases the physical barrier of the cuticle and in the cell walls through the polymerization process to prevent pathogens from penetration (Debona *et al.,* 2017). Second, Si enhances the activities of plant defensive enzymes, which leads to increases in the accumulation of defensive compounds such as phenolics and phytoalexins to improve plant resistance to biotic and abiotic stresses (Reynolds *et al.,* 2016). Third, Si can induce systemic resistance in plants. For example, potassium silicate increased the activity of chitinase and b-1,3-glucanase in pea seeds in pea

plants that have been inoculated with *Mycosphaerella pinodes* (Dann and Muir, 2002).

## 5.2 Aims and objectives

The aim of this study was to investigate the effect of sodium silicate concentrations (0, 10, 20, 30, 40 and 50 mM) on disease severity of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) on tomato under glasshouse conditions. In addition, experiments were undertaken to confirm the presence of DNA of the pathogens in treated plants using LAMP assays and quantification of the pathogens' DNA using qPCR assays.

## 5.3 Materials and Methods

### 5.3.1 The effect of sodium silicate on *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*).

#### 5.3.1.1 *In vitro* culture experiments with sodium silicate

The dual culture technique was used to evaluate the effect of a range of concentrations (0, 10, 20, 30, 40, and 50 mM) of sodium silicate (metasilicate $Na_2SiO_3$) on mycelial growth of *Fol* and *Forl* under laboratory

conditions (Fig. 5.1). The sodium silicate concentrations were made through addition to 500 ml autoclaved PDA and 5 mm diameter discs of 7 days old FOL and FORL cultures were placed in the middle of the sodium silicate+PDA Petri dishes. For control treatments, a mycelial disc of tested pathogens was placed on the PDA medium alone. The plates were incubated at 27$^{o}$C for 5 days. Radial growth of tested pathogens was measured, and percent inhibition of average radial mycelial growth were calculated in relation to growth of the control. Four replicates of each treatment were measured, and percentage inhibition calculated according to the formula:

Percentage inhibition= (C-E)/Cx100, where C=colony diameter on the control, E= colony diameter on the experimental Petri dishes.

Completely randomized experimental design was used for four Petri dishes of each treatment.

### 5.3.1.2 Effect of sodium silicate solution on the severity of fusarium wilt and crown and root rot diseases

#### 5.3.1.2.1 Preparation of sodium silicate solution and treatment method

Sodium silicate solutions were prepared by dissolving the reagent of sodium silicate (Sigma-Aldrich) in distilled water (DW). The concentrations were adjusted to 0, 10, 20, 30, 40, and 50 mM. Each silicate aqueous solution (30 mL) was applied to the plants as a soil drench at three time points within a week (every two days) (Elsharkawy *et al*., 2015).

#### 5.3.1.2.2 Plant material

Seeds of the tomato cv. Moneymaker were planted in 1 L pots of Levington M3 compost and grown in a heated and, ventilated glasshouse. The lighting, heating and ventilation were as stated in section 2.5.

#### 5.3.1.2.3 Inoculum preparation and inoculation

*Fol* and *Forl* inoculations were performed on tomato cv. Moneymaker seedlings 22 days after planting. Plants were inoculated with spore solutions of $10^6$ $mL^{-1}$ as was stated in section 2.4.

#### 5.3.1.2.4 LAMP analysis

LAMP analysis was performed for DNA of all treated plants with different concentrations of sodium silicate as stated in section 4.3.4.

#### 5.3.1.2.5 Quantification of *Fol* and *Forl* DNA in treated plants

Absolute Quantification was used to quantify the concentration of *Fol* and *Forl* DNA in treated plants using a standard curve generated with samples of a known quantity as stated in section 4.3.5.

### 5.3.2 Experimental design and data analysis

A completely randomised experimental design within the glasshouse was applied to study the effect of five concentrations (0, 10, 20, 30, 40, and 50 mM) of sodium silicate on disease development for *Fol* and *Forl*. Two independent experimental replicates were performed with a total of 12 biological replicates per treatment group. Data was analysed in SPSS as stated in section 4.3.6.

## 5.4 Results and discussion

### 5.4.1 *In vitro* effect of sodium silicate on mycelial growth of *Fol* and *Forl*

#### 5.4.1.1 *In vitro* culture experiments with sodium silicate

The suppression capabilities of different sodium silicate concentrations were assessed by the inhibition of *Fol* and *Forl* mycelial growth through the dual culture test (Fig. 5.1). The low concentrations (10 and 20 mM) of sodium silicate recorded low inhibition in the mycelial growth of *Fol* 15.02, 20.30 %, and 21.25, 30.00% for *Forl* respectfully. The higher concentrations (30, 40 and 50 mM) showed significant reduction in the mycelial growth of both *Fol* and *Forl*. They recorded the highest inhibition values and the main effects ranged between 45 to 98%, with 30, 40 and 50 mM concentrations recording 57.23, 73.77 and 96.25% reduction for *Fol*, and 46.25, 81.99 and 97.73% for *Forl* respectively (Table 5.1 and 5.2).

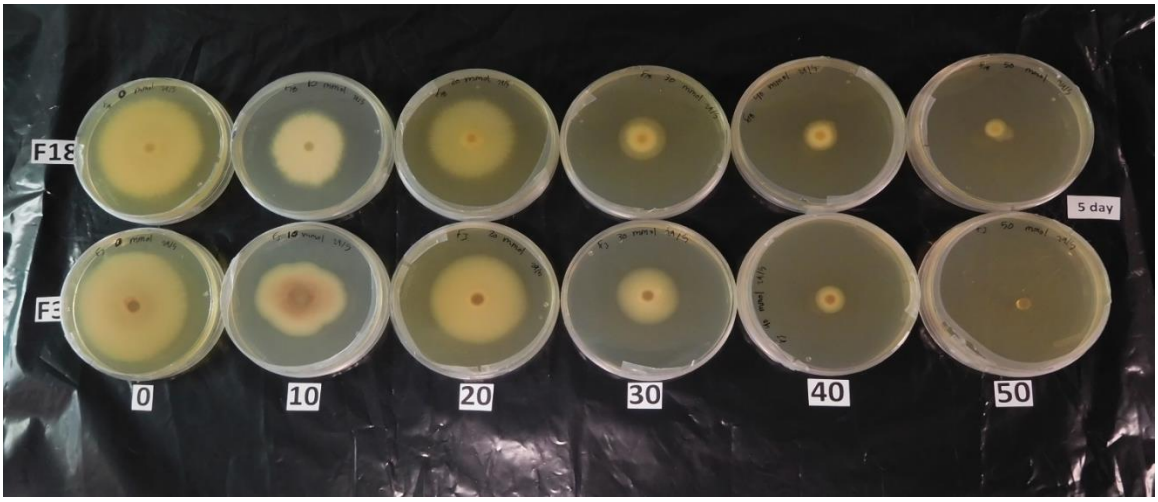


**Figure 5.1.** Inhibition zone of *Fol* and *Forl* mycelia at different concentrations of sodium silicate. F18: *Fol* and F3: *Forl*.

**Table 5.1 The effect of different concentrations of sodium silicate on mycelial growth of *Fol***

| Sodium silicate concentration | Radial growth (mm) | % inhibition** |
|---|---|---|
| 0 mM | 55.52*[a] | 0 |
| 10 mM | 47.37[b] | 15.02 |
| 20 mM | 44.69[b] | 20.30 |
| 30 mM | 23.27[c] | 57.23 |
| 40 mM | 14.38[d] | 73.77 |
| 50 mM | 1.87[e] | 96.25 |

*Mean values are average of 4 replicates

**Inhibition % was calculated according to the following formula: (C-E)/Cx100, where C=colony diameter of the control, E= colony diameter of the experiment Petri dishes.

-Mean values followed by the same letters are not significantly different at P<0.05.

**Table 5.2 The effect of different concentrations of sodium silicate on mycelial growth of *Forl***

| Sodium silicate concentration | Radial growth (mm) | % inhibition** |
|---|---|---|
| 0 mM | 57.63*[a] | 0 |
| 10 mM | 46.01[b] | 21.25 |
| 20 mM | 39.97[b] | 30.00 |
| 30 mM | 30.44[c] | 46.25 |
| 40 mM | 10.07[d] | 81.99 |
| 50 mM | 1.65[e] | 97.73 |

*Mean values are average of 4 replicates

**Inhibition % was calculated according to the following formula: (C-E)/Cx100, where C=colony diameter of the control, E= colony diameter of the experiment Petri dishes.

-Mean values followed by the same letters are not significantly different at P<0.05.

### 5.4.2 *In vivo* effect of sodium silicate on reducing disease severity of *Fol* and *Forl* under glasshouse conditions.

All plants derived from tomato seeds that were treated with 0, 10, 20, 30, 40, and 50 mM concentrations of sodium silicate and inoculated with *Fol* and *Forl* were scored for their symptoms after 30 days from inoculation. Different symptoms including stunting, wilting, necrosis, vascular browning, and yellowing of leaves were observed in some treatments (Figs 5.2, 5.3, 5.4 and 5.5). The results of the effect of sodium silicate in reducing disease severity of *Fol* and *Forl* in vivo showed that 30, 40, and 50 mM concentrations were highly significant compared to the other treatments. The disease severity was 56.0, 50.0, and 46.6% respectively for *Fol* after converting scores to disease severity, and there was a reduction in disease severity by 17.3, 23.3, and 26.7% respectively compared to the control (Table 5.3). In addition, 30, 40, and 50 mM concentrations of sodium silicate showed significant differences compared to others for *Forl*. The disease severity was 45.83, 41.66, and 29.16%, and there was a reduction in disease severity of 16.67, 20.84, and 33.34% respectively compared to the control (Table 5.4).


**Figure 5.2** Stunting and wilting symptoms in different sodium silicate concentrations caused by *Fol*.


**Figure 5.3** Different levels of vascular browning in the stems of treated plants with different sodium silicate concentrations and inoculated with *Fol*.


**Figure 5.4** No clear foliar symptoms caused be *Forl* on treated plants.


**Figure 5.5** Different levels of necrosis on the crown and roots of treated plants with different sodium silicate concentrations and inoculated with *Forl*.

**Table 5.3** Disease scores and disease severity percentage on tomato Moneymaker inoculated with *Fol* and treated with sodium silicate

| Sodium silicate concentration | Disease Severity Index (1-5) | Disease Severity % |
|---|---|---|
| 0 mM | 3.75[a]* | 73.3 |
| 10 mM | 3.41[b] | 70.0 |
| 20 mM | 3.41[b] | 70.0 |
| 30 mM | 2.91[c] | 56.0 |
| 40 mM | 2.50[c] | 50.0 |
| 50 mM | 2.16[d] | 46.6 |

*Values represent the mean of twelve replicates.

-Mean values followed by the same letters are not significantly different at P<0.05.

**Table 5.4** Disease scores and disease severity percentage on tomato Moneymaker inoculated with *Forl* and treated with sodium silicate

| Sodium silicate concentration | Disease Severity Index (0-4) | Disease Severity % |
|---|---|---|
| 0 mM | 2.41[a]* | 62.50 |
| 10 mM | 2.08[b] | 54.16 |
| 20 mM | 1.99[b] | 54.16 |
| 30 mM | 1.74[c] | 45.83 |
| 40 mM | 1.66[c] | 41.66 |
| 50 mM | 1.24[d] | 29.16 |

*Values represent the mean of twelve replicates.

-Mean values followed by the same letters are not significantly different at P<0.05.

### 5.4.3 Detecting *Fol* and *Forl* DNA by using LAMP assay

The results of LAMP assays showed that DNA from roots, crown and stems of all treated plants with sodium silicate were detected using SIX1 and Sprl LAMP primers (Figs 5.6 and 5.7). Table 5.5 and 5.6 show times of amplification and annealing temperature for the detection assays.

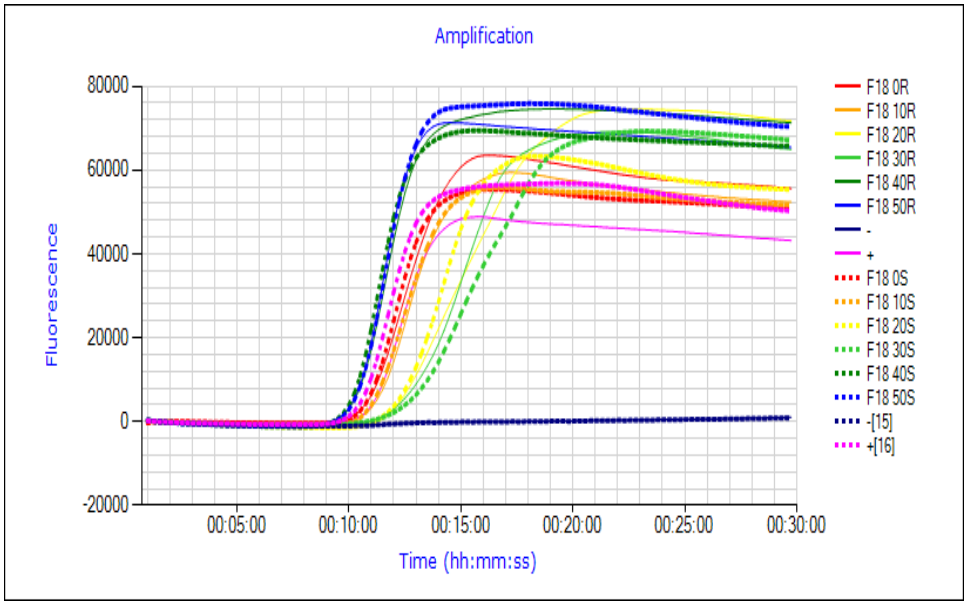


**Fig 5.6** The amplification plots for *Fol* DNA from roots and stems of 0, 10, 20, 30, 40 and 50mM concentrations of sodium silicate in treated plants with SIX1 LAMP primers. + and - stand for positive and non-template control respectively. R: Roots and S: Stems.

**Table 5.5** Amplification time and annealing temperature for *Fol* DNA from treated plants with sodium silicate concentrations with SIX1 LAMP primers.

| Well N0. | Well Name | Amplification time Mm:ss | Anneal Derivative ˚C |
|---|---|---|---|
| 1 | F18 0 ROOT | 12:00 | 88.0 |
| 2 | F18 10 ROOT | 12:30 | 88.1 |
| 3 | F18 20 ROOT | 13:30 | 88.1 |
| 4 | F18 30 ROOT | 14:45 | 88.2 |
| 5 | F18 40 ROOT | 11:30 | 88.2 |
| 6 | F18 50 ROOT | 11:15 | 88.1 |
| 7 | - | - | - |
| 8 | + | 12:15 | 87.9 |
| 9 | F18 0 STEM | 12:00 | 87.9 |
| 10 | F18 10 STEM | 12:15 | 88.1 |
| 11 | F18 20 STEM | 14:00 | 88.2 |
| 12 | F18 30 STEM | 14:45 | 88.2 |
| 13 | F18 40 STEM | 11:00 | 88.2 |
| 14 | F18 50 STEM | 11:15 | 88.2 |
| 15 | - | - | - |
| 16 | + | 11:15 | 88.1 |

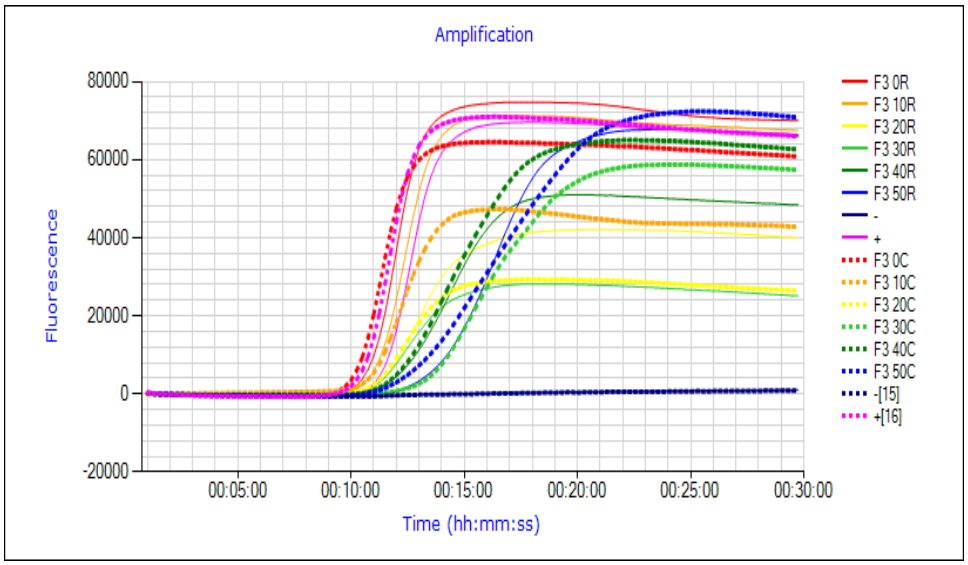


**Fig 5.7** The amplification plots for *Forl* DNA from roots and crowns of 0, 10, 20, 30, 40 and 50mM concentrations of sodium silicate in treated plants with Sprl LAMP primers. + and - stand for positive and non-template control respectively. R: Roots and C: Crowns.

