VARIABILITY IN PLANT GROWTH PROMOTING PROPERTIES AMONG CLINICAL AND ENVIRONMENTAL ISOLATES OF *STENOTROPHOMONAS MALTOPHILIA*

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Title

Variability in plant growth promoting properties among clinical and environmental isolates of *Stenotrophomonas maltophilia*

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MASTER OF SCIENCE

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ABSTRACT

Stenotrophomonas maltophilia has both negative and positive attributes by being a human pathogen and plant growth promoting rhizobacterium. This study sought to determine if environmental and clinical isolates of *S. maltophilia* are phenotypically distinct. A total of 18 *S. maltophilia* isolates from clinical and environmental sources were investigated. Under normal growing conditions, *S. maltophila* isolates did not enhance growth of canola seedlings. However, under sodium chloride stress (6 decisiemens per meter or 0.33% NaCl), canola seedlings inoculated with *S. maltophilia* isolates had significantly ($P < 0.05$) higher number of root branches (isolate D457), root length (D457, CDC 2004-33-01-01 and CDC 2007-23-08-03) and stem length (D457, CDC 2005-37-11-04 and CDC 2011-01-42) than the "no bacteria" control. A number of *S. maltophilia* isolates protected canola plants from the growth limiting effects of *Leptosphaeria maculans* and *Burkholderia cenocepacia*. No clear evidence was found between clinical and environmental isolates based on phenotypic data.

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DEDICATION

To the memory of my beloved younger brother, **ERIC KAFUI WORNYO**

(June 23 1984 - September 22 2007), an energetic and promising final year student of North Georgia College and State University who passed on in Dahlonega, near Atlanta, Georgia.

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INTRODUCTION

Soil bacteria that associate with plants may confer neutral, beneficial, or inhibitory effects on their host. Plant growth promotion involves different bacterial relationships. Some of these relationships include symbiosis, colonization of plant intercellular tissues and cyanobacteria (Glick, 2012). Bacteria plant growth promotion could be either direct or indirect. Directly, they help in the acquisition of resources as well as plant growth hormone levels regulation. On the other hand, they promote plant growth and development by protecting the plant from harmful agents (Glick, 2012). The use of beneficial microorganisms in Agriculture has gained prominence recently. This is due to concerns over the harmful effects chemicals used on food produce may pose (Whips, 2001).

Stenotrophomonas play an important role in nature, particularly in biogeochemical cycling, and in recent times, they have also been used in applied microbiology and biotechnology (Ikemoto *et al*., 1980). The biotechnological interest in *S. maltophilia* stems from their potential plant growth promoting properties, their use in biological control of plant fungal pathogens, their capability to degrade xenobiotic compounds and their potential to decontaminate the soil (Suckstorff and Berg 2003, Hayward *et al*. 2009).

The ability of *Stenotrophomonas* isolates to control fungal infections in plants has been widely documented (Dunne *et al*., 1997, Suckstorff and Berg 2003, Hayward *et al*. 2009, Zhang and Yuen, 2000). For example, *S. maltophilia* strain W81, isolated from the rhizosphere of field-grown sugar beet, produced the extracellular enzymes chitinase and protease and inhibited the growth of the phytopathogenic fungus *Pythium ultimum in vitro*, resulting in reduction of damping-off of soil-grown sugar beet (Dunne *et al*., 1997). Zhang and Yuen (2000) also reported *S. maltophilia* isolate C3 to be a biological control agent, active in part through chitinase activity.

This bacteria strain was effective in inhibiting germination of conidia of *Bipolaris sorokiniana* (Sacc.). In the Nile Delta of Egypt, some strains of *S. maltophilia* (PD3532, PD3533, PD3534) have been demonstrated to have the potential to control brown rot of potato caused by the bacterium *Ralstonia solanacearum* (Messiha *et al*., 2007). Suckstorff and Berg (2003) found that three strains of *S. maltophilia* could enhance plant growth in a dose-dependent manner in strawberry seedlings. They also found a positive correlation between plant growth enhancement and the production of the plant hormone, indole-3-acetic acid (IAA) in plants grown in vitro.

