CHAPTER 5

SUPPRESSION OF *RALSTONIA SOLANACEARUM* IN SOIL USING HERBAL PLANT MATERIAL

ABSTRACT

Due to its extremely wide host range and its ability to survive for prolonged periods in the soil, control of *R. solanacearum* is complicated. No single method exists for the control of this pathogen and different options should be considered in an integrated program to effectively control bacterial wilt. Due to the detrimental effect of pesticides on human health and the environment, agricultural industries have been forced to consider alternative disease control strategies that is safer and more in line with natural farming systems. One such option is the use of natural plants or microbes to prevent disease development. One component of biological control is the incorporation of plant material into soil, providing a biofumigation effect to control soilborne pathogens. Mustard (*Brassica juncea* L.) and other cruciferous species have previously been shown to be effective in reducing *R. solanacearum* populations in soil. In this study, thirteen herbal plant species were evaluated as biofumigation agents to suppress *R. solanacearum* in soil. The capability of these species acting as host plants for *R. solanacearum* (biovar 2 and 3) was also determined. Crude extracts were prepared from non-host species and tested *in vitro* for possible inhibition of the pathogen. Plant material from non-host species was incorporated into artificially inoculated soil and a glasshouse pot trail was conducted to determine whether or not *R. solanacearum* could be suppressed in soil. Marjoram, nasturtium and parsley acted as hosts for *R. solanacearum* (biovar 2), while chive, coriander, marjoram, mustard, nasturtium and parsley were hosts for *R. solanacearum* (biovar 3). Crude extracts from non-host species were unable to suppress the pathogen *in vitro*. Difficulties were experienced with soil inoculation and biofumigation could not be applied successfully.

1. Introduction

Once *Ralstonia solanacearum* has been detected and identified in a field, various control measures can be implemented to exclude the pathogen from further spread and infection to subsequent or alternate crops. Some of these methods include; crop rotation and intercropping with non-host plant species (Hayward, 1991; Hartman and Elphinstone, 1994), soil amendments with organic and inorganic material (Chellemi *et al.*, 1992; Hartman and Elphinstone, 1994), using resistant potato cultivars (Tung *et al.*, 1990), chemical control (Shekhawat *et al.*, 1992) and biological control (Hartman *et al.*, 1993).

Due to its wide host range (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 2000) and ability to survive for prolonged periods in the soil (McCarter, 1976; Graham *et al.*, 1979), control of *R. solanacearum* is complicated and several methods should be combined in an integrated program to effectively manage the disease. The primary objective of an integrated control strategy would be to limit the survival and multiplication of the pathogen and eliminate alternative hosts and interacting nematodes by means of cost-effective programs (Elphinstone and Aley, 1993). The effectiveness of any integrated approach will depend on factors such as climatic conditions, soil type, pathogen strain present, farming system as well as socio-economic situation of a specific area.

Interest in the field of biological control as an alternative method for the control of weeds, insects and plant pathogens has grown due to the global move towards more environmental friendly and natural farming systems (Lydon and Duke, 1989; Lampkin, 1990). Traditionally biological control has been defined as the total or partial destruction of plant pathogen populations by other microorganisms (Agrios, 1997). However, plant material or extracts can also be used for the suppression of pathogens and are also classified as biological control (Lampkin, 1990). Plant material has been used successfully as green manure by incorporating it into the soil for use as a biofumigant to control soilborne diseases (Akiew *et al.*, 1996). Interest in the use of biofumigation has particularly increased recently due to the prohibition of certain synthetic soil fumigants such as methyl bromide (Kirkegraad *et al.*, 1998). According to Kirkegraad and Sarwar (1998), biofumigation refers to the suppression of soilborne pests and pathogens by biocidal compounds released in soil when certain plant material is broken down. During the decomposition of plant residues, substances such as glucosinolates can be hydrolysed to release a range of volatile compounds known as isothiocyanates and other secondary compounds such as

oxazolidinethiones, nitriles and thiocyanates (Akiew *et al.*, 1996; Kirkegraad and Sarwar, 1998). The type of isothiocyanate released is specific to the type of glucosinolate present in the tissue. Isothiocyanates are the same class of chemical compounds produced by the decomposition of metham sodium (vapam), a commercial soil fumigant (Akiew *et al.*, 1996). Since some of the substances released by biofumigation have broad biocidal activity, including insecticidal, nematicidal, fungicidal, antibiotic and phytotoxic effects, it can be used as part of an integrated control program to suppress soilborne pathogens (Kirkegraad and Sarwar, 1998).