**Table 5.6** Amplification time and annealing temperature for *Forl* DNA from treated plants with sodium silicate concentrations with Sprl LAMP primers.

| Well N0. | Well Name | Amplification time Mm:ss | Anneal Derivative ˚C |
|---|---|---|---|
| 1 | F18 0 ROOT | 11:45 | 88.0 |
| 2 | F18 10 ROOT | 12:15 | 88.0 |
| 3 | F18 20 ROOT | 12:45 | 87.8 |
| 4 | F18 30 ROOT | 12:30 | 87.9 |
| 5 | F18 40 ROOT | 14:00 | 88.1 |
| 6 | F18 50 ROOT | 16:00 | 88.3 |
| 7 | - | - | - |
| 8 | + | 12:30 | 88.0 |
| 9 | F18 0 CROWN | 11:15 | 88.0 |
| 10 | F18 10 CROWN | 12:00 | 88.1 |
| 11 | F18 20 CROWN | 12:15 | 88.2 |
| 12 | F18 30 CROWN | 15:00 | 88.3 |
| 13 | F18 40 CROWN | 13:45 | 88.3 |
| 14 | F18 50 CROWN | 15:00 | 88.3 |
| 15 | - | - | - |
| 16 | + | 11:30 | 88.2 |

### 5.4.4 qPCR analysis

Real-time qPCR amplification was used to quantify the *Fol* and *Forl* DNA present in samples from roots, crowns and stems collected from different treatments. DNA fragments of *Fol* and *Forl* from root, stem and crown samples in treated plants were successfully amplified using specific primers SIX1 and Sprl as stated in Table 4.3 (Figs 5.8, 5.10, 5.14 and 5.16). Standard regression lines were generated from root and stem samples for *Fol* and from root and crown samples for *Forl* using genomic DNA ranging from 1ng to 0.1 pg/µl (Figs 5.9, 5.11, 5.15 and 5.17). The regression equation for the DNA standard curve for *Fol* from roots and stems were y= -2.66x+ 20.99 and ($R^2$=0.9857) and y= -2.38x+ 26.36 and ($R^2$=0.9733) respectively. In tested root samples for *Fol*, the results indicated that the quantity of DNA extracted from 0, 10, 20, 30, 40, 50mM treatments were 1.8593, 1.7933, 1.1266, 1.4910, 0.8698 and 0.8522 ng/µl, whereas in tested stem samples the DNA quantities were 64.9161, 22.7466, 1.5388, 1.0380, 0.0024 and 0.0058 ng/µl respectively (Tables 5.7, 5.8 and Figs 5.12, 5.13). The regression equations for *Forl* from roots and crowns were y= -1.37x+ 18.40 and ($R^2$=0.5662) and y= -2.65x+ 17.98 and ($R^2$=0.9966) respectively and in tested root samples for *Forl*, the results indicated that the quantity of DNA extracted from 0, 10, 20, 30, 40, 50mM treatments were 9.9990, 8.8616, 5.5600, 5.1723, 1.9076 and 1.2346 ng/µl, whereas in tested crown samples the DNA quantities were 2.2853, 1.2705, 1.3236,

1.2692, 0.7113 and 0.3854 ng/µl respectively (Tables 5.9, 5.10 and Figs 5.18, 5.19).

The results showed that there was a correlation between the reduction in the disease severity by 40 and 50 mM concentration treatments of sodium silicate and the DNA quantity in the root, crown and stem samples for both *Fol* and *Forl*.

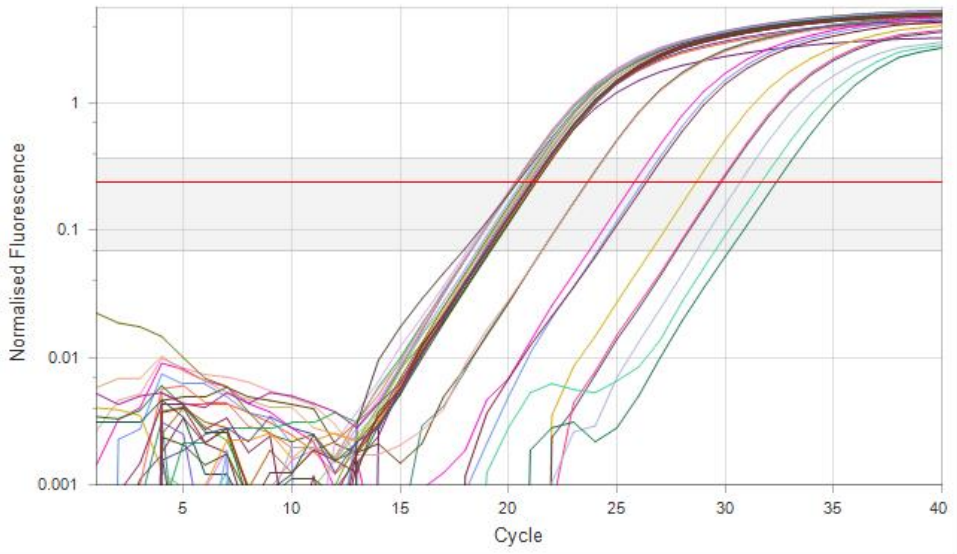


**Fig. 5.8** Amplification curves of roots DNA samples of *Fol* with SIX1 primer.

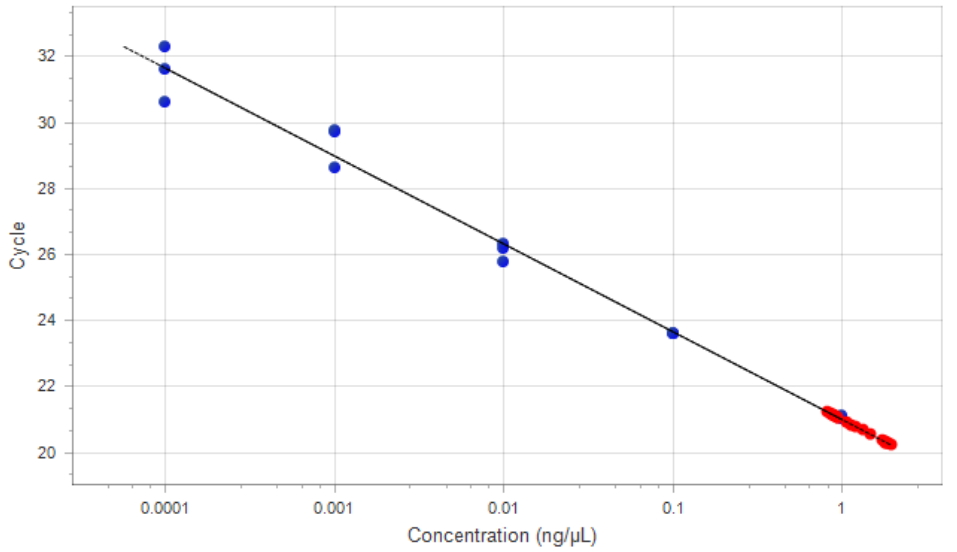


**Fig. 5.9** Standard regression lines of 10-fold serial dilution of *Fol* culture DNA (blue dots) and DNA quantity of root samples (red dots).

**Table 5.7** Ct values and DNA quantity of root samples of *Fol*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µl)** |
| 16 | 0R | 20.24 | 1.927 |
| 17 | 0R | 20.28 | 1.855 |
| 18 | 0R | 20.32 | 1.795 |
| 19 | 10R | 20.32 | 1.788 |
| 20 | 10R | 20.33 | 1.782 |
| 21 | 10R | 20.31 | 1.81 |
| 22 | 20R | 20.93 | 1.06 |
| 23 | 20R | 20.85 | 1.131 |
| 24 | 20R | 20.79 | 1.189 |
| 25 | 30R | 20.37 | 1.709 |
| 26 | 30R | 20.68 | 1.31 |
| 27 | 30R | 20.56 | 1.454 |
| 28 | 40R | 21.13 | 0.8931 |
| 29 | 40R | 21.15 | 0.8751 |
| 30 | 40R | 21.19 | 0.8413 |
| 31 | 50R | 21.15 | 0.8711 |
| 32 | 50R | 21.16 | 0.8697 |
| 33 | 50R | 21.23 | 0.816 |
| 34 | + | 21.10 | 0.9088 |
| 35 | + | 21.07 | 0.9389 |
| 36 | + | 21.08 | 0.9275 |
| 37 | N | - | - |
| 38 | N | - | - |
| 39 | N | - | - |
| 40 | N | - | - |
| 41 | N | - | - |
| 42 | N | - | - |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | N | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

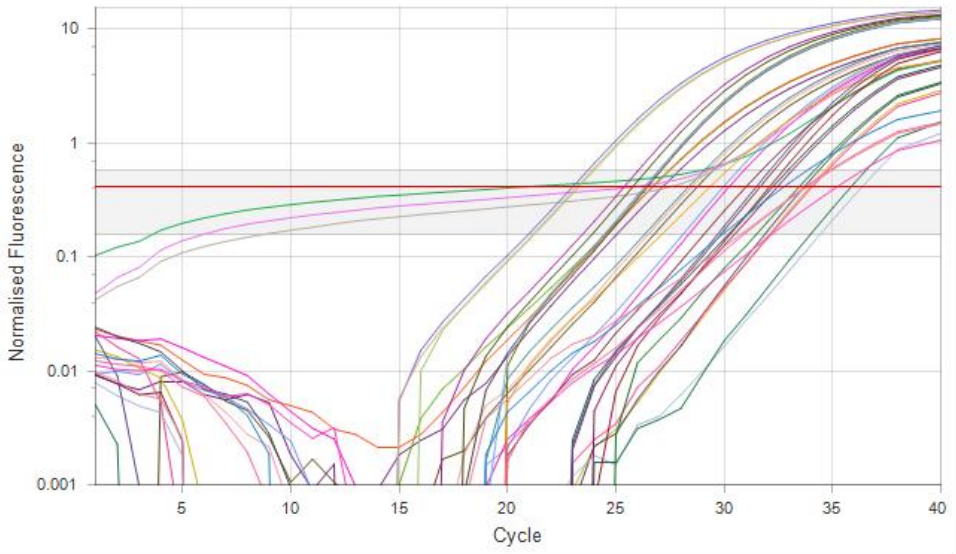


**Fig. 5.10** Amplification curves of stem DNA samples of *Fol* with SIX1 primer.

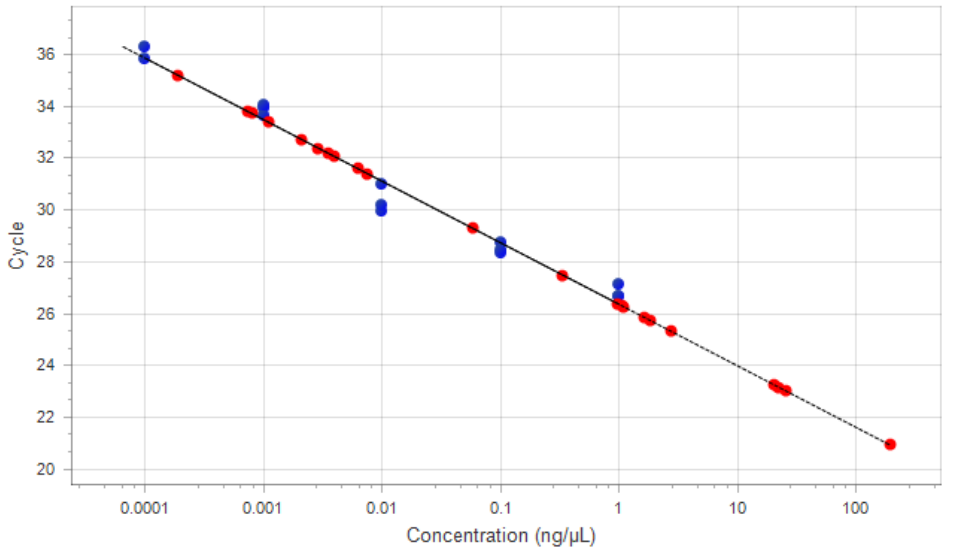


**Fig. 5.11** Standard regression lines of 10-fold serial dilution of *Fol* culture DNA (blue dots) and DNA quantity of stem samples (red dots).

**Table 5.8** Ct values and DNA quantity of stem samples of *Fol*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µL)** |
| 19 | 0S | 20.94 | 192.8 |
| 20 | 0S | 25.87 | 1.615 |
| 21 | 0S | 27.50 | 0.3335 |
| 22 | 10S | 23.01 | 25.83 |
| 23 | 10S | 23.17 | 22.06 |
| 24 | 10S | 23.26 | 20.35 |
| 25 | 20S | 25.33 | 2.721 |
| 26 | 20S | 29.31 | 0.05764 |
| 27 | 20S | 25.74 | 1.838 |
| 28 | 30S | 26.40 | 0.9681 |
| 29 | 30S | 26.30 | 1.067 |
| 30 | 30S | 26.29 | 1.079 |
| 31 | 40S | 32.20 | 0.003486 |
| 32 | 40S | 32.40 | 0.002888 |
| 33 | 40S | 33.41 | 0.001085 |
| 34 | 50S | 32.07 | 0.003952 |
| 35 | 50S | 31.60 | 0.006266 |
| 36 | 50S | 31.42 | 0.007468 |
| 37 | + | 32.73 | 0.002086 |
| 38 | + | 33.73 | 0.0007895 |
| 39 | + | 35.20 | 0.0001907 |
| 40 | + | 33.80 | 0.0007432 |
| 41 | N | - | - |
| 42 | N | - | - |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | w | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

S=Stems, +=Positive and N=Negative.

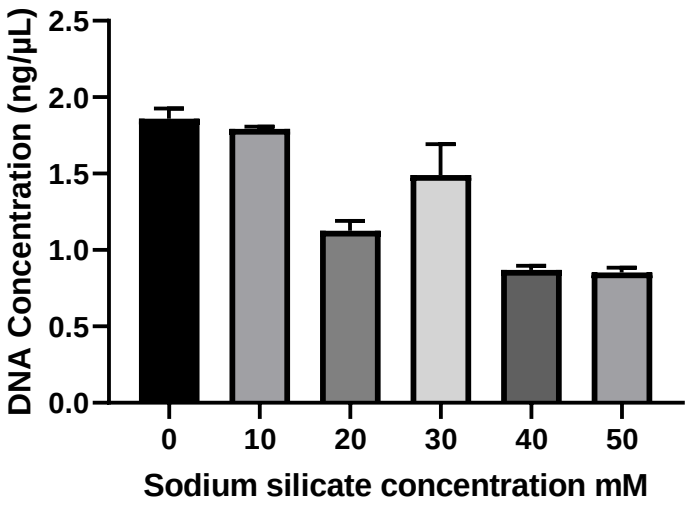


**Figure 5.12** The effect of sodium silicate concentrations on DNA quantity of root samples caused by *Fol*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

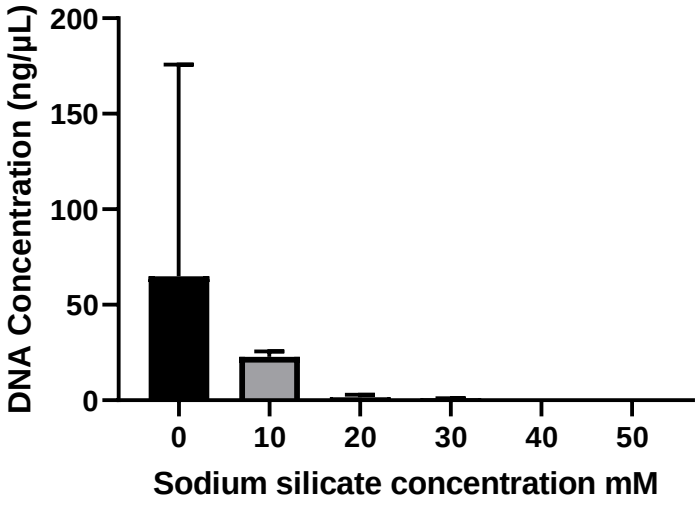


**Figure 5.13** The effect of sodium silicate concentrations on DNA quantity of stem samples caused by *Fol*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

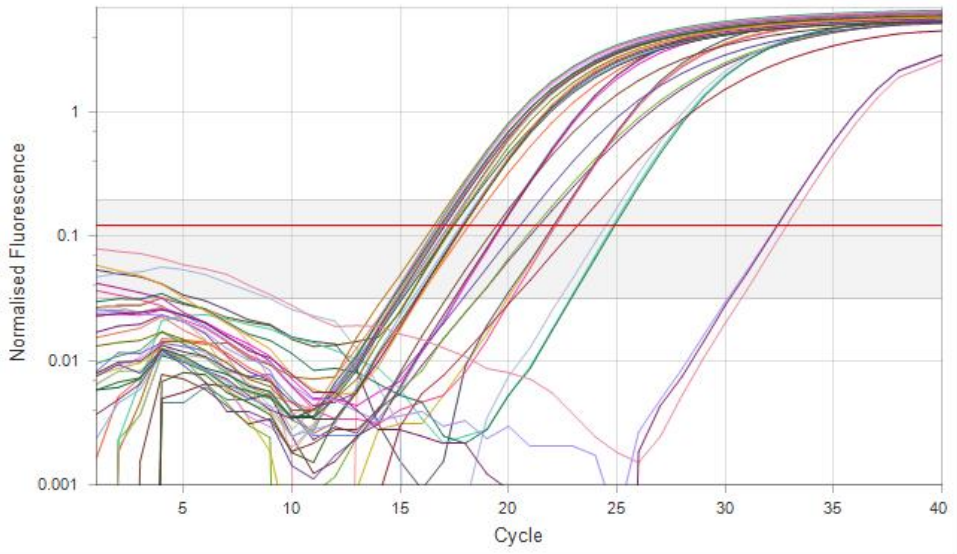


**Fig. 5.14** Amplification curves of roots DNA samples of *Forl* with sprl primer.

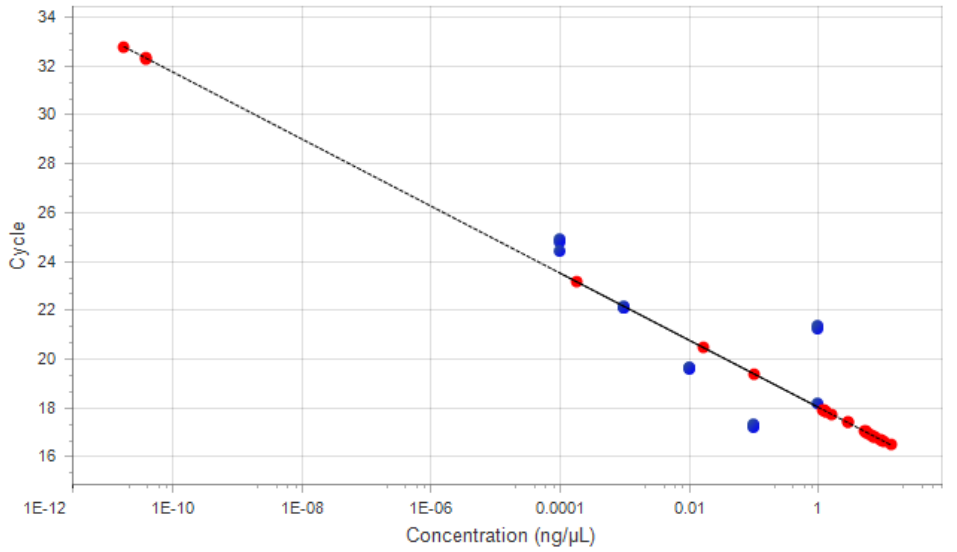


**Fig. 5.15** Standard regression lines of 10-fold serial dilution of *Forl* culture DNA (blue dots) and DNA quantity of stem samples (red dots).