On the other hand, some *S. maltophilia* isolates cause disease in humans, including pneumonia, bacteremia, urinary tract infections, wound infections, bronchitis, endocarditis, meningitis, eye infections and catheter-associated infections (Schaumann *et al*., 2001; Brooke *et al*., 2007; Brooke, 2012). This pathogen has been shown to be an opportunistic bacterium usually associated with respiratory infections in immunocompromised individuals (Brooke, 2012) and has become a microbe of concern in hospitals in recent years. *S. maltophilia* is the third most common nosocomial non-fermenting Gram-negative bacilli after *Pseudomonas aeruginosa* and *Acenitobacter* species in patients in intensive care units. In addition, there are many multi-drug resistant strains of *S. maltophilia* that affect humans. More cases of drug-resistant *S. maltophilia* infections have been reported in the last decade, and high case/fatality ratios in susceptible populations have characterized these cases (Sader and Jones, 2005). Although *S. maltophilia* is naturally an environmental bacterium, its transition to an important nosocomial pathogen has likely been driven by natural selection and adaptation.

Extensive studies have been conducted on *S. maltophilia*-plant interactions but only a few of them have focused on using phenotypic data to compare clinical and environmental isolates. The overarching goal of this research was therefore to determine if environmental isolates of *S.*

maltophilia are phenotypically distinct from clinical isolates. One of the ways to achieve this goal was to determine if the clinical isolates retain or lose their plant growth promotion properties once they leave the hospital environment through experiments conducted under normal and sodium chloride stress conditions. In addition, the variability among *S. maltophilia* isolates in their ability to protect canola seedlings against the harmful effects of the blackleg fungus, *Leptosphaeria maculans* and the plant pathogenic bacterium *Burkholderia cenocepacia* (K56-2) was investigated. In order to generate information on genetic relatedness of the isolates used in this study, phylogenetic analysis was conducted.

LITERATURE REVIEW

History and clinical significance of *Stenotrophomonas maltophilia*

First isolated in 1943, *S. maltophilia* was initially called *Bacterium bookeri* and was subsequently classified as a member of the genus *Pseudomonas* in 1961 (Denton and Kerr, 1998, Looney *et al*., 2009) and then *Xanthomonas* in 1983 (Palleroni and Bradbury, 1993) and finally *Stenotrophomonas* in 1993 (Denton and Kerr, 1998). The genus *Stenotrophomonas* currently consists of four species, but only *S. maltophilia* is known to cause infection in human beings (Looney *et al*., 2009). It has become an important hospital related pathogen which has resulted in crude mortality rates. These mortality rates have been reported to range from 14 to 69% in patients who develop bacteremia (Yang *et al*., 1992, Victor *et al*., 1994). *S. maltophilia* has been associated with a variety of diseases, including pneumonia, bacteremia, urinary tract infections, wound infections, bronchitis, endocarditis, meningitis, eye infections and catheter-associated infections (Schaumann *et al*., 2001; Brooke, 2012). However, the two most common diseases caused by *S. maltophilia* are bacteremia and pneumonia with infection occurring through an in-dwelling catheter or ventilator, respectively (Senol, 2004).

S. maltophilia has over the years been described to have built resistance against many antibiotics. It has been isolated from aqueous environments in and outside the hospital. *S. maltophilia* has been recovered from a wide range of sources such as soils, plant roots, animals, invertebrates, water treatment distribution systems, waste water plants, sink holes, bottled water, tap water, rivers, washed salads, faucets, ice machines and hand washing soap (Brooke, 2008, Berg, 2009, Berg *et al*., 2005, Denton *et al*., 1998, Ivanov *et al*., 2005, Nakatsu *et al*., 1995). In the hospital environment, *S. maltophilia* has been associated with surfaces of materials used in intravenous cannulae, prosthetic devices, dental unit waterlines and nebulizers (Denton *et al*.,

2003, Hutchinson *et al*., 1996, Lidsky *et al*., 2002, O'Donnell *et al*., 2005). Susceptible individuals (both children and adults) can be infected through direct contact with the source (Brooke, 2012), and even the hands of health care personnel have been reported to transmit nosocomial *S. maltophilia* infection in an intensive care unit (Schable *et al*., 1991).