Akiew *et al.* (1996) incorporated mustard (*Brassica juncea* L.) into *R. solanacearum* infested soil and were able to suppress the pathogen population to an undetectable level after a four to five week incubation period. Kirkegraad and Sarwar (1998) were also able to successfully suppress pathogen populations with the incorporation of cruciferous green manures.

The medicinal and antimicrobial properties of herbal plant species have been recognised since before 2000 B.C. (Craker *et al*, 1986; Roberts, 1999). Various studies have since been conducted on the medicinal use and antimicrobial properties of herbs. Thanassoulopous *et al.* (1997) used essential oils from different herbs for biological control of *Botrytis cinerea.* It was therefore decided to conclude this study by evaluating various readily available, fast growing herbal plant species as green manure for biofumigation properties to suppress *R. solanacearum* in artificially inoculated soils.

2. Materials and Methods

2.1. Cultures

For the first part of this study the South African isolates, 111 (biovar 2) and 117 (biovar 3) were randomly selected (Appendix 1). For the subsequent biofumigation studies only isolate 111 (biovar 2) was used. To prepare fresh inoculum the techniques described in Chapter 3 were used.

2.2. Plant Material

Thirteen herbal species were selected for this study: *Ocimum basilicum* L. (basil), *Borago officinalis* L. (borage), *Apium graveolens* L. (celery), *Matricaria recutita* L. (chamomile), *Foeniculum vulgare* Mill. (fennel), *Melissa officinalis* L. (lemon balm), *Anethum graveolens* Linn. (dill), *Allium tuberosum* Rottler (chive), *Coriandrum sativum* L. (coriander), *Origanum majorana* L. (marjoram), *Brassica alba* L. (mustard), *Tropaeolum majus* L. (nasturtium) and *Pertoselinum crispum* L. (parsley). These species were selected according to availability, rapid germination and the production of large amounts of plant material within a relatively short period. Commercial seed was bought from a local nursery and germinated in seedling trays containing 2:1 sand-peat mixture. Seedling trays were placed in a dark growth cabinet at 20°C until seedlings were 2 to 3 cm long. Trays were then moved to a glasshouse where seedlings were hardened off for three to four weeks at 25/20°C day/night temperatures.

2.3. Host Determination

After four weeks, plants were transplanted in 2 *l* pots containing 2 kg of a sterile 1:1 clay-bark mixture (v/v). Sterile vermiculite was soaked in inoculum of either isolate 111 (biovar 2) or 117 (biovar 3) at 1 x 10^6 cfu ml^{-1} for 2 h (1:2 m/v) where after 40 g vermiculite was added to each pot and mixed with the top third of the soil. Pots were thoroughly watered and plants evaluated for symptom development each week for eight consecutive weeks. Each treatment (herbal plant species) consisted of six pots inoculated with isolate 111 (biovar 2) and six pots inoculated with isolate 117 (biovar 3).

Each plant was tested individually for the presence of the pathogen according to the technique described by Swanepoel (1992). Plants were removed from the pots and the roots and bottom 10 cm of the stems washed in tap water to remove excess soil. This material was surface sterilised in 1% sodium hypochlorite for five minutes and then rinsed three times in sterile distilled water. Prepared plant material was placed into sterile Petri dishes containing 9 ml sterile Ringers solution and cut into fine pieces with a sterile scalpel. After 30 minutes diffusion time a dilution series were prepared, plated out on TZC medium (Kelman, 1954), incubated and evaluated for the presence of *R. solanacearum* according to the methods described in Chapter 3.