**Table 5.9** Ct values and DNA quantity of root samples of *Forl*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µl)** |
| 16 | 0R | 16.70 | 9.434 |
| 17 | 0R | 16.50 | 13.26 |
| 18 | 0R | 16.85 | 7.303 |
| 19 | 10R | 16.65 | 10.27 |
| 20 | 10R | 16.70 | 9.418 |
| 21 | 10R | 16.89 | 6.897 |
| 22 | 20R | 16.97 | 5.981 |
| 23 | 20R | 17.04 | 5.32 |
| 24 | 20R | 17.03 | 5.379 |
| 25 | 30R | 17.41 | 2.847 |
| 26 | 30R | 17.06 | 5.191 |
| 27 | 30R | 16.84 | 7.479 |
| 28 | 40R | 17.89 | 1.29 |
| 29 | 40R | 17.41 | 2.86 |
| 30 | 40R | 17.77 | 1.573 |
| 31 | 50R | 17.88 | 1.307 |
| 32 | 50R | 17.94 | 1.181 |
| 33 | 50R | 17.92 | 1.216 |
| 34 | + | 23.16 | 0.0001835 |
| 35 | + | 19.40 | 0.1014 |
| 36 | + | 20.48 | 0.01665 |
| 37 | N | - | - |
| 38 | N | - | - |
| 39 | N | - | - |
| 40 | N | - | - |
| 41 | N | - | - |
| 42 | N | - | - |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | N | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

R=Roots, +=Positive and N=Negative.

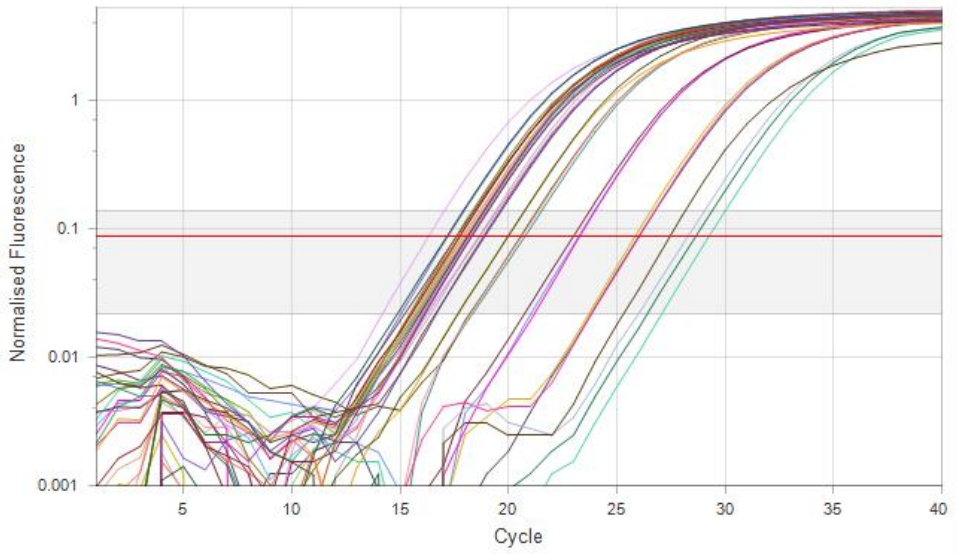


**Fig. 5.16** Amplification curves of crow DNA samples of *Forl* with sprl primer.

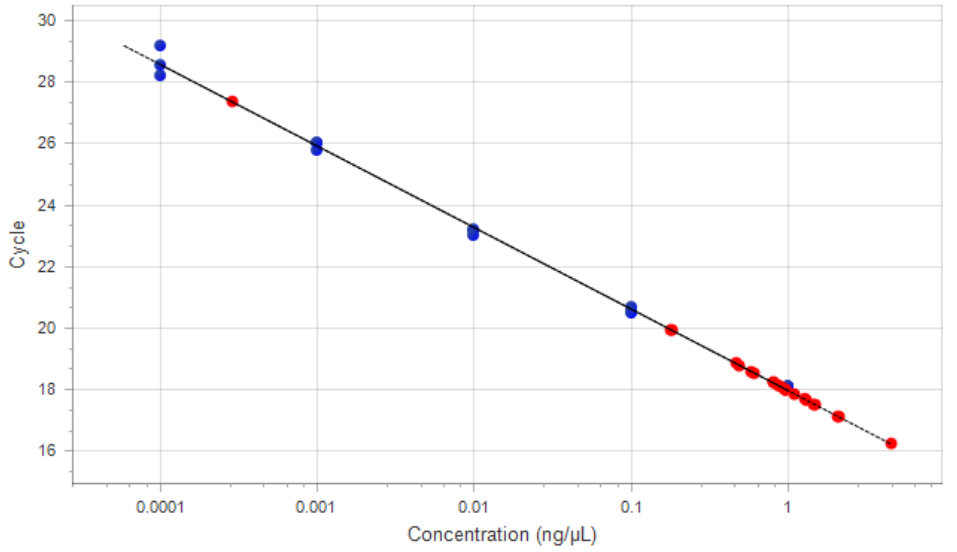


**Fig. 5.17** Standard regression lines of 10-fold serial dilution of *Forl* culture DNA (blue dots) and DNA quantity of crown samples (red dots).

**Table 5.10** Ct values and DNA quantity of crown samples of *Forl*.

| Sample Results | | | |
|---|---|---|---|
| **Well** | **Sample Name** | **Cq** | **Calculated Concentration (ng/µl)** |
| 16 | 0C | 16.25 | 4.492 |
| 17 | 0C | 17.70 | 1.264 |
| 18 | 0C | 17.86 | 1.1 |
| 19 | 10C | 17.54 | 1.461 |
| 20 | 10C | 17.51 | 1.499 |
| 21 | 10C | 18.16 | 0.8516 |
| 22 | 20C | 17.15 | 2.052 |
| 23 | 20C | 18.02 | 0.9576 |
| 24 | 20C | 18.02 | 0.9612 |
| 25 | 30C | 17.12 | 2.106 |
| 26 | 30C | 18.09 | 0.9048 |
| 27 | 30C | 18.24 | 0.797 |
| 28 | 40C | 18.03 | 0.9563 |
| 29 | 40C | 18.62 | 0.5733 |
| 30 | 40C | 18.55 | 0.6043 |
| 31 | 50C | 18.23 | 0.7995 |
| 32 | 50C | 19.95 | 0.18 |
| 33 | 50C | 19.97 | 0.1767 |
| 34 | + | 17.66 | 1.31 |
| 35 | + | 18.80 | 0.4889 |
| 36 | + | 18.87 | 0.461 |
| 37 | N | - | - |
| 38 | N | - | - |
| 39 | N | - | - |
| 40 | N | - | - |
| 41 | N | - | - |
| 42 | N | - | - |
| 43 | N | - | - |
| 44 | N | - | - |
| 45 | N | - | - |
| 46 | N | - | - |
| 47 | N | - | - |
| 48 | N | - | - |

C=Crown, +=Positive and N=Negative.

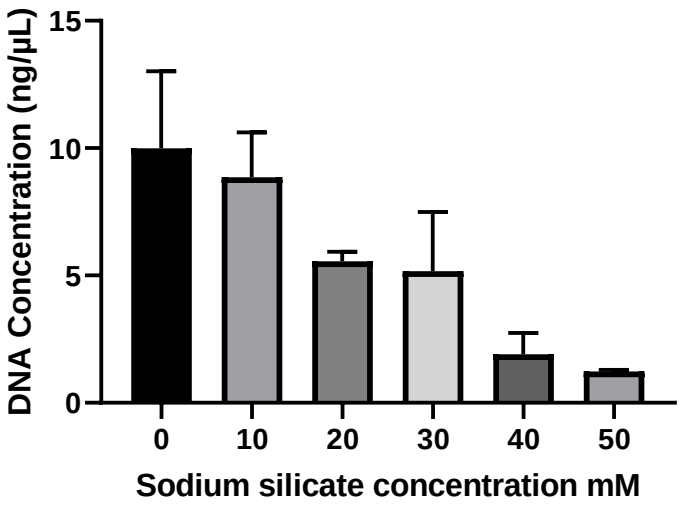


**Figure 5.18** The effect of sodium silicate concentrations on DNA quantity of root samples caused by *Forl*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

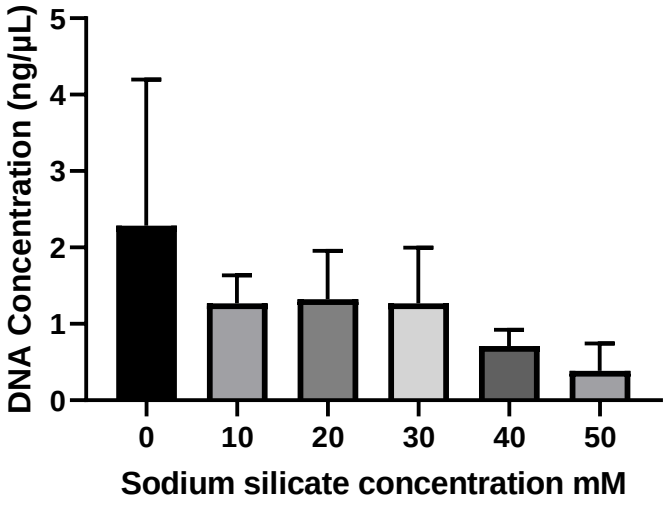


**Figure 5.19** The effect of sodium silicate concentrations on DNA quantity of crown samples caused by *Forl*. Labelling indicates statistical significance at $p < 0.05$. Means sharing the same label are not significantly different from each other. N=6.

## 5.5 Discussion

Different concentrations ((0, 10, 20, 30, 40, 50mM) of sodium silicate have been evaluated to study their effects in reducing the disease severity of fusarium wilt and crown and root rot diseases caused by *Fol* and *Forl* respectively. For the in vitro experiments, the results showed that the high concentrations (30, 40 and 50mM) of sodium silicate were able to suppress the mycelial growth of *Fol* and *Forl* significantly compared to the control. This study indicated that the growth of *Fol* and *Forl* were significantly inhibited by treatment with sodium silicates. In this study, all Petri dishes containing different concentrations of sodium silicate could reduce the mycelial growth of both *Fol* and *Forl* in vitro, and their effect seemed to be increasing along with their increasing concentrations. This result was in agreement with Mahdikhani *et al.* (2015) who also studied the effect of silicon on mycelial growth of *Fusarium oxysporum* in vitro.

For the In vivo experiments to study the ability of different concentrations of sodium silicate to reduce disease severity of *Fol* and *Forl,* the results demonstrated that 30, 40, and 50 mM concentrations were highly significant compared to the other treatments. The disease severity was 17.3, 23.3, and 26.7% respectively compared to the control for *Fol* and 16.67, 20.84, and 33.34% respectively compared to the control for *Forl*.

The results of this study demonstrated the reduction in disease severity by applying high concentrations of sodium silicate under glasshouse

conditions which supported the results of others (Dannon and Wydra, 2004; French-Monar *et al.,* 2010; Huang *et al.,* 2011) who showed that silicon amended in the soil decreased the severity of diseases caused by *Pythium ultimum* and *P. aphanidermatum*, *Ralstonia solanacearum*, *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Phytophthora capsica*.

The finding of this study also showed reduction in *Fol* and *Forl* DNA with high concentrations of sodium silicate compared to the control. These results are in agreement with the results of Elsharkawy and Mousa (2015) who found that silicon application to cucumber plants significantly reduced the severity of Papaya ring spot virus and its accumulation in leaves.

# Chapter 6. The expression of resistance genes in tomato induced by UV-C, sodium silicate and *Trichoderma* against *Fusarium oxysporum* f.sp. *lycopersici* and *Fusarium oxysporum* f.sp. *radicis-lycopersici*

## 6.1 Introduction

Induction of a systemic resistance is one of the mechanisms plants can use to reduce the severity of disease caused by pathogens. Plants can produce or synthesize biochemical substances in responses to fungal infection (Abo-Elyousr *et al*., 2008). Phenolic compounds are considered as one of the most important biochemical products for disease resistance and changes in the activity of phenol-oxidizing enzymes play a role in the regulation of metabolic pathways in diseased plants (Pradeep and Jambhale, 2002).

Several papers have supported the ability of *Trichoderma* spp. to induce resistance in treated plants. Yedidia *et al*. (2000) demonstrated that Pathogen-Related (PR) proteins and a number of hydrolytic enzymes were induced in treated plants when inoculated with *Trichodeerma harzianum.* They also stated that the defence responses induced by T. *harzianum* were similar to the resistance induced by the chemical inducer 2, 6-dichloroisoncotinic acid. Ojha and Chatterjee (2012) investigated the effect of salicylic acid (SA) and *T. harzianum* (TH) on the induction of

phenolic accumulation content and defence enzymes in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici.* Different concentrations of SA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) and TH were used to treat tomato plant infected with *Fol*. The results showed significant increases in the activities of both peroxidase and polyphenol oxidase enzymes at a 1.5 mM concentration of SA and both enzymes reached their maximum activities on the 28th day. The combined application of SA (1.5 mM) and TH in infected tomato plants also showed higher activities of peroxidase and polyphenol oxidase enzymes up to the 14th day from inoculation, and after that the activities decreased or remained more stable.

ElKom *et al*. (2016) investigated the ability of two strains of *T. asperellum* (TS-12 and TS-39) to trigger tomato defence mechanisms against *Fol.* The results showed the expression levels of the defence-related genes chitinase (SlChi3), β-1,3-glucanase (SlGluA) and PR-1 (SlPR-1a) were significantly increased in the stems and roots of *Trichoderma* + *Fol* treatment plants compared with *Fol* only infected ones.

## 6.2 Aims and objectives

The aim of this study was to investigate the expression of some representative pathogen related genes GluA (acidic extracellular b-1,3-glucanase), GluB (basic intracellular b-1,3-glucanase), Chi3 (acidic extracellular chitinase), Chi9 (basic intracellular chitinase), PR-1a (PR-1 protein isoform PR-P6) and PAL (phenylalanine ammonia-lyase) in tomato induced by UV-C, sodium silicate, and *Trichoderma* spp., against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*).

## 6.3 Materials and Methods

### 6.3.1 *Trichoderma* isolates

Ten isolates of *Trichoderma* spp. were obtained from the fungal collection maintained at 4° C in the Plant and Crop Sciences lab., University of Nottingham. These isolates were maintained on potato dextrose agar (PDA) plates and were evaluated for their ability to inhibit the mycelial growth of *Fol* and *Forl* (Fig. 6.1).


**Figure 6.1** Colony morphology of four of the *Trichoderma* isolates used in this study.

### 6.3.1.2 Molecular identification of *Trichoderma* isolates using Polymerase Chain Reaction (PCR) analysis

DNA was extracted from approximately 100 mg of 7 days old mycelia of all ten *Trichoderma* isolates as stated in section 2.2. All DNA samples were subjected to PCR using the universal primers ITS1 and ITS4 performed as stated in section 2.3. The amplified PCR products of *Trichoderma* isolates were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocols and quantified on a Nanodrop

spectrophotometer. The purified PCR products were sequenced by Eurofins Genomics as stated in section 3.3.3.

### 6.3.1.3 *In vitro* screening of antagonistic effect of *Trichoderma* isolates against *Fol* and *Forl*.

#### 6.3.1.3.1 Dual culture experiment

The dual culture technique was used to study the effect of four *Trichoderma* isolates on mycelial growth of *Fol* and *Forl* under laboratory conditions (Fig. 6.2). Five millimetre diameter discs of 7 days old *Fol*, *Forl* and *Trichoderma* isolate cultures were placed at each edge of PDA Petri dishes, and for control treatments, a mycelial disc of the tested pathogens was placed on the PDA medium alone. The plates were incubated at 27 $^{o}$C for 5 days. Radial growth of the tested pathogens was measured, and percent inhibitions of average radial mycelial growth were calculated in relation to growth of the control. Four replicates of each treatment were measured, and percentage inhibitions calculated according to the formula:

Percentage inhibition= (C-E)/Cx100, where C=colony diameter on the control, E= colony diameter on the experimental Petri dishes.