S. maltophilia is the third most common nosocomial non-fermenting Gram-negative bacilli (Sader and Jones, 2005) in the intensive care unit (ICU). Lockhart (2007) reported that from 1993 to 2004, *S. maltophilia* was one of the organisms most isolated from patients in the intensive care unit in the US and formed 4.3% of a total of 74,394 Gram-negative bacillus isolates. *S. maltophilia* also accounted for 0.6% to 0.9% of all bloodstream infections reported from the United States, Canada and Latin America from 1997 through 1999 (Diekema, 1999). In another study, it was shown that chronic obstructive pulmonary disease (COPD) patients with severe acute exacerbation were commonly infected with MDROs and *S. maltophilia* was isolated from 3.0 % of this cohort. Further, it has been demonstrated that this pathogen accounts for 6.75 % and 1.11 % of the cases of ventilator-associated pneumonia and hospital-acquired pneumonia, respectively (Weber *et al*., 2007).

S. maltophilia **in relation to other microbes**

Phylogenetically, *S. maltophilia* belongs to the family, Xanthomonadaceae, which is made up of five genera, *Xylella*, *Xanthomonas*, *Pseudoxanthomonas*, *Lysobacter* and *Stenotrophomonas* which are closely related but are of diverse origin. Of these genera, *Xanthomonas* and *Xyllela* are known to cause diseases in plants. *Pseudoxanthomonas* has been associated with breaking down of hydrocarbon-contaminated soils into a form which is not toxic (Nayak *et al*. 2009). *Lysobacter* and *Stenotrophomonas* on the other hand, have been described

to promote plant growth. They colonize the rhizosphere of plants and have the ability to produce antibiotics that have been applied in biological control of plant diseases (Hayward *et al*., 2009).

Microbiology of *S. maltophilia*

S. maltophilia is a Gram-negative obligate aerobe and a bacillus with polar flagella (Brooke, 2012). *S. maltophilia* cells occur singly or in pairs and may be straight or slightly curved with non-sporulating bacilli that are 0.5 to 1.5 mm long (Denton and Kerr, 1998). *S. maltophilia* colonies are smooth and are white to pale yellow in color (Denton and Kerr, 1998). Although standard microbiological data refer to *S. maltophilia* as an oxidase-negative bacterium, recent data analysis of a collection of 766 isolates indicated that approximately 20 % of these strains were actually oxidase positive (Carmody *et al*., 2011). At a low temperature of 5°C or a high temperature of 40°C, *S. maltophilia* does not grow. The optimal temperature for its growth is 35°C (Denton and Kerr, 1998). Most strains of *S. maltophilia* require methionine or cysteine for growth (Ikemoto *et al*., 1980, Marraro and Mitchell, 1974). In nutrient-poor aqueous environments, the bacterium has the ability to survive (Denton and Kerr, 1998).

Identification of *S. maltophilia* can be challenging and complex because this bacterium is commonly co-isolated with other microorganisms (e.g., *P. aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumanii*, *Escherichia coli*, *Enterobacter* species and *Candida albicans*) in samples recovered from patients (Araoka *et al*., 2010, Gülmez and Hasçelik, 2005, Tseng *et al*., 2009). To improve the isolation of this pathogen from polymicrobial cultures or environmental specimens, a number of selective media have been designed (Denton *et al*., 2000, Kerr *et al*., 1996). Vancomycin, imipenem, amphotericin B (VIA) medium is one medium which has been suggested for isolation of *S. maltophilia* from environmental and clinical sources (Kerr *et al*., 1996) to replace the *Xanthomonas maltophilia* Selective Medium (XMSM) described for

isolation of *Xanthomonas maltophilia* from bulk soil and plant rhizosphere (Juhnke and des Jardin 1989). The preparation of XMSM medium is complicated and expensive. In addition, it is time-consuming and above all, not specific for environmentally recovered isolates of *S. maltophilia* (Kerr *et al*., 1996). VIA medium has been shown to be less inhibitory to *S. maltophilia.* However, in preventing the growth of other bacteria like maltose fermenters, they are more selective than XMSM. *S. maltophilia* from various sources have been cultured using VIA medium.