2.4. *In Vitro* Tests with Crude Extracts

The basil, borage, celery, chamomile, fennel, lemon balm and dill were selected for *in vitro* testing of crude extracts from the original 13 species based on their non-host characteristics. Crude extracts were prepared according to the method described by Terblanche and de Villiers (1998). Plant material (roots, stems and leaves) was removed, surface sterilised in a 1% sodium hypochlorite solution for five minutes and then rinsed three times in sterile distilled water. Crude extracts were prepared by homogenising plant material in sterile distilled water (1:1 v/v). Seeded TZC agar plates were prepared by pouring an additional thin layer of cool TZC medium, to which *R. solanacearum* inoculum (1 x 10^4 cfu ml^{-1}) was added, to standard TZC agar plates (Stander, 2001). Four wells were punched in each agar plate using a sterile cork bore and each well was filled with 100µl suspension. Treatments (suspensions) included a 1% sodium hypochlorite solution and sterile water that acted as controls, crude extracts and sterile extracts prepared from the selected herbal plant species. Crude extracts were sterilised with the aid of a Millipore filter. Each treatment was repeated three times. Plates were incubated at 28°C and formation of inhibition zones evaluated and measured after 72 h.

2.5. Soil Suppression (a)

Seed from the seven species selected in 2.4. was sown and seedlings raised according to the method described in 2.2. After four weeks, six plants from each species were transplanted in 2 *l* pots containing 2 kg sterile 1:1 clay-bark mixture (v/v) and kept in a glasshouse for six to eight weeks. Plants from each species were removed, rinsed and the whole plant cut into 1 to 2 cm pieces.

Class 1, BP1 potato tubers (generation 1) were taken from cold storage 48 h prior to planting and 50 to 69 g tubers selected. A 2:1 clay-bark soil mixture (v/v) was steam sterilised, added to 1 part non-sterilised bark, thoroughly mixed and 2 kg mixture weighed off into 2 *l* pots. Each treatment was divided into two parts and consisted of five replicates. For part 1, vermiculite was soaked in biovar 2 inoculum (refer to 2.3) and together with 20 g herbal material, were added to each pot and thoroughly mixed. For part 2, the vermiculite was omitted and only 20 g of cut plant material was added to each pot and thoroughly mixed. Control treatments were also included where herbal plant material was omitted. Tubers were planted 10 cm deep, pots were placed in a

glasshouse and watered regularly. Glasshouse temperatures ranged between 10 to 12°C min and 20 to 22°C max (winter temperatures). After emergence, potato plants were weekly evaluated for typical wilt symptoms.

After six weeks, plants were removed and tissue isolations made on TZC medium from the bottom 10 cm of stems to determine the presence of *R. solanacearum* (refer to 2.3). Tubers were harvested, weighed and cut open to evaluate possible ring symptoms in the vascular ring (Harrison, 1961). Developing tubers smaller than 1 cm were discarded. The average amount of tubers per plant, average mass of tubers per plant and average mass per tuber were statistically analysed. Variance of analysis to separate means were carried out followed by Duncan's multiple range test used at the 5% significance level to determine the possible effect of incorporated plant material on tuber production (SAS Institute, 1999).

2.6. Stem Inoculation

To regain possible loss of virulence, *R. solanacearum* isolate 111 (biovar 2) was inoculated into potato plants and re-isolated according to the technique described by Kelman and Sequeira (1965). Mature potato plants were inoculated by piercing the stem with a sterile needle through a drop of bacterial suspension (10^7 cfu ml^{-1}) placed on a leaf axil at the third node below the stem tip. After inoculation plants were transferred to a glasshouse and temperatures maintained between 25 and 30°C. After first symptoms of wilt appeared, plants were removed, tissue isolations prepared and the pathogen re-isolated on TZC medium.

2.7. Soil Suppression (b)

Soil inoculation was repeated but instead of soaked vermiculite, 50 ml isolate 111 inoculum (1 x 10^5 cfu ml^{-1}) was added to each pot after tubers were planted (Martin and Nydegger, 1982). Isolate 111 inoculum (1 x 10^6 cfu ml^{-1}) was again added to each pot after a week. Pots were placed over drip trays and watered daily. Glasshouse temperatures ranged between 18 to 20°C min and 30 to 35°C max (summer temperatures). After emergence, potato plants were weekly evaluated for typical wilt symptoms. After six weeks, plants were removed, tissue isolations made and plates evaluated for positive *R. solanacearum* growth as described in 2.5.