Completely randomized experimental design was used for the four Petri dishes of each treatment.

### 6.3.2 Investigation into the expression of resistance-related genes in tomato induced by UV-C, sodium silicate, and *Trichoderma* against *Fol* and *Forl*.

#### 6.3.2.1 Plant material

Seeds of the tomato Moneymaker were planted in 1 L pots of Levington M3 compost and grown in a heated and ventilated glasshouse. The lighting, heating and ventilation were as stated in section 2.5.

#### 6.3.2.2 Inoculum preparation and inoculation

FOL and FORL inoculations were performed on cv. Moneymaker seedlings 22 days after planting. Conidial suspensions of *Fol*, *Forl* and *Trichoderma* were prepared from 7-day-old cultures grown on PDA at 28 °C. Inoculum was prepared as stated in section 2.4. The quantity of the inoculum to be used was calculated to achieve a final concentration of $10^3$ conidia/g soil for FOL and FORL. For the *Trichoderma pseudokoningii* strains that had shown good inhibition in the dual-plate assays the quantity used was $10^6$ conidia/g of soil (Barakat and Al-Masri, 2009). The soil was infested with the *Fol* and *Forl* pathogen inoculum ($10^3$ conidia/g soil) and left for one week for pathogen establishment. Then, the inoculum of the antagonistic fungi (*Trichoderma*) was applied ($10^6$ conidia/g of soil) in the *Fol* and *Forl* infested soil and left for one more week for *Trichoderma* establishment.

Seedlings were transplanted, one per pot and 12 replicates (pots) were used for each treatment. The treatments were: (1) healthy control (no pathogen); (2) soil infested with *Fol*; (3) UV-C dose (6 KJ $m^{-2}$) + *Fol*; (4) 50 mM sodium silicate + *Fol*; (5) *T. pseudokoningii* + *Fol*; (6) UV-C dose (6 KJ $m^{-2}$) + 50 mM sodium silicate + *T. pseudokoningii* + *Fol*; (7) soil infested with *Forl*; (8) UV-C dose (6 KJ $m^{-2}$) +*Forl*; (9) 50 mM sodium silicate + *Forl*; (10) *T. pseudokoningii* + *Forl*; (11) UV-Cdose (6 KJ $m^{-2}$) + 50 mM sodium silicate + *T. pseudokoningii* + *Forl*. Treated plants were scored and then converted to disease severity as stated in section 3.3.6.

### 6.3.2.3 Sampling, RNA extraction and cDNA synthesis

Root, stem and crown materials from three plants from each treatment were sampled at 2, 4, 6, 8, 10 and 20 days after inoculation (dai). The sampled roots, stems and crowns were washed and briefly dried, and 100mg samples were placed into microcentrifuge tubes and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until use. A single 4 mm steel bead (Qiagen) was cooled in liquid nitrogen and added to each microcentrifuge tube and the samples were homogenised using two runs of aTissuelyser II (Qiagen) at 30Hz for 1 minute. Homogenised samples were stored at -80 °C until RNA extraction. RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's guidelines. RNA purity and yield were assessed via NanoDrop (Thermo Scientific). All

samples were then diluted to a concentration of ≤ 50 ng/µl. cDNA was synthesized using 20 µl reverse transcription reaction using the High-Capacity RNA-to-cDNA kit (Applied Biosystems) following the manufacturer's protocol. The resulting cDNA was stored at -20°C until use.

### 6.3.2.4 qPCR

The expression profiles of pathogenesis-related genes, chitinase (Chi3 and chi9), β-1,3-glucanase (GluA and Glub), PAL and PR-1 (PR-1a), were evaluated using quantitative real-time PCR (qPCR). The forward and reverse primers for the tested genes are shown in table 6.1. The qPCR reaction mixture was composed of 25 ng of cDNA, 1 µl of each primer (10 µM), 10 µl of SYBR green master mix (Applied Biosystems) and RNase-free water in a final volume of 20 µl. RNase-free water was used as the negative control. Reactions were run on a LightCycler 480 ® (Roche) with a two-step amplification cycle. The cycle was as follows; a pre-incubation of 10 minutes at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 58 °C and extension for 30 s at 72 °C. Ct values were calculated utilising the second derivative maximum method. The qPCR reactions were performed twice. The actin gene was used as a reference gene, and the relative expression of the gene of interest was calculated according to the method of Pfaffi (2001) using the following Formula:

$$RQ = \frac{(E_{target})^{\Delta Ct(target)}}{(E_{reference})^{\Delta Ct(reference)}} = \frac{2^{\Delta Ct(target)}}{2^{\Delta Ct(reference)}}$$

$$= 2^{-[Ct(target,test) - Ct(target,calibrator)] - [Ct(ref,test) - Ct(ref,calibrator)]}$$

$$= 2^{-\Delta\Delta Ct}$$

A melting curve was run between 90°C and 60°C following the amplification to allow for checking of product specificity. Primers were optimised utilising a pooled sample and a 5-point 5-fold dilution series from which efficiency was calculated.

### 6.3.2.5 Experimental design and data analysis

The experiments were arranged in a completely randomized design, and the statistical analyses of all experiments were performed using the GraphPad prism 8 software system, LLC (GraphPad prism 8.3.0 (538), 2019). All experiments were conducted twice. Analyses of variance (ANOVA) were performed for all data sets. The least significant difference (LSD) at $P<0.05$ was applied to detect differences among different treatments (Gomez and Gomez, 1984).

**Table 6.1**. Primers used in real-time quantitative RT-PCR analyses of tomato defense-related genes.

| No | Primer | Sequence Nucleotide sequence (5´-3´) | Amplicon Size bp | Reference |
|---|---|---|---|---|
| 1 | Actin | F. AGGCACACAGGTGTTATGGT<br>R. AGCAACTCGAAGCTCATTGT | 177 | Aime et al., 2008 |
| 2 | LECHI3 | F. TGCAGGAACATTCACTGGAG<br>R. TAACGTTGTGGCATGATGGT | 250 | Aime et al., 2008 |
| 3 | LECHI9 | F. GAAATTGCTGCTTTCCTTGC<br>R. CTCCAATGGCTCTTCCACAT | 235 | Aime et al., 2008 |
| 4 | LEGLUCA | F. GGTCTCAACCGCGACATATT<br>R. CACAAGGGCATCGAAAAGAT | 202 | Aime et al., 2008 |
| 5 | LEGLUCB | F. TCTTGCCCCATTTCAAGTTC<br>R. TGCACGTGTATCCCTCAAAA | 246 | Aime et al., 2008 |
| 6 | LEPR1A | F. TCTTGTGAGGCCCAAAATTC<br>R. ATAGTCTGGCCTCTCGGACA | 248 | Aime et al., 2008 |
| 7 | PAL | F. ATTGGGAAATGGCTGCTGATT<br>R. TCAACATTTGCAATGGATGCA | 652 | Bovy et al., 2002 |

## 6.4 Results and discussion

### 6.4.1 Identification of *Trichoderma* isolates

From the 10 *Trichoderma* isolates recovered from culture, the ITS region was successfully amplified from DNA of four isolates using the fungal specific universal primer pairs ITS1 and ITS4. The lengths of sequences as determined by capillary electrophoresis ranged from 600 bp to 650 bp (Fig. 6.2). The BLAST analysis of the ITS rDNA sequence data showed the closest match (98–100% similarity) in the NCBI GenBank database to be with *T. harzianum*, *T. pseudokoningii, T. atroviride and T. asperellum* and supported the morphological identifications (Table 6.2).

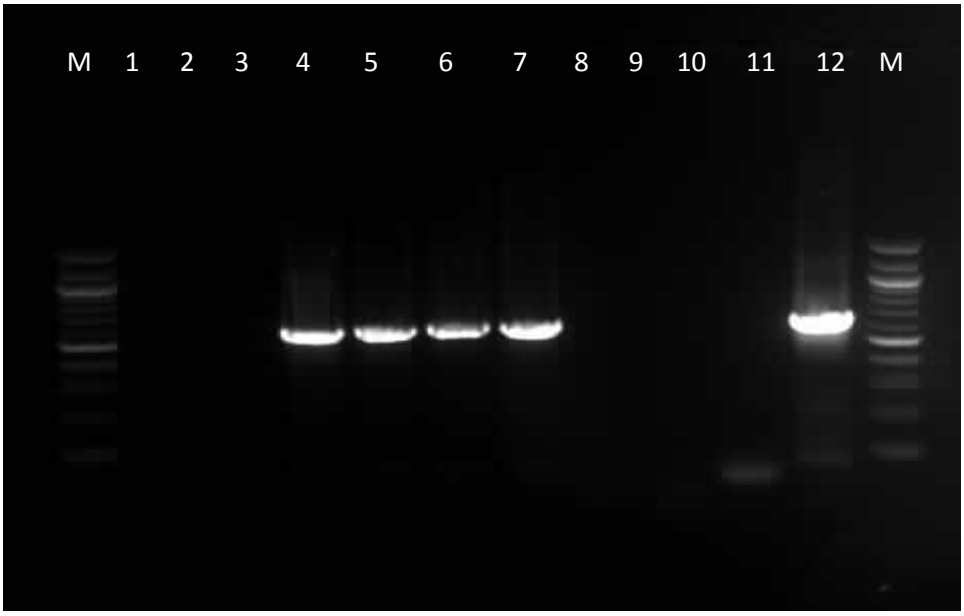


**Figure 6.2** ITS PCR products amplified from different *Trichoderma* isolates on 1.5% agarose gel. M, 100-bp marker. Lanes 1 through 10 show ITS amplicons from *Trichoderma* codes (MJD147, MJD160, MJD164, MJD174, MJD194, MJD184, MJD196, MJD195, MJD171, MJD188). Lane 11 represents negative control. Lane 12 represent positive control.

**Table 6.2 Identification of the *Trichoderma* isolates using PCR method with the gene targeted, identity (%) and accession number.**

| code | Isolate name | gene | Identity% | Accession No. |
|---|---|---|---|---|
| MJD174 | *Trichoderma harzianum* | rRNA | 100 | MK871264.1 |
| MJD194 | *Trichoderma pseudokoningii* | rRNA | 99.46 | EU280097.1 |
| MJD184 | *Trichoderma atroviride* | rRNA | 99.62 | MN533771.1 |
| MJD196 | *Trichoderma asperellum* | rRNA | 100 | KM456217.1 |

### 6.4.2 Antagonistic effect of *Trichoderma* isolates against *Fol* and *Forl*.

#### 6.4.2.1 Dual culture experiment

The antagonistic effects of the four *Trichoderma* isolates, *Trichoderma harzianum, T. pseudokoningii, T. atroviride* and *T. asperellum* were assessed by the inhibition of *Fol* and *Forl* growth through the dual test (Figs 6.3 and 6.4). All four *Trichoderma* isolates caused significant reduction in the mycelial growth of *Fol* and *Forl*, which indicated potential for their use as biocontrol agents against the tested fungal pathogens. The inhibition values for *T. harzianum, T. pseudokoningii, T. atroviride* and *T. asperellum* against *Fol* were 40.57, 51.55, 54.72 and 40.47% respectively, whereas against *Forl* the values were 43.15, 43.47, 36.49 and 38.99% respectively (Table 6.3 and 6.4).

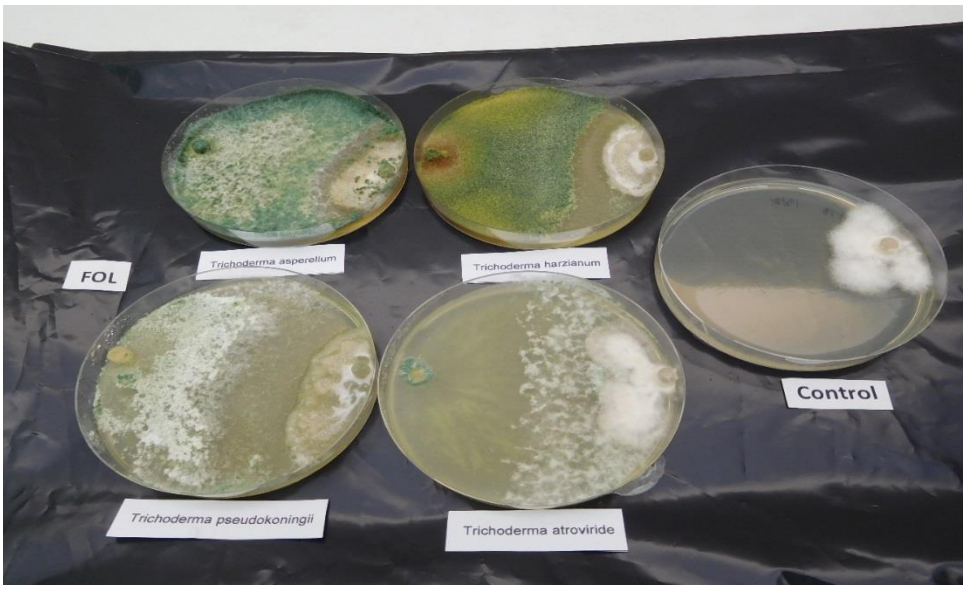


**Figure 6.3** Antagonistic effect of *Trichoderma harzianum, T. pseudokoningii, T. atroviride* and *T. asperellum* against *Fol* after five days of inoculation at 27° C.

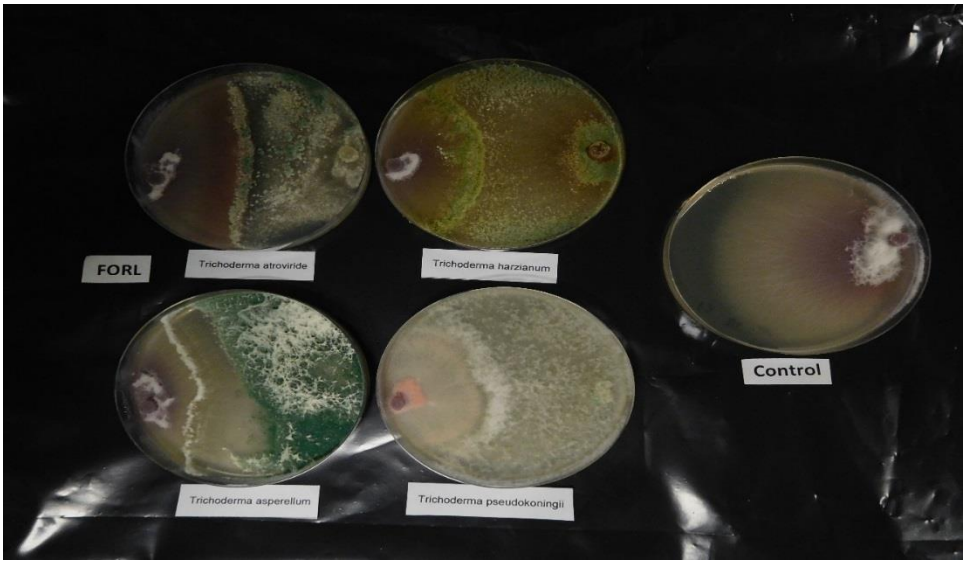


**Figure 6.4** Antagonistic effect of *Trichoderma harzianum, T. pseudokoningii, T. atroviride* and *T. asperellum* against *Forl* after five days of inoculation at 27° C.

**Table 6.3 Antagonistic effects of *Trichoderma* spp. isolates against *Fol*.**

| *Trichoderma* isolates | Radial growth (mm)* | % inhibition** |
|---|---|---|
| Control | 52.99[a] | 0 |
| *Trichoderma harzianum* | 31.49[b] | 40.57 |
| *Trichoderma pseudokoningii* | 25.67[c] | 51.55 |
| *Trichoderma atroviride* | 23.99[c] | 54.72 |
| *Trichoderma asperellum* | 31.54[b] | 40.47 |

*Mean values are average of 8 replicates

**Inhibition % was calculated according to the following formula: (C-E)/Cx100, where C=colony diameter of the control, E= colony diameter of the experiment Petri dishes.

-Mean values followed by the same letters are not significantly different at P<0.05.

**Table 6.4 Antagonistic effects of *Trichoderma* spp. isolates against *Forl*.**

| *Trichoderma* isolates | Radial growth (mm)* | % inhibition** |
|---|---|---|
| Control | 61.73[a] | 0 |
| *Trichoderma harzianum* | 35.09[b] | 43.15 |
| *Trichoderma pseudokoningii* | 34.89[b] | 43.47 |
| *Trichoderma Aatroviride* | 39.20[b] | 36.49 |
| *Trichoderma asperellum* | 37.73[b] | 38.99 |

*Mean values are average of 8 replicates

**Inhibition % was calculated according to the following formula: (C-E)/Cx100, where C=colony diameter of the control, E= colony diameter of the experiment Petri dishes.

-Mean values followed by the same letters are not significantly different at P<0.05.

### 6.4.3 The effect of combination of UV-C seed treatment, sodium silicate and *Trichoderma pseudokoningii* in reducing disease severity of *Fol* and *Forl*.