Pathogenicity of *S. maltophilia*

The *S. maltophilia* genome sequence of suggests that this pathogen is not highly virulent but has capacity for environmental adaptations and these presumably contribute to its persistence in vivo (Crossman *et al*., 2008). Factors that may promote the ability of this bacterium to colonize the respiratory tract and plastic surfaces (e.g., catheters and endotracheal tubes) including positively charged surface are flagella and fimbrial adhesins, with the latter being associated with biofilm formation (Jucker *et al*., 1996, Waters *et al*., 2007).

A bacterial biofilm consists of a microbial community embedded in an extracellular polysaccharide matrix or extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). Biofilms have been reported to form about 65% of nosocomial infections (Potera, 1999). For biofilms to form, bacterial cells adherence is needed. A study has shown that the *S. maltophilia,* SM33 needs 2 h after inoculation to adhere to polystyrene surfaces and form biofilms within 24 hours (Di Bonaventura, 2004). *S. maltophilia* can form biofilms on its own or together with other species and once growing in biofilms, it is more resistant to phagocytes and antibiotics (Martinez and Baquero, 2002). *S. maltophilia* engages in cell-to-cell signaling (quorum sensing) through the diffusible signaling factor (DSF), a molecule found in the

Xanthomonas and *Xylella* signaling systems (Crossman *et al*., 2008, Fouhy *et al*., 2007). Disruption of DSF signaling leads to diminished biofilm development, loss of motility, reduced production of extracellular proteases, and increased susceptibility to certain antibiotics and heavy metals (Fouhy *et al*., 2007).

Other factors which may contribute to *S. maltophilia* colonization or infection of host include the production of protease and lipase, shown to be involved in bacterial pathogenesis in other genera, and several other extracellular enzymes including DNase, RNase, gelatinase, esterase and proteinase (Travassos *et al*., 2004, O'Brien and Davis, 1982, Travis *et al*., 1995). Evidence suggests that proteinases are not only to provide a source of free amino acids or simple sugars for bacterial survival and growth (Travis *et al*., 1995) but are also capable of destroying host proteins (Windhorst *et al*., 2002). The *S. maltophilia* protease coded for by the *StmPr1* gene is able to breakdown the protein components of collagen, fibronectin, and fibrinogen and thus may contribute to local tissue damage and hemorrhage (Windhorst *et al*., 2002).

Lipopolysaccharide (LPS) is a major component of the outer membrane of most Gram-negative bacteria and serves as a virulence determinant. *S. maltophilia* has been shown to have LPS. The LPS is made up of lipid A, core oligosaccharide and O-antigen (Wang and Quinn, 2010). A bi-functional enzyme has been shown to be encoded in the *spgM* gene in *S. maltophilia*. This enzyme has both phosphoglucomutase and phosphomannomutase activities. These activities are necessary for O-polysaccharide chain assembly (Brooke *et al*., 2008). Consequently, *spgM* mutants exhibit a modest increase in susceptibility to diverse antimicrobials and prove to be completely avirulent in infection experiments performed with animal host models (Brooke, 2012).

Another feature that supports the development of infectious manifestations is the immunostimulatory effect of *S. maltophilia*. Stimulation by the lipid A component of lipopolysaccharide of peripheral-blood monocytes and alveolar macrophages to produce tumor necrosis factor α (TNFα) plays a part in the pathogenesis of airway inflammation (Waters *et al*., 2007). *S maltophilia* also induces interleukin-8 expression and polymorphonuclear leucocyte recruitment (Waters *et al*., 2007). Flagella are highly immunostimulatory (Prince, 2008) and it is thought that decreased expression of flagella may protect the bacteria from the host immune response. Loss of motility, likely due to attenuated expression of flagella, appears to be a common mechanism of adaptation to the cystic fibrosis airways for *S. maltophilia*.