3. Results

3.1. Host Determination

None of the 13 selected herbal plant species developed typical wilt symptoms but the pathogen was re-isolated from several species (Table 1).

Table 1 Host determination of the different herbal plant species (results from individual replicates not included)

Herbal specie	Host determination	
	Biovar 2	Biovar 3
Ocimum basilicum L. (basil)	-	-
Borago officinalis L. (borage)	-	-
Apium graveolens L. (celery)	-	-
Matricaria recutita L. (chamomile)	-	-
Foeniculum vulgare Mill. (fennel)	-	-
Melissa officinalis L. (lemon balm)	-	-
Anethum graveolens Linn. (dill)	-	-
Allium tuberosum Rottler (chive)	-	+
Coriandrum sativum L. (coriander)	-	+
Origanum majorana L. (marjoram)	+	+
Brassica alba L. (mustard)	-	+
Tropaeolum majus L. (nasturtium)	+	+
Pertoselinum crispum L. (parsley)	+	+

3.2. *In Vitro* Tests with Crude Extracts

After incubation, *R. solanacearum* colonies developed evenly throughout the seeded TZC plates and clear zones could be measured around all wells inoculated with the 1% sodium hypochlorite solution. Although some degree of suppression was noted around wells inoculated with non-filtered crude extracts, no clear inhibition zones developed. Bacterial growth (not *R. solanacearum*) developed in and around these wells and suppression was possibly as result of these endophytes and not because of antibacterial properties of the plant extracts. No inhibition zones developed around wells inoculated with filter-sterilised crude extracts or sterile distilled water.

3.3. Soil Suppression (a)

No wilt symptoms developed during the six-week period after emergence. All tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. There was no significant difference in the average amount of tubers produced per treatment, the average mass of tubers produced per treatment or the average mass per tuber of the different treatments

3.4. Stem Inoculation and Soil Suppression (b)

First wilt symptoms appeared after twelve days and *R. solanacearum* was successfully re-isolated from stem inoculated potato plants and used in the second soil inoculation trial.

No wilt symptoms developed during the six-week period after emergence. All tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. Due to a very low percentage emergence (28%) no statistical analysis could be performed on the tuber production between the different treatments.

4. Discussion

Although only 13 herbal species were tested in this study, the general ability of the pathogen to survive in alternative hosts was confirmed. Biovar 3 was able to infect more herbal species than biovar 2. This coincided with previous observations that biovar 3 has a wider host range than biovar 2 (Swanepoel, 1992). Strains of *R. solanacearum* were differentiated into 5 races according to host range (Buddenhagen *et al.*, 1962; Walker and Stead, 1993). South African biovar strains could be designated to race 1 (Swanepoel and Young, 1988) that affected a wide range of host plants including solanaceous hosts, diploid bananas and a variety of non-solanaceous plants (Buddenhagen *et al.*, 1962). Biovar 2 could be designated as race 3 (Buddenhagen and Kelman, 1964). This race is restricted to potato, to a lesser extent tomato and a few solanaceous weed species (Buddenhagen *et al.*, 1962). *Origanum majorana* L. (marjoram), *Tropaeolum majus* L. (nasturtium) and *Pertoselinum crispum* L. (parsley) belonged to the

families *Labiatae*, *Tropaeolaceae* and *Umbelliferae* respectively. These results did not coincide with previous findings that biovar 2 (race 3) only infected solanaceous plants (Buddenhagen *et al.*, 1962) and should be further investigated.

No wilt symptoms developed during the six-week period after emergence, all tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. From these results it could be speculated that the pathogen was unable to successfully infect the potato plants. This can be attributed to various factors such as low inoculum concentration, low soil temperatures or low soil water content (Kelman and Sequeira, 1965; Ciampi *et al.*, 1980; Chen and Echandi, 1984). Although *R. solanacearum* has the ability to survive for periods of several years under field conditions (McCarter, 1976; Graham *et al.*, 1979) its viability in soil under controlled conditions is known to be poor (Granada and Sequeira, 1983; Shekhawat and Perombelon, 1991). This was confirmed by our findings.