All plants that were treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate and *T. pseudokoningii* and inoculated with *Fol* and *Forl* were scored for their symptoms after 30 days from inoculation. Stunting, wilting, necrosis, vascular browning, and yellowing leaves were observed in some treatments (Figs 6.5 and 6.6). The results showed that there were significant reductions in disease severity for plants inoculated with *Fol* and *Forl* and treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination compared to the control. The disease severity values were 46.6, 40.0, 33.6 and 26.6% respectively whereas the disease severity values for *Forl* were 58.3, 41.6, 33.3 and 25.0% respectively (Tables 6.5 and 6.6).


**Figure 6.5** Stunting and wilting symptoms on plants inoculated with *Fol* and treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination compared to the control.


**Figure 6.6** Different levels of necrosis on the crown and root plants inoculated with *Forl* and treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination compared to the control.

**Table 6.5** Disease severity for *Fol* on Moneymaker tomato variety treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination (UV+S.S+*T.p*).

| Treatments | Disease Severity Index (1-5) | Disease Severity % ** |
|---|---|---|
| Cont. | 3.6[a]* | 73.3 |
| UV-C doses (6 KJ $m^{-2}$) | 2.3[b] | 46.6 |
| 50 mM sodium silicate | 2.1[c] | 40.0 |
| *Trichoderma pseudokoningii* | 1.6[d] | 33.6 |
| Combination (UV+S.S+*T.p*) | 1.3[e] | 26.6 |

*Values represent the mean of six replicates.

**Disease scores were converted to disease severity by using the following formula (Weitang *et al*., 2004):

$$\frac{\sum (\text{scale} \times \text{number of plants infected})}{\text{highest scale} \times \text{total number of plants}} \times 100$$

-Mean values followed by the same letters are not significantly different at P<0.05.

**Table 6.6** Disease severity for *Forl* on Moneymaker tomato variety treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination (UV+S.S+*T.p*).

| Treatments | Disease Severity Index (0-4) | Disease Severity % ** |
|---|---|---|
| Cont. | $3.3^{a}$* | 83.3 |
| UV-C doses (6 KJ $m^{-2}$) | $2.3^{b}$ | 58.3 |
| 50 mM sodium silicate | $1.5^{c}$ | 41.6 |
| *Trichoderma pseudokoningii* | $1.3^{d}$ | 33.3 |
| Combination (UV+S.S+*T.p*) | $1.0^{e}$ | 25.0 |

*Values represent the mean of six replicates.

**Disease scores were converted to disease severity by using the following formula (Weitang *et al*., 2004):

$$\frac{\sum (\text{scale} \times \text{number of plants infected})}{\text{highest scale} \times \text{total number of plants}} \times 100$$

-Mean values followed by the same letters are not significantly different at $P<0.05$.

### 6.4.4 Gene expression analysis with real-time RT-PCR

UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combinations of these treatments were screened for their ability to induce tomato systemic resistance against fusarium wilt and crown and root rot diseases. The defence related genes encoding chitinase (LeChi3 and Lechi9), β-1,3-glucanase (LeGluA and LeGluB), PAL and LePR1a were studied. Expression levels of these genes were determined in tomato plants at 2, 4, 6, 8, 10 and 20 days after inoculation (dpi) with *Fol* and *Forl*.

As shown in Figure 6.7, there was no accumulation of LeChi3 transcripts in root, stem and crown tissues of the control plants, whereas in plants inoculated with *Fol* and *Forl* only, the LeChi3 transcript increased gradually over time. However, in plants inoculated with *Fol* and *Forl* and treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and a combination of these treatments, LeChi3 expression was significantly higher compared to that of the plants inoculated with *Fol* and *Forl* only. In root samples for the plants inoculated with *Fol*, *T. pseudokoningii* and the combination treatments, SlChi3 expression was higher especially at 10 and 20 dpi compared to other treatments, whereas in stem samples, it was higher in all treatments at 10 and 20 dpi compared to *Fol* only. In root and crown samples for the plants inoculated with *Forl*, LeChi3 expression was significantly higher in all treatments, and it showed a progressive

accumulation from 2 to 20 dpi compared to the plants inoculated with *Forl* only (Fig. 6.8).

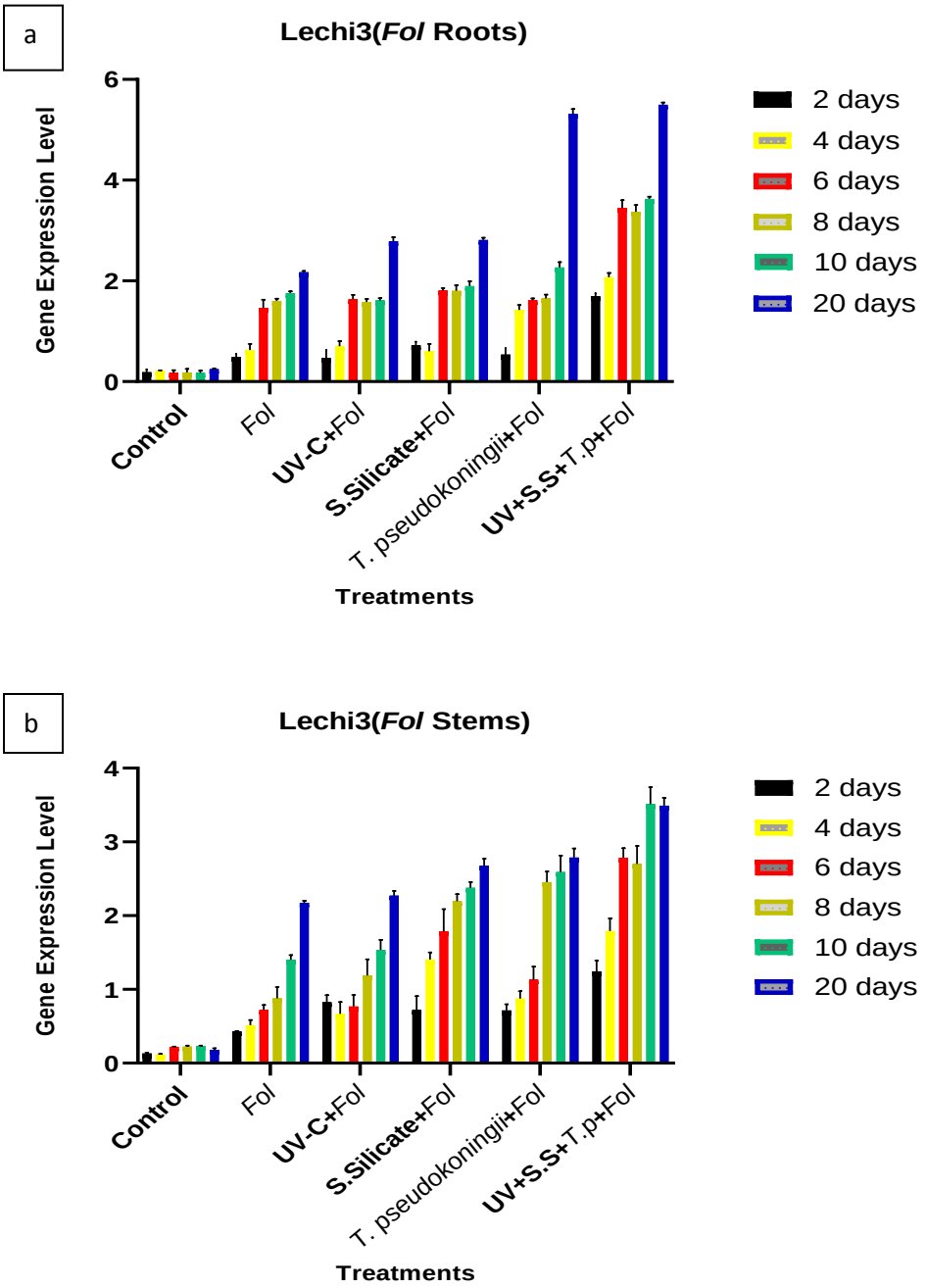


**Figure 6.7** Gene expression analysis of the defence-related gene encoding acidic extracellular chitinase (LeChi3) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

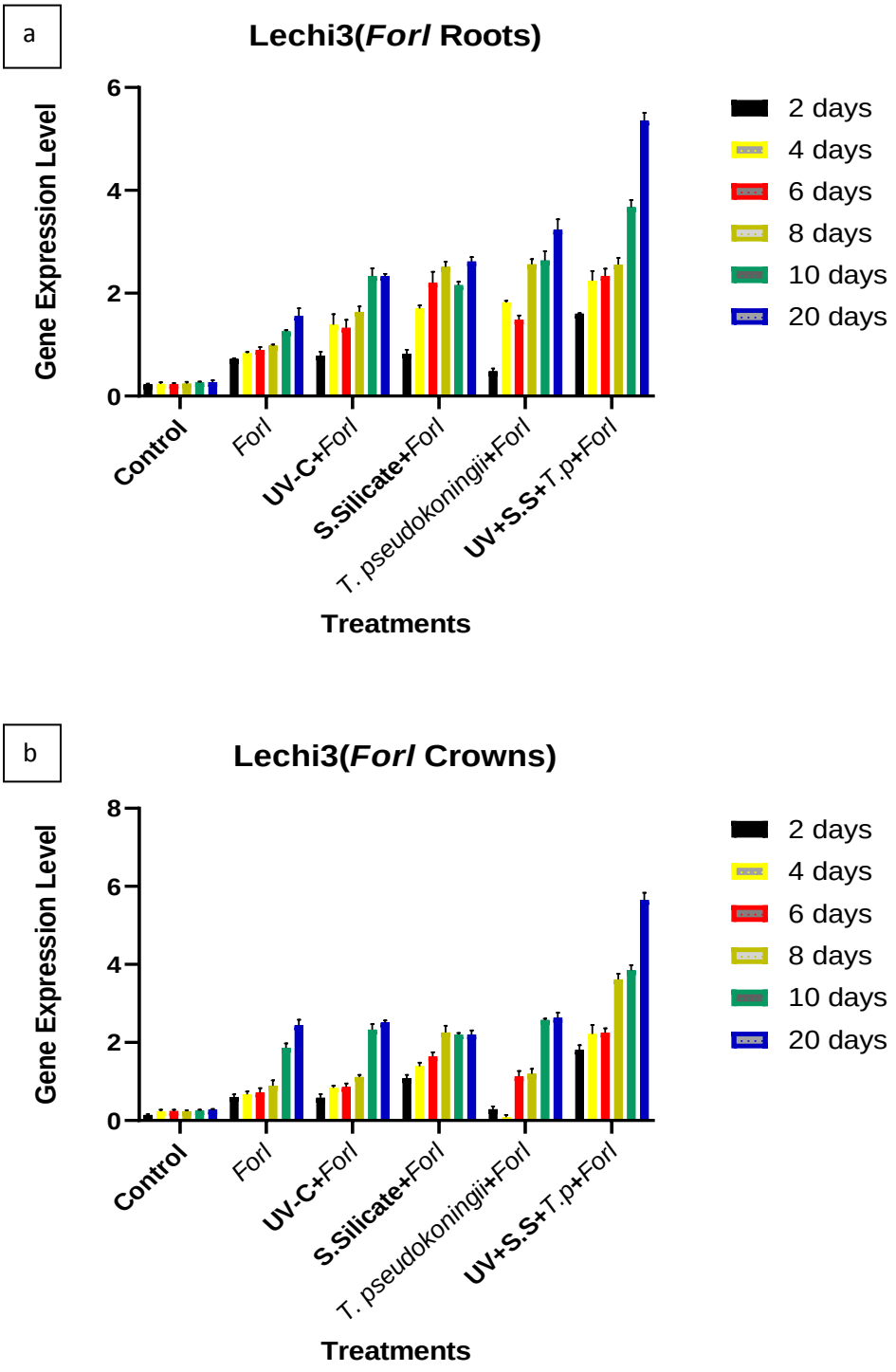


**Figure 6.8** Gene expression analysis of the defence-related gene encoding acidic extracellular chitinase (LeChi3) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

LeChi9 transcripts showed some accumulation in the healthy plants and those inoculated with *Fol* and *Forl* only in root, stem and crown samples. However, its expression was clearly increased gradually at 8 dpi in UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination treatments. In the plants inoculated with *Forl*, the expression of LeChi9 in crown samples was less than its accumulation in all treatments in root samples (Figs 6.9 and 6.10).

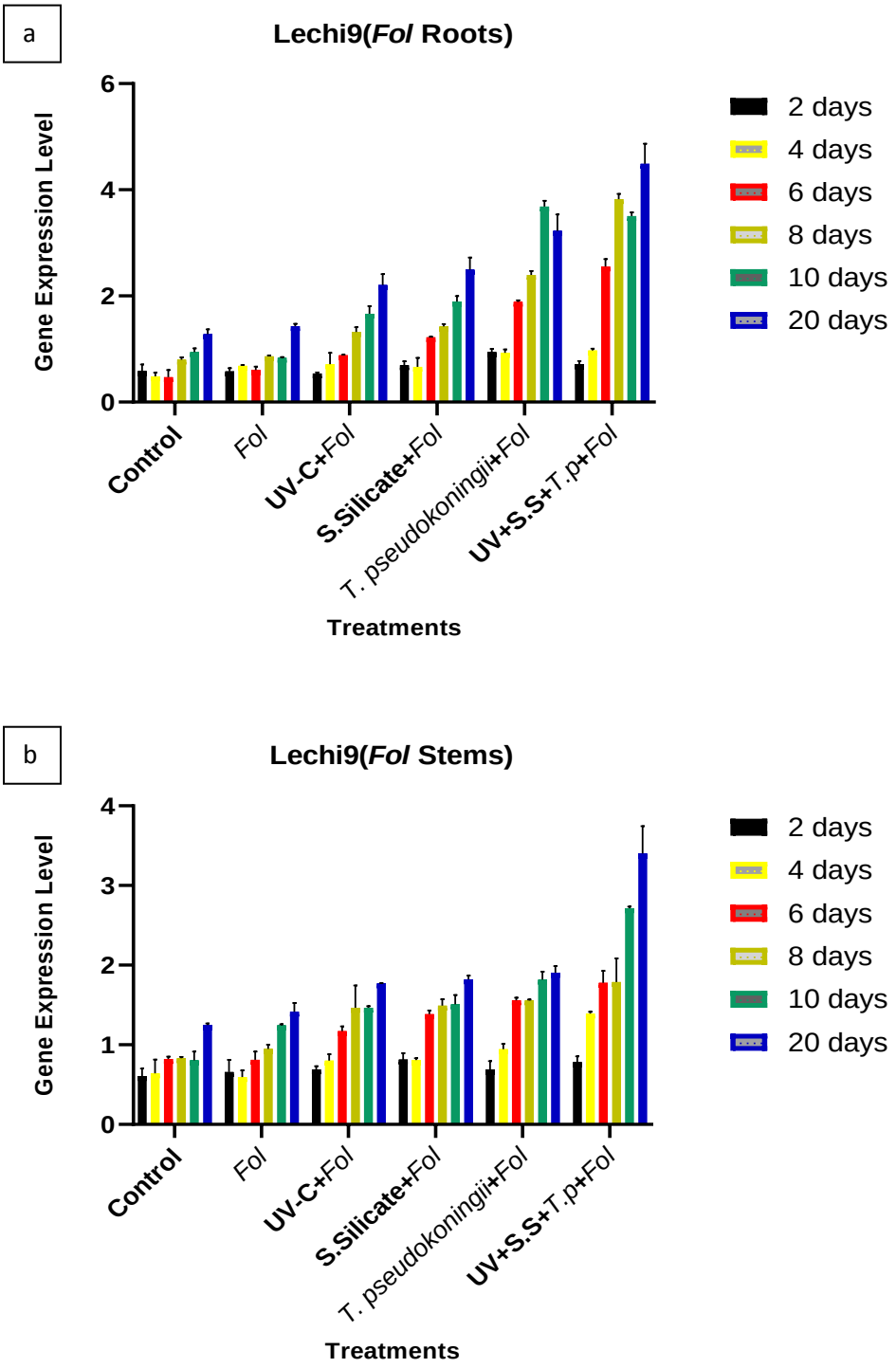


**Figure 6.9** Gene expression analysis of the defence-related gene encoding basic intracellular chitinase (LeChi9) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