Epidemiology and risk factors

Notwithstanding the fact that *S. maltophilia* comes second after *P. aeruginosa* as the most common non-fermentative gram-negative bacteria isolated from clinical specimens, its isolation was considered unusual in the diagnostic microbiology laboratory until recently

(Blazevic, 1976, Holmes *et al*., 1979). There have also been reports of *S. maltophilia* carriage in humans. For example, a fecal carriage rate of 6.0% (14 out of 218 stool samples) was detected in outpatients with diarrheal illness or people they came into contact with (Von Graevenitz and Bucher, 1983).

The rate of *S. maltophilia* isolation has been increasing since the early 1970s as revealed by surveys in several continents and this may be a reflection of an increasing population of patients at risk and the result of advances in medical technologies and treatment (Tan *et al*., 2008, Rolston *et al*., 2005). *S. maltophilia* isolation rate varies between hospitals and geographic regions (Fedler *et al*., 2006). In England and Wales, the annual number of blood isolates increased between 2000 and 2006 by 93% to 773 cases, and a Taiwanese tertiary-care hospital

reported an 83% increase from 5.3 to 9.8 episodes per 10,000 discharges from 1999 to 2004 (Tan *et al*., 2008). A German study in 34 intensive-care units (ICUs) between 2001 and 2004 showed an increasing rate of *S. maltophilia* infections in some units and a decrease in others (Meyer *et al*., 2006). A Spanish study (late 1990s) reported isolation rates between 3.4 and 12.1 per 10,000 admissions (DelToro *et al*., 2002). The rate of *S. maltophilia* isolations rose from less than 2 in 1972 to 8 in 1984 per 10,000 admissions at the M.D. Anderson Cancer Center (Houston, Texas) (Elting and Bodey, 1990). At the Mayo Clinic in 1987, incidence rates of *S. maltophilia* infection was from 12.8 in 1984 to 37.7 per 10,000 patient discharges (Marshall *et al*., 1989) while, the annual isolation rate doubled from 7.1 to 14.1 per 10,000 patient discharges at the University of Virginia Hospital from 1981 to 1984 (Denton and Kerr, 1998).

Risk factors associated with *S. maltophilia* include underlying malignancy (Calza *et al*., 2003), the presence of in-dwelling medical devices (Metan *et al*., 2006), prolonged hospitalization, ICU stay, chronic respiratory disease (Waters *et al*., 2013; Waters *et al*., 2011), and compromised host immune system (Calza *et al*., 2003). Long-term therapy involving use of broad-spectrum antibiotics has also been found to be an independent risk factor for stenotrophomonad infection (Paez and Costa, 2008).

Comparison between clinical and environmental isolates of *S. maltophilia*

To determine the mechanisms responsible for the *S. maltophilia* pathogenicity in humans, recent studies have looked into the differences between environmental and clinical isolates of the bacterium (Brooke, 2012) and also to characterize those which may have biotechnological potential. In one of such studies, 40 *S. maltophilia* isolates from clinical and environmental settings were investigated. In this study, various phenotypic and genotypic fingerprinting methods were used to characterize and distinguish the variability among clinical and

environmental isolates of *S. maltophilia* (Berg *et al*., 1999). They noticed a great diversity among S. *maltophilia* isolates studied and their findings agreed with other previous studies. This study reported a wide range of heterogeneity (Minkwitz and Berg, 2001) in physiological parameters, which was confirmed by genotypic studies (Chatelut *et al*., 1995; Hueben *et al*., 1997). High intraspecific diversity was revealed, but various DNA-based fingerprinting methods of the phenotypic and genotypic features did not reveal any clustering patterns by origin. Minkwitz and Berg (2001), however, were able to distinguish between clinical and environmental (aquatic and plant-associated) strains using 16S rDNA sequencing.

In a study to analyze the global distribution of mutation frequencies in 174 *S. maltophilia* samples from clinical as well as non-clinical environments, Kerr *et al*. (1996) found that low mutation frequencies were common among environmental *S. maltophilia* strains whereas strong mutators were found only among isolates with a clinical origin. These results indicate that clinical environments might select bacterial populations with high mutation frequencies, likely by second-order selection processes, a natural selection process in which certain locations on a genome affect the expression of genes rather than coding for proteins.