According to Ciampi *et al.* (1980), potatoes grown under cool conditions (12-22°C) produced no visually infected tubers and no stem infection could be detected. Swanepoel (1990) however found that BP1 potato plants could develop symptoms where temperatures were as low as 14 to 16°C. To promote rapid disease development, glasshouse temperatures were maintained at 26 to 34°C during the day and 22 to 28°C during the night (Graham *et al.*, 1979). The best growth of inoculated *R. solanacearum* took place between 30 and 35°C (Shekhawat and Perombelon, 1991) and Gallegly and Walker (1949) also found that bacterial wilt increased with an increase in temperature. When temperatures were higher during our second soil inoculations, symptoms again did not developed and it can therefore be concluded that insufficient infection was not due to unfavourable temperatures maintained in the glasshouse.

Although wounding of roots is not absolutely essential for infection (Chen and Echandi, 1984) cutting the lateral roots with a scalpel along one side of the plant to a depth of approximately 4 cm prior to soil inoculation can enhance infection (Winstead and Kelman, 1952). We decided against root cutting since uniform wounding could not be ensured. However, since no apparent infection occurred in this study, wounding should be considered for future studies.

Kelman and Sequeira (1952) observed that where wounding did not occur, infection could only take place if inoculum concentration were higher than 10^4 to 10^5 cfu ml^{-1} soil sample. They did however not specify whether or not these concentrations were the initial inoculum concentrations

of the final concentrations after soil inoculation. Chen and Echandi (1984) found that there was a direct correlation between the inoculum concentration and the number of cells that adhered to roots. They used inoculum concentrations of 10^6 to 10^7 cfu g^{-1} of soil and concluded that wilt severity increased as inoculum concentration increased. Although inoculum concentrations of 10^5 to 10^6 cfu ml^{-1} inoculum were used in our trial, the dilution factor of the soil was not taken into account and possibly resulted in too low concentrations after soil inoculum for successful infection.

Survival of *R. solanacearum* is most efficient in wet, but well drained soils and survival is affected by soil desiccation and flooding (Kelman, 1953; Buddenhagen and Kelman, 1964). High soil moisture should be maintained for the duration of the trial (Kelman and Sequeira, 1965) but during our first soil inoculations, pots were not watered immediately after inoculation and allowed to dry out between each watering. According to Nesmith and Jenkins, 1979) highest populations of the pathogen were found in saturated (but not flooded) soils. According to them, colony counts declined rapidly in flooded or dry soil. This could explain why infection was again unsuccessful during both the soil inoculations. Soil should not have been allowed to dry out between watering during the first soil inoculations and was probably too wet during the second trial. This wet conditions probably attributed to very poor plant emergence (28%) and most tubers rotted within a week after planting.

According to Kelman (1953), *R. solanacearum* has the ability to loose its virulence under certain conditions, resulting in a reduced ability to cause wilt in host plants. This phenomenon is also referred to as phenotypic conversion (PC) and is thought to be regulated by environmental signals. Exactly what these environmental signals are and how they interact are not yet clear (Denny *et al.*, 1998).

It has been hypothesised that *R. solanacearum* normally exists as the PC-type in soil (Denny *et al.*, 1994). When conditions are suitable, the pathogen will switch to its wild type to cause infection and induce wilt symptoms in the host. Although the environment may play a role in this switching (Denny *et al.*, 1998) pathogen concentration may also play an important role (Schell, 1996). According to him, phenotypic conversion occurs when some endogenous inducer, probably 3-hydroxypalmitic acid methyl ester (3-OH PAME) exceeds a critical concentration. When pathogen concentrations in the soil are too low, sufficient 3-OH PAME cannot accumulate and the pathogen would not be able to cause wilt symptoms in its host. A much longer incubation

period will then be necessary to ensure sufficient cell multiplication and therefore high enough levels of 3-OH PAME to switch on virulence.

This could explain why infection was not successful in this study and should be taken into consideration in future studies.

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