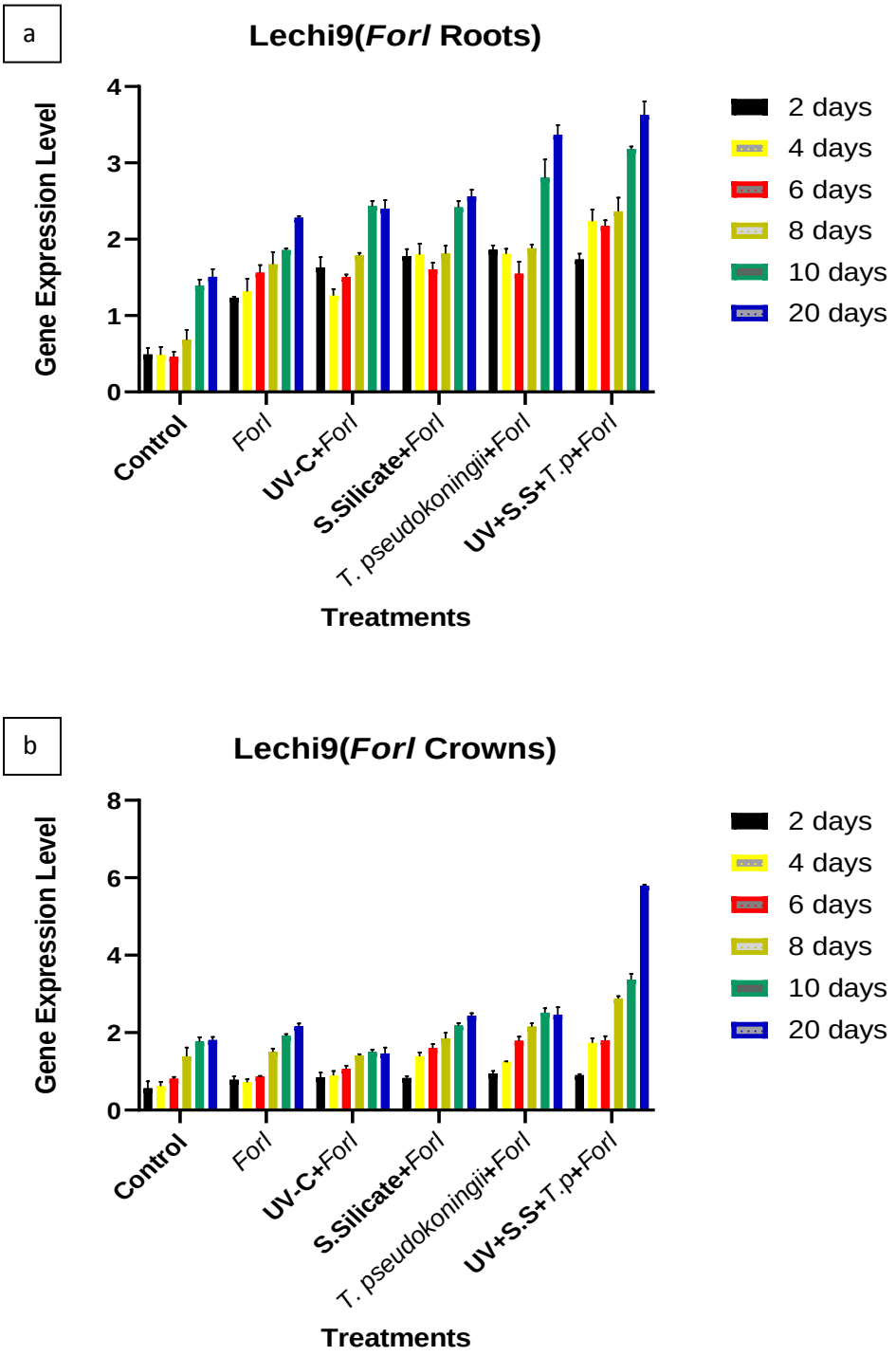


**Figure 6.10** Gene expression analysis of the defence-related gene encoding basic intracellular chitinase (LeChi9) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

For LeGluA, no expression was detected in the control healthy plants at all stem sampling time points but in root samples, there was a clear accumulation at 20 dpi. However, the *Fol* only inoculated plants showed a weak accumulation of LeGluA transcripts at 10 and 20 dpi (Fig. 6.11). The expression of LeGluA in UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination treatments was significantly higher. In plants inoculated with *Forl*, the expression of LeGluA showed also a weak accumulation in healthy and *Forl* only at 20 dpi. However, UV-C doses (6 KJ $m^{-2}$) showed higher expression compared to 50 mM sodium silicate and *T. pseudokoningii* treatments in root samples (Fig. 6.12).

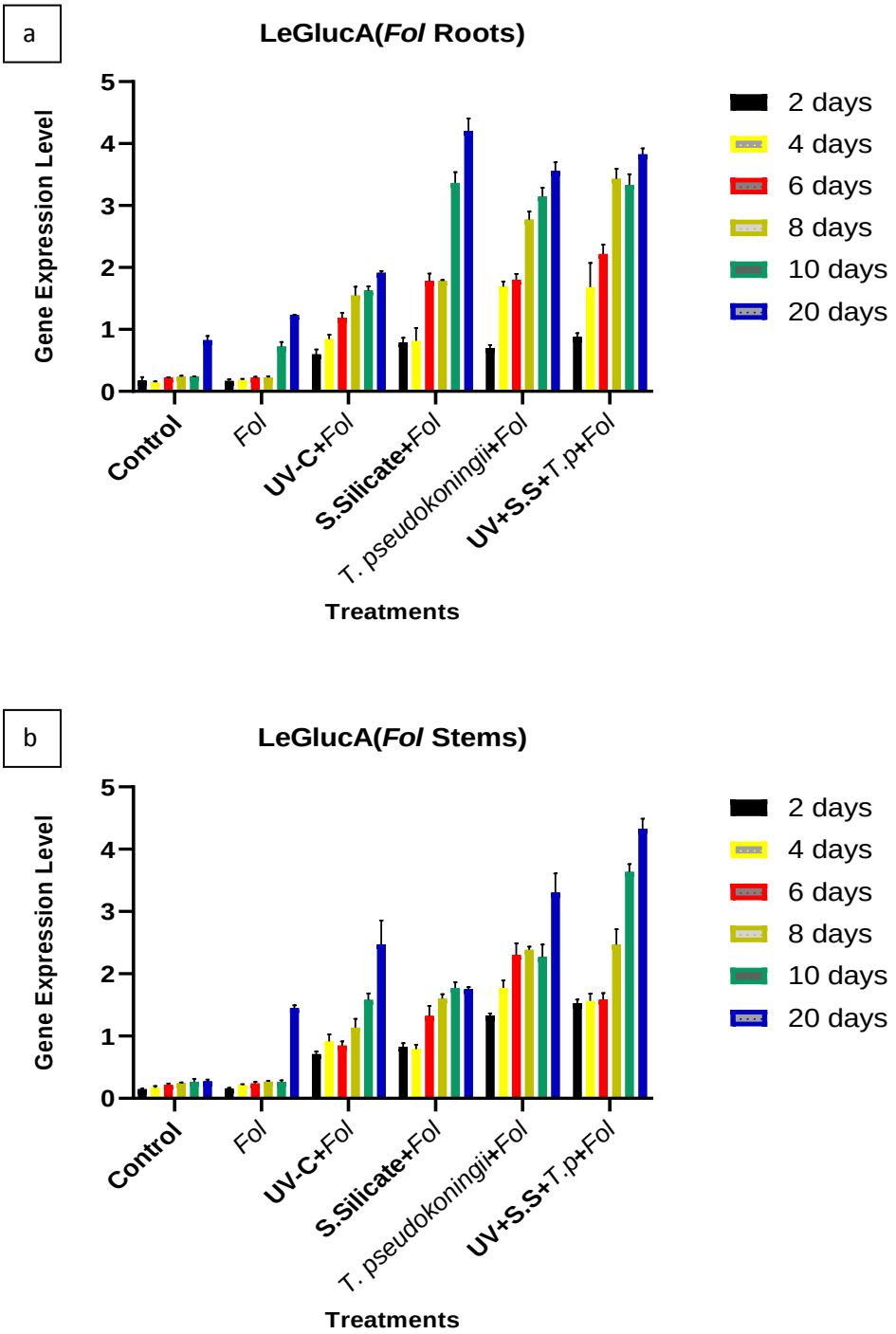


**Figure 6.11** Gene expression analysis of the defence-related gene encoding acidic extracellular b-1,3-glucanase (LeGluA) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

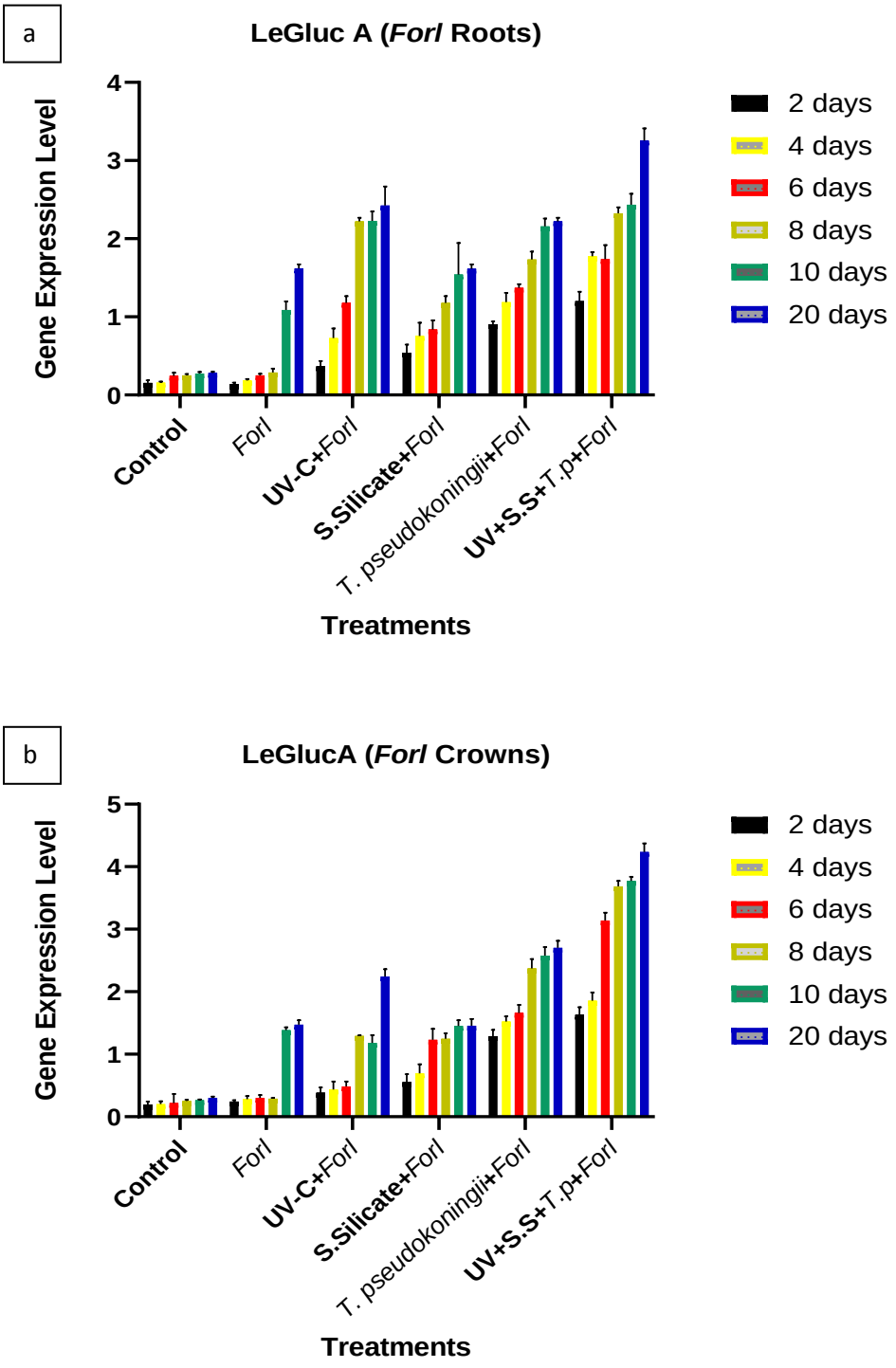


**Figure 6.12** Gene expression analysis of the defence-related gene encoding acidic extracellular b-1,3-glucanase (LeGluA) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

The accumulation of LeGluB was similar to the expression of LeGluA in root, stem and crown samples in plants inoculated with *Fol* and *Forl*. All treatments were significantly higher compared to the plants inoculated with *Fol* and *Forl* only. In plants inoculated with *Fol*, the accumulation of LeGluB in root and stem samples showed no big differences in UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate and *T.pseudokoningii* (Fig 6.13), whereas in root and crown samples in plants inoculated with *Forl* the accumulation of LeGluB was different in all treatments; in UV-C doses (6 KJ $m^{-2}$) and 50 mM sodium silicate treatments the accumulation was higher than *T. pseudokoningii* in root samples, whilst *T. pseudokoningii* treatment was higher in crown samples (Fig. 6.14).

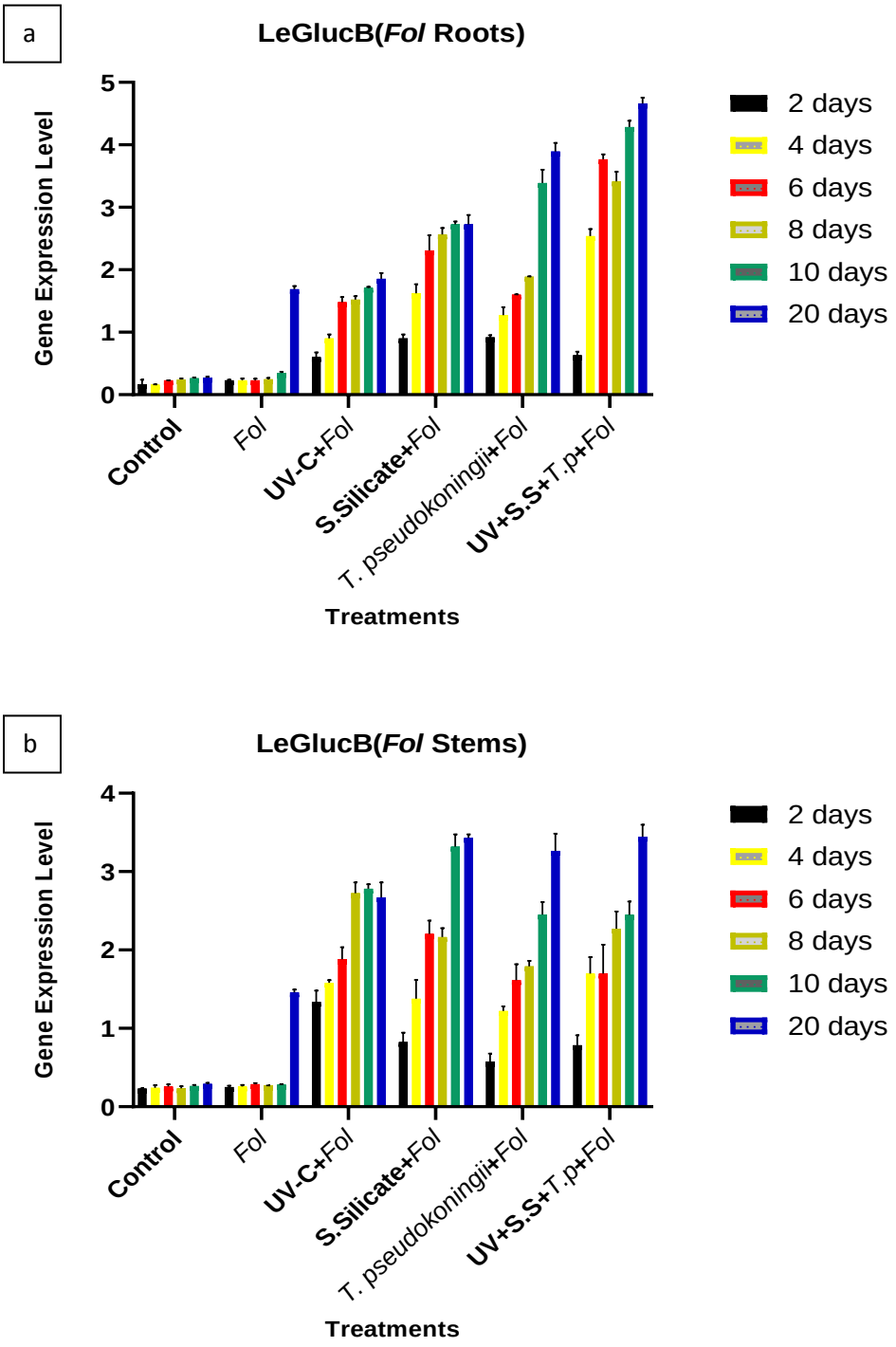


**Figure 6.13** Gene expression analysis of the defence-related gene encoding basic intracellular b-1,3-glucanase (LeGluB) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