Many studies have reported species specific antifungal features between environmental and clinical isolates. Environmental isolates tend to have higher antifungal activity *in vitro* than clinical isolates (Minkwitz and Berg, 2001, Suckstorff and Berg, 2003). To further demonstrate the differences between environmental and clinical isolates of *S. maltophilia*, two isolates, K279A (clinical isolate from a cystic fibrosis patient) and R551-3 (environmental isolate of plant origin), whose genomes have been sequenced (Brooke, 2012), were explored. Highly variable content of genomic islands has been revealed by sequencing data for *S. maltophilia* isolates K279a and R551-3 (Rocco *et al*., 2009). In K279a, 41 genomic islands have been identified

while R551-3 harbors 36 islands. The two isolates have been reported to possess different gene islands with corresponding genes that interact differently with the environment (Rocco *et al*., 2009). The two isolates have some genes with the same function (Brooke, 2012).

The antimicrobial and plant growth promoting properties of *S. maltophilia*

In nature, *Stenotrophomonas* species play a very important role. They are mostly involved in the bio-geochemical element cycle. In addition, in recent years, they have been applied in microbiological and biotechnological fields (Ikemoto *et al*., 1980). The biotechnological interest in *S. maltophilia* stems from their potential plant growth promotion effects, their use in biological control of plant fungal pathogens, their capability to degrade xenobiotic compounds and their potential to decontaminate the soil (Berg *et al*. 1994, Hayward *et al*. 2009). In recent times, the call for use of biological control methods is on the ascendancy. This is as a result of public concerns over the use of chemicals in plant propagation. In order to address this, new ways which are less harmful are needed in control of plant diseases. There are different ways in which biological control occurs such as antibiosis, siderophores production, competition for colonization sites and nutrients, induction of plant resistance mechanisms, inactivation of pathogen germination factors, degradation of pathogenicity factors such as toxins and parasitism that may involve the production of extracellular cell wall-degrading enzymes (chitinase and β-1,3 glucanase that can lyse pathogen cell walls) (Keel and Defago, 1997). Plant growth promoting rhizobacteria (PGPR) enhance plant growth indirectly, either by suppression of pathogens or reduction of their deleterious effects; associative N_2 fixation; promoting mycorrhizal function; regulating ethylene production and releasing phytohormones and decreasing metal toxicity (Whips, 2001).

The ability of *Stenotrophomonas* strains to suppress fungal pathogens associated plant diseases has been shown in many studies (Berg *et al*. 1994, Berg, 1996, Minkwitz and Berg, 2001, Dunne *et al*., 1997, Hayward *et al*. 2009, Zhang and Yuen, 2000). *S. maltophilia* has been found to produce extracellular enzymes such as chitinases and proteases which inhibit fungal growth.

Canola production and distribution

Canola (*Brassica napus* L., and *Brassica rapa* L.) is one of the major oilseed crops grown in Europe, Asia, Australia, Canada and the United States. Canola is the most important oilseed crop in Canada and is now second only to soybean as the most important source of vegetable oil in the world (Raymer, 2002). Canola is considered a new crop in the United States with *B. napus* L. being the most dominant, grown largely as a spring-planted crop. More than 90% of *B. napus* L. production in the United States occurs in North Dakota (Del Río *et al*., 2007).

Diseases of canola

Blackleg (phoma stem canker) caused by *Leptoshaeria maculans* (Desmaz) and Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, are the two major fungal diseases that affect canola production (Del Rio *et al*., 2007). Other pathogens which cause diseases in canola include *Rhizoctonia solani* Kühn, *Fusarium avenaceum* (Corda:Fr.) Sacc., and *Alternaria brassicae* (Berk.) Sacc. (Beatty and Jensen, 2002). The effects of blackleg include lodging, early senescence and seedling death. Blackleg is widespread in most regions where canola is grown. Since 1950, blackleg has caused serious economic losses in canola and rapeseed production in Europe and this disease virtually caused the collapse of the rapeseed and canola industry in Western Australia in 1972 (Beatty and Jensen, 2002).