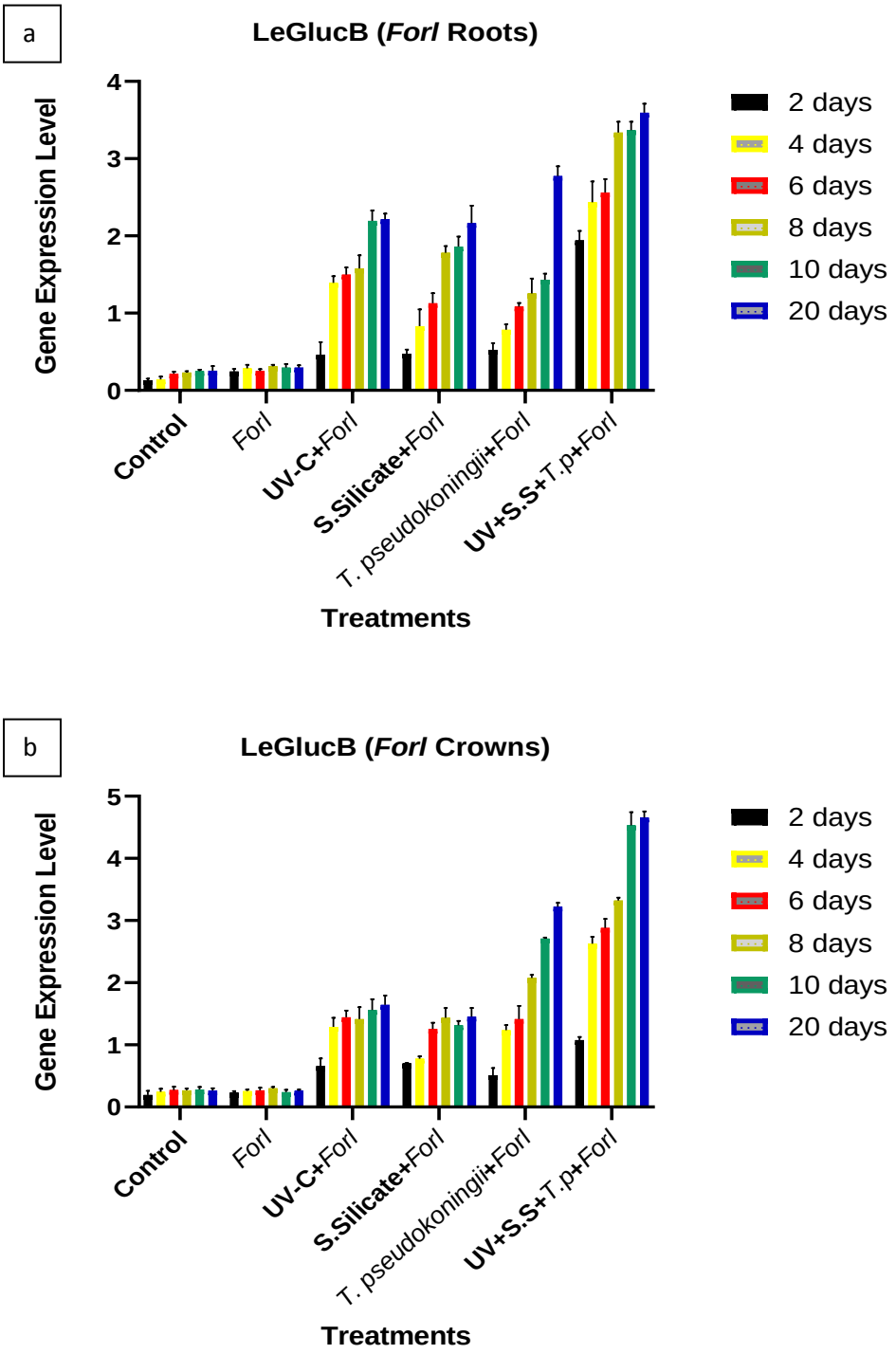


**Figure 6.14** Gene expression analysis of the defence-related gene encoding basic intracellular b-1,3-glucanase (LeGluB) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

LePR1a was observed only in root, stem and crown tissues of *Fol* and *Forl* inoculated tomato plants that were treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination treatments. No LePR1a expression was detected in either the healthy control or *Fol* and *Forl* only inoculated plants at all sampling time points (Fig. 6.15). LePr1a expression was significantly higher in all treatments, and it showed a progressive accumulation from 2 to 20 dpi compared to the plants inoculated with *Fol* and *Forl* only. However, in plants that were inoculated with *Forl* the accumulation of LePR1a was less in crown samples than root samples (Fig. 6.16).

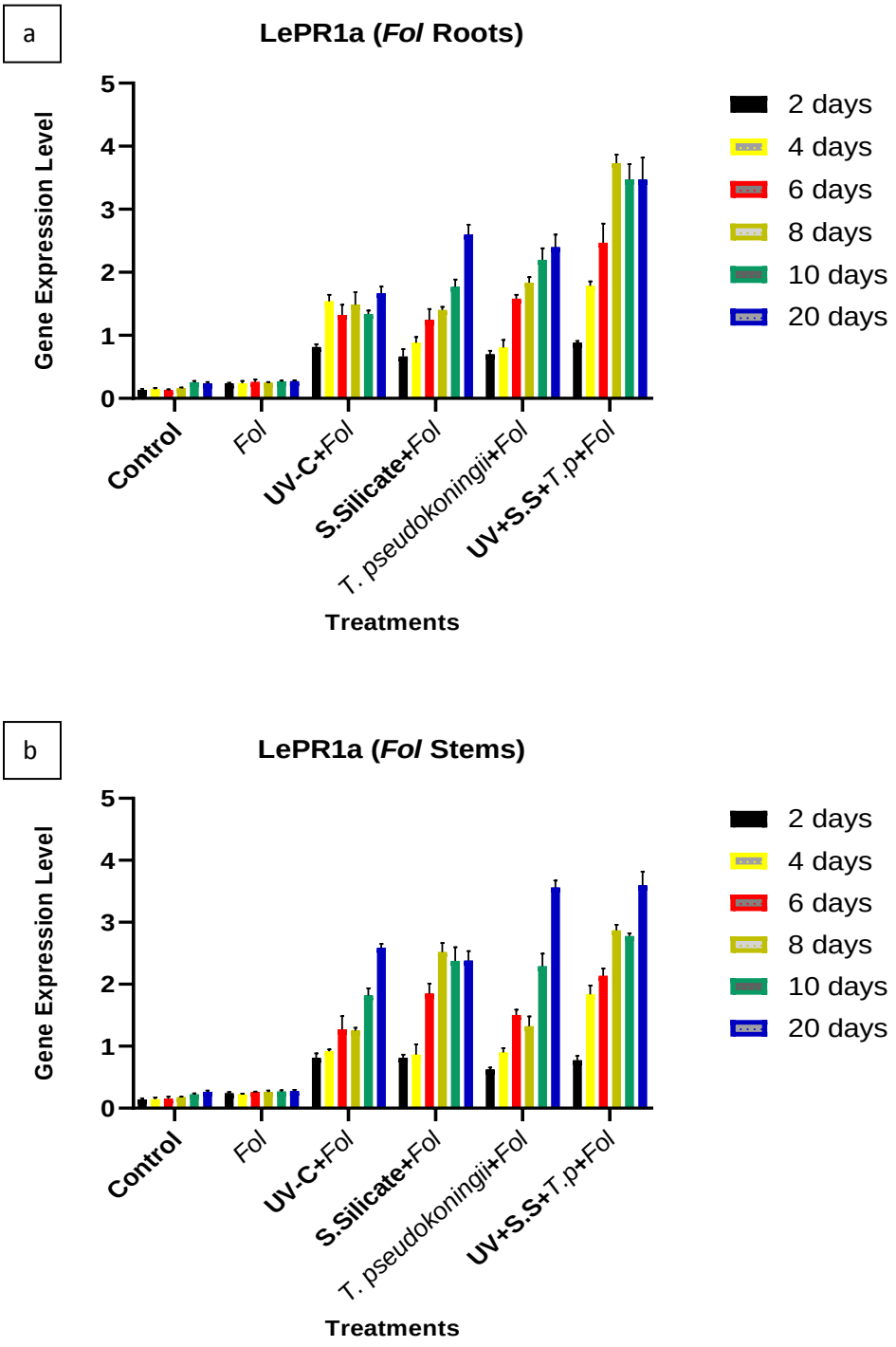


**Figure 6.15** Gene expression analysis of the defence-related gene encoding PR-1 protein isoform PR-P6 (LePR1a) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

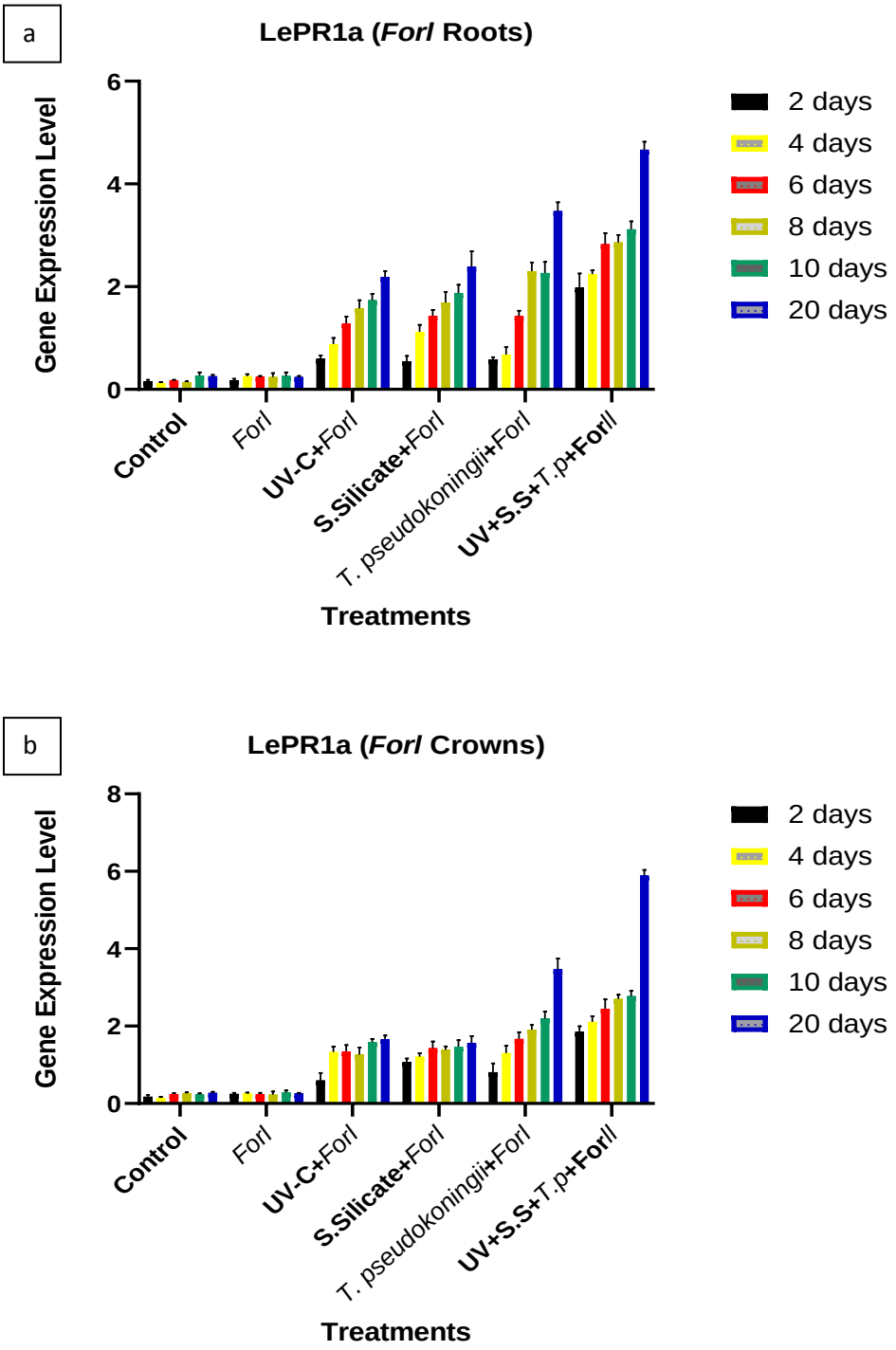


**Figure 6.16** Gene expression analysis of the defence-related gene encoding PR-1 protein isoform PR-P6 (LePR1a) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

PAL accumulation in plant samples inoculated with *Fol* and *Forl* showed that there was some accumulation in the healthy and plants inoculated with *Fol* and *Forl* only in root, stem, crown samples. All treatments were significantly higher than plants inoculated with *Fol* and *Forl* only. PAL accumulation reached its peak at 10 dpi and then declined in all samples for both *Fol* and *Forl* (Figs 6.17 and 6.18).

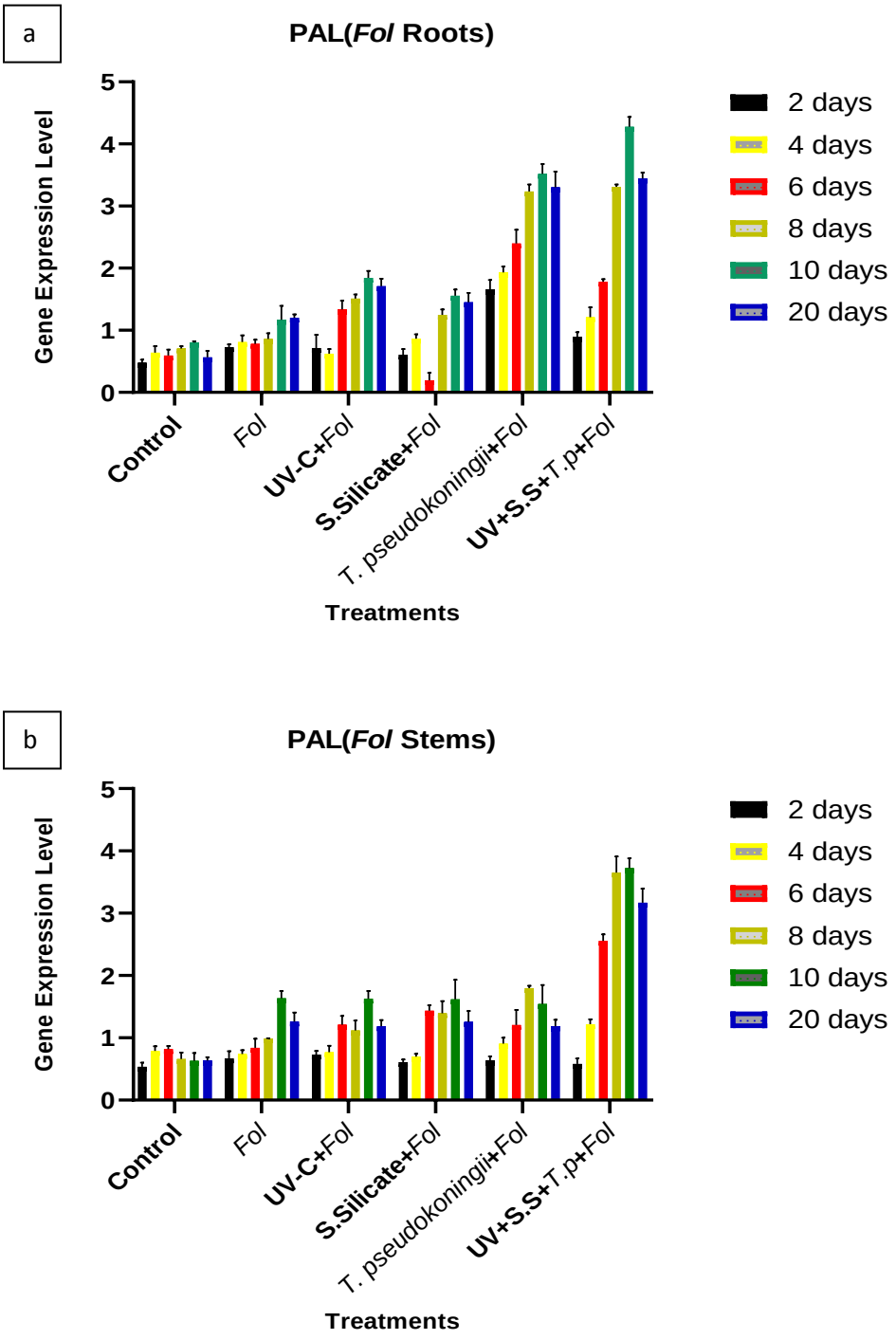


**Figure 6.17** Gene expression analysis of the defence-related gene encoding phenylalanine ammonia-lyase (PAL) in roots **(a)** and stems **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Fol* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

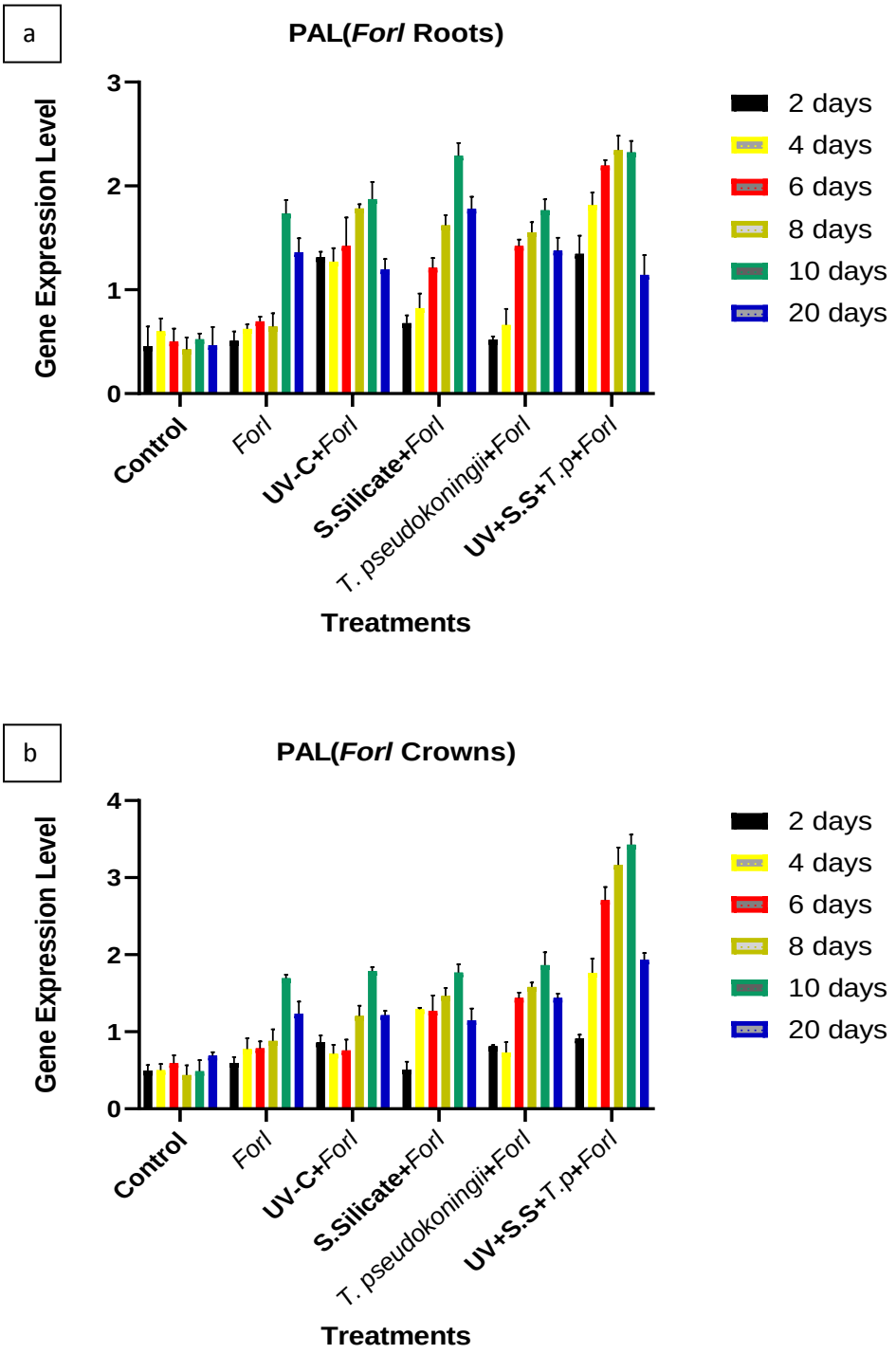


**Figure 6.18** Gene expression analysis of the defence-related gene encoding phenylalanine ammonia-lyase (PAL) in roots **(a)** and crowns **(b)** of tomato plants grown in soil treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination at different time points after inoculation with the *Forl* pathogen. Actin was used as the reference gene. Each data point represents the average for two independent biological replicates ±standard error.

In this study, the combination treatment recorded high accumulation of all tested genes among other treatments. LeChi3 and LeChi9 accumulation were higher in roots samples in plants inoculated with *Fol*. whilst in plants inoculated with *Forl*, LeChi3, LeChi9 and LePR1a accumulation were higher in root and crown samples. PAL was less expressed in all treated samples in both *Fol* and *Forl* compared to other genes.

IN UV-C (6 KJ $m^{-2}$) treatments, the results showed that Lechi3 and LeChi9 were higher expressed in root samples, whereas LeGlucA and LePR1a were higher in stem samples compared to other genes in plants inoculated with *Fol*. For *Forl* inoculated plants, LeChi3 and LeGlucA were higher expressed in both root and crown samples, while LeChi9, LeGlucB and LePR1a were higher in root samples only.

In 50 mM sodium silicate treatments, for *Fol* inoculated plants, the expression of Lechi3, LeGlucB and LePR1a was high in root and stem samples, wheras LeChi9 and LeGlucA were high in root samples only. In plants inoculated with *Forl*, Lechi3 and LeChi9 were highly expressed in root and crown samples, whereas LeGlucB and LePR1a were high in root samples only. In plants treated with the biocontrol agent *T. pseudokoningii,* all genes tested in this study showed high expression in root and stem samples except LeChi9 which recorded less accumulation in stem samples and the PAL gene which recorded less accumulation in both root and stem samples in plants inoculated with *Fol*. For *Forl* inoculated

plants, all tested genes were highly expressed in both root and crown samples except PAL which was less expressed in both samples.

## 6.5 Discussion

In this chapter, four (UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *Trichoderma pseudokoningii* and combination treatments) inducers have been investigated to study their ability in inducing systemic resistance in tomato against fusarium wilt and crown and root rot diseases. The results showed that UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination treatments reduced the disease severity by 26.7, 33.3, 39.7 and 46% respectively in plants inoculated with *Fol*, whereas in plants inoculated with *Forl* the reduction was 25.0, 41.7, 50.0 and 58.3% respectively.

The expression profile of tomato resistance-related genes (LeChi3, LeChi9, LeGlucA, LeGlucB, PAL and LePR1a) were investigated in roots, stem and crown tissues using qPCR. Tomato plants were inoculated with *Fol* and *Forl* and then treated with UV-C doses (6 KJ $m^{-2}$), 50 mM sodium silicate, *T. pseudokoningii* and combination and sampled at 2, 4, 6, 8, 10 and 20 (dpi). The results showed that the expression of all tested genes varied in all treatments. However, they were significantly different compared to the controls.

β -1,3-Glucanases play many important roles in plants, from regulating germination to defending the plant against pathogen attack. Pathogenesis-related chitinases, which include acidic extracellular chitinase (LeChi3) and basic intracellular chitinase (LeChi9) are involved in the break-down of glycosidic bonds in the cell wall of fungal pathogens. Here we observed significant expression of acidic extracellular b-1,3-glucanase (LeGlucA) and basic intracellular b-1,3-glucanase (LeGlucB) in the *T. pseudokoningii* treatment in comparison with the control. This finding was in agreement with Aimee *et al*. (2008) and ElKomy *et al*. (2016) who demonstrated increases in the accumulation of these genes in plants that were treated with protective *Trichoderma* strains against *Fol*.

The results also demonstrated the impact of biological control using *T. pseudokoningii* in enhancing resistance genes in the root, stem and crown tissues against *Fol* and *Forl* pathogens. This action of factors was presumably due to its ability to secrete enzymes such as chitinases and glucanases which work to break down chitin and glucan chains in the cell walls of the wilt fungi. These results supported the study of Ojha and Chatterjee (2012) who demonstrated the ability of salicylic acid (SA) and a biocontrol agent, *T. harzianum* (TH) on the induction of phenolic accumulation content and defence enzymes in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* through increases in the

activities of both peroxidase and polyphenol oxidase enzymes which play a role in disease resistance against fungal pathogens.

# Chapter 7. General discussion

Tomato production is threatened by attack by many fungal diseases and fusarium wilt and crown and root rot are among these serious diseases of tomato, causing huge losses in tomato production. They can destroy tomato plants at all developmental stages and affect the quality and quantity of fruit production (Biondi *et al*., 2004). Although using traditional methods such as the use of chemical fungicides and soil fumigators are considered effective ways to control plant disease, they have resulted in dramatic increases in pathogen resistance to the chemicals and the accumulation of toxic chemicals in the environment. Alternative ways of disease control are needed. Recently, physical and biocontrol agents have been becoming more widely used to manage a variety of soilborne pathogens. Hormetic UV-C treatments may be one of these new strategies that have been recently used to control plant diseases.

The present work was conducted to evaluate the potential of using UV-C seed treatments, sodium silicate and *Trichoderma* spp. to reduce disease severity of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) on tomato. Furthermore, studies were undertaken on the capacity of these methods as inducers to elicit host defence genes against these pathogens. In this study, the effect of a range of UV-C seed treatments (0, 2, 4 and 6 kJ/$m^2$) on disease severity has been investigated. The glasshouse experiment result showed that 4 and 6

kJ $m^{-2}$ doses significantly decreased disease severity on the plants inoculated with *Fol*. The percentage disease severity score was decreased from 87.5% (control) to 75.0% and 66.6% respectively. In the plants inoculated with *Forl*, only the 6 kJ $m^{-2}$ dose resulted in a significant reduction in disease severity, which was decreased from 69.4% (control) to 50.0%. The results of this study are in agreement with the findings of Brown *et al.* (2001) who tested different UV-C doses (1.3 to 7.5 kJ $m^{-2}$) seed treatments on cabbages stored for 2 days, 1, 5, and 8 months against black rot disease caused by *Xanthomonas campestris pv. campestris. They stated that* the effect of storage time at room temperature on disease incidence of black rot of cabbage from seeds treated with a low hormetic UV-C dose of 3.6 kJ $m^{-2}$ was 90%, 40%, 60% and 60% reduction of black rot in plants from UV-C treated seeds stored for 2 days, 1, 5, and 8 months, respectively.

The present investigation is also in line with the findings of Siddiqui *et al.* (2011) who found that treating seeds of mung bean and groundnut with ultraviolet (UV-C) radiation for 0, 5, 10, 15, 20, 30 and 60 minutes altered growth parameters and reduced infection by root infecting fungi like *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* spp. They observed that both crop plants showed increases in shoot weight, shoot length, root length and root weight, leaf area and number of nodules when treated with UV-C for 10, 15, 30 and 60 minutes. They also observed that

the maximum reductions of *R. solani*, *M. phaseolina* and *Fusarium* spp. on mung bean roots were recorded when seeds were treated with UV-C for 5, 10, 15 and 20 minutes, whilst for groundnut seeds treatments for 20, 30 and 60 minute intervals showed maximum reduction in infection of *R. solani* and *M. phaseolina.*

The results of this study are also in accordance with the findings of Scott *et al*., (2018) who reported that hormetic UV-C treatments of tomato seed can control disease caused by *Botrytis cinerea.* They tested the effect of 2, 4 and 6 kJ $m^{-2}$ UV-C doses on disease incidence and progression of *B. cinerea* on cv. Shirley. The results showed that plants grown from seed treated with a 4 kJ $m^{-2}$ dose showed a statistically significant decrease in disease incidence of 10.3% when compared to both the control and 4 kJ $m^{-2}$ dose treatment. The 4 kJ $m^{-2}$ treatment also showed a significant reduction in the median disease progression (11.4%) in comparison to the control.

It has been reported in previous studies that application of silicon materials such as potassium silicate and sodium silicate improved growth and yield of plants as well as enhanced plant resistance against abiotic and biotic stresses (Seebold *et al*., 2001). In the present study the beneficial effects of sodium silicate to reduce disease severity of fusarium wilt and crown and root rot diseases have been evaluated. Different concentrations (0, 10, 20, 30, 40, and 50 mM) of sodium silicate were tested for their

ability to reduce disease severity of *Fol* and *Forl* under glasshouse conditions. The results showed that 30, 40, and 50 mM concentrations were highly significant compared to the other treatments. The disease severity was 56.0, 50.0, and 46.6% respectively for *Fol* and was 45.83, 41.66, and 29.16%, for *Forl*. These results are in agreement with findings of Rahman *et al.* (2015) who demonstrated the ability of three different levels of calcium silicate (1, 5, or 10 t/ha) in comparison to the unamended soil as a control in reduction of disease incidence and severity of gray leaf spot disease on perennial ryegrass caused by *Magnaporthe oryzae* under field conditions. They found that 5 t/ha calcium silicate was found to significantly suppress gray leaf spot in perennial ryegrass, including a significant reduction of disease incidence (39.5%) and disease severity (47.3%).

Several reports indicate that silicates effectively control plant diseases, and the reduction of disease progression is correlated with the increases of sodium silicate concentrations. Li *et al*. (2009) investigated different concentrations (0, 25, 50, 100, and 200 mM) of sodium silicate to reduce dry rot infection of potato tubers caused by *Fusarium sulphureum.* They found that sodium silicate treatments at 100 and 200 mM significantly decreased the diameter of dry rot lesions in tubers inoculated with *F. sulphureum* with inhibition rates of 44% and 45%, respectively. The ability of sodium silicate to control dry rot was enhanced when the sodium

silicate concentration was increased from 25 to 200 mM. These results support the findings of this present study, which showed the relationship between the rate of disease reduction and increasing the sodium silicate concentrations in *in vivo* experiments.

Different sources of silicon have been investigated to study their ability in reducing disease incidence and severity. Franaz *et al.* (2012) tested silica gel in infested soil and sodium silicate as a foliar spray at the rates of 0, 60, 120, 180 g/5 kg soil and 0, 1, 2, 3 ml/l respectively against the rice blast disease caused by *Magnaporthe oryzae.* Silica gel application at the rate of 120 g consistently reduced disease severity by as much as 75% compared to the control. In both silica gel and sodium silicate treatments, greater reduction in disease severity was observed with increasing rates of silicon up to 120 g and 2 ml/l. However, applications of 120 g and 180 g of silica gel and 2 ml/l and 3 ml/l of sodium silicate didn't show any significant reduction in disease severity between them. Moreover, they found that disease severity reduction was higher with silica gel application than sodium silicate treatment at all rates compared to the control treatment.

In general, UV stimulates transcription of genes important for defense, including those encoding pathogenesis-related proteins such as *β*-1,3-glucanase, chitinase, Phenylalanine ammonia lyase (PAL) and stilbene synthase (Borie *et al.*, 2004; Sävenstrand *et al*., 2004; Maeda *et al*., 2005). Here, we investigated the expression of the pathogenesis related genes

*Chi3, Chi9, GluA, GluB, PR-1a* and *PAL* in tomato induced by UV-C, sodium silicate, *Trichoderma pseudokoningii* and combination of these treatments against *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *radicis-lycopersici.* The results demonstrated that the expression of all tested genes changed in all treatments. However, they were significant compared to the control. In this study, the combination treatment recorded high accumulation of all the tested genes compared to other treatments. LeChi3 and LeChi9 accumulations were higher in root samples in plants inoculated with *Fol*, whilst in plants inoculated with *Forl*, LeChi3, LeChi9 and LePR1a accumulation were higher in root and crown samples. PAL was less expressed in all treated samples in both *Fol* and *Forl* compared to other genes. With UV-C doses (6 KJ $m^{-2}$) as individual treatment, the results showed that Lechi3 and LeChi9 were more highly expressed in root samples, whereas LeGlucA and LePR1a were more highly expressed in stem samples compared to other genes in plants inoculated with *Fol*. For *Forl* inoculated plants, LeChi3 and LeGlucA were more highly expressed in both root and crown samples, while LeChi9, LeGlucB and LePR1a were more highly expressed in root samples only. These findings are in accordance with Jin *et al*. (2017) who investigated the effect of ultraviolet C treatment on controlling gray mold caused by *Botrytis cinerea* in strawberry fruit. They found UV-C treatment significantly reduced lesion diameter and enhanced activities of β-1, 3-glucanase, chitinase (CHI),

phenylalanine ammonia-lyase (PAL), peroxidase (POD) and polyphenoloxidase (PPO) in strawberry fruit. Moreover, these results suggested that UV-C treatment directly play a major role in activation of disease resistance against gray mold caused by *B. cinerea* in strawberry fruit.

In this study, when 50 mM sodium silicate was used as an individual treatment, it was found that the expression of Lechi3, LeGlucB and LePR1a was high in root and stem samples, whereas LeChi9 and LeGlucA were high in root samples only for plants inoculated with *Fol*. In plants inoculated with *Forl*, Lechi3 and LeChi9 were highly expressed in root and crown samples, whereas LeGlucB and LePR1a were more highly expressed in root samples only. Li *et al.* (2010), who investigated the effect of sodium silicate at 100 mM to reduce dry rot disease caused by *Fusarium. sulphureum* and to induce defense responses in potato, found that the activity of peroxides (POD), polyphenoloxidase (PPO), β-1, 3-glucanase (GLU), phenylalanine ammonialyase (PAL) and the production of total phenolics in tuber tissue after treatment with 100 mM of sodium silicate were not higher than that of controls. However, the activity of POD, PPO, PAL and GLU and the production of total phenolics dramatically increased after challenging with *F. sulphureum.* These results are in agreement with the findings of this study that revealed the increases of the expression of the targeted genes.

*Trichoderma* spp. can produce antimicrobial peptaibols that play a pivotal role in controlling plant disease caused by fungi, virus, and bacteria which can involve upregulation of the expression of pathogenesis-related protein genes in host plants. Here, we have tested the expression of some defence genes that are induced by using *Trichoderma pseudokoningii against Fol* and *Forl* pathogens in tomato. In plants treated with the biocontrol agent *T. pseudokoningii,* all genes tested in this study showed higher expression in root and stem samples except LeChi9 which recorded less accumulation in stem samples and PAL gene which recorded less accumulation in both root and stem samples in plants inoculated with *Fol.* For *Forl* inoculated plants, all tested genes were highly expressed in both root and crown samples except PAL which was less expressed in both samples. The present results are in the line with the findings of Li *et al.* (2014) who evaluated the control effect of Trichokonins, antimicrobial peptaibols from *T. pseudokoningii* SMF2, on soft rot disease of Chinese cabbage caused by *Pectobacterium carotovorum* subsp. *carotovorum.* They found Trichokonin treatment led to increases in the production of the reactive oxygen species hydrogen peroxide and the superoxide radical, and a significant enhancement in the activities of pathogenesis-related enzymes catalase, polyphenoloxidase and peroxidase. In addition, there was upregulation of the expression of the pathogenesis-related protein gene acidic *PR-1a* in Chinese cabbage.

To conclude, this study has demonstrated the efficacy of using new strategies to control fusarium wilt and crown and root rot diseases in tomato. Firstly, the use of UV-C seed treatments has been shown to work as a physical method to reduce disease severity and induce tomato resistance against root pathogens. The findings of this study have concurred with other studies on the effects of UV-C seed treatment on disease resistance and plant physiology that have been published and proved the ability of UV-C hormesis to reduce disease incidence and progression against fungal pathogens. Further investigation of different UV-C doses more than 6 kJ $m^{-2}$ and their role in inducing disease resistance, and on the longer-term effects of these treatments should also be undertaken.

Secondly, the use of sodium silicate as a source of silicon has been confirmed in controlling fungal pathogens. The present study revealed that 40 and 50 mM concentrations were able to inhibit the mycelial growth of *Fol* and *Forl* significantly in *in vitro* experiments and reduce disease progression of these pathogens under glasshouse conditions. Moreover, the findings confirm that silicon might be applied as an alternative tool to chemical fungicides for controlling fusarium wilt and crown and root rot diseases and to promote the defense responses in tomato. Further evaluation, especially under field conditions is required to know the

efficacy of sodium silicate concentrations against fungal pathogens on different crops.

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