Blackleg is caused by a complex of at least two species of *Leptosphaeria*: *L. maculans* (Desmaz.) Ces. & de Not. (anamorph Phoma lingam (Tode:Fr.) Desmaz.), and *L. biglobosa* Shoemaker and Brun. (Howlett *et al.*, 2001, Williams and Fitt, 1999). *Leptosphaeria maculans* is by far the more damaging of the two and has been reported from almost all canola and oilseed rape growing regions of the world except China (Fitt *et al*., 2006). The two pathotypes are differentiated by their ability to produce pigment in culture, growth rate of colony, molecular characteristics and disease reaction on *B. napus* (Howlett *et al*., 2001; Williams and Fitt, 1999). These species were previously referred to as highly and weakly virulent forms of a single pathogen (*L. maculans*), until the latter was determined to be a different species (Howlett *et al*., 2001). Isolates of *L. maculans* have been subdivided based on their reaction with differential cultivars Westar, Glacier and Quinta, into pathogenicity groups PG2, PG3 and PG4 (Howlett *et al*., 2001). In North Dakota, PG1 (*L. biglobosa*), PG2, PG3, PG4 and PGT, have been identified, with PG2 isolates causing 92% of *L. maculans* infections between 2002 and 2004 (Markell *et al.*, 2008).

Symptoms and signs of blackleg

The blackleg pathogen usually induces its first symptoms on the leaves of the host. Lesions may appear at the seedling stage or anytime until the crop matures (Markell *et al*., 2008). As leaf lesions expand, small black fruiting bodies called "pycnidia" are formed. Lesions also form on stems, usually, near where an infected leaf was attached. The most vulnerable stage of the stem to blackleg infection is the four-to-six leaf stage (Markell *et al*., 2008). Stem lesions show a sunken appearance and may rapture and girdle the stem resulting in the characteristic "blackleg" symptoms (Markell *et al*., 2008).

Disease cycle

Blackleg on canola is usually considered a monocyclic disease (West *et al*., 2001) initiated by sexual spores (ascospores) produced by fruiting bodies called pseudothecia on infected canola stubble. Infection of seedlings occurs through wounds or stomata on young leaves or cotyledons and the pathogen then moves into the intercellular spaces between the mesophyll cells. Following ascopore infection, pycnidia are produced which in turn produce asexual spores called conidia (Markell *et al*., 2008). The conidia are rain-dispersed and are responsible for localized spread of blackleg.

Management of blackleg

Currently, blackleg of canola is managed mainly through the use of fungicides and crop rotations since there are no commercially available resistant cultivars (Bradley *et al*., 2006). Other management options include the use of certified disease-free seed and control of weed hosts such as volunteer canola and wild mustard (Markell *et al*., 2008).

MATERIALS AND METHODS

Bacterial isolates

18 clinical and environmental isolates of *Stenotrophomonas maltophilia* were used (Table 1). *Burkholderia cenocepacia* (K 56-2) and *Escherichia coli* (DH5α) were used as positive and negative controls respectively. The CDC *S. maltophilia* isolates were provided by Dr. Judith Noble-Wang while the ATCC isolates were purchased from the American Type Culture Collection, Manassas, VA, USA.

Fungal isolate and plant material

The fungal strain used in this study was *Leptosphaeria maculans*, the causal pathogen of blackleg in canola. *L. maculans* belongs to the pathogenicity group (PG) 4, a subdivision of pathotype A (the aggressive and highly virulent cause of stem cankers in canola). Westar, a commercially planted *Brassica napus* (canola) cultivar which is susceptible to blackleg was used. Seeds of Westar and inoculum of *L. maculans* were kindly provided by Dr. Luis Del Rio Mendoza of the Plant Pathology Department of North Dakota State University.

Table 1: Name, source and origin of *S. maltophilia* isolates and other bacterial strains used in this study

Isolate Name [y]	**Origin**	**Source**
ATCC 700475	Patient	ATCC
BAA 2423 (K279a)	Patient	ATCC
BAA 84 (D457)	Patient	ATCC
CDC 2007-07-01	Patient, Blood	CDC
CDC 98-43-10	Patient	CDC
CDC 2011-09-42	Patient	CDC
CDC 2013-11-01	Patient	CDC
CDC 2004-33-31	Patient, Trachea	CDC
CDC 2004-33-01-01	Sublingual Sensor (Device)	CDC
CDC 2005-37-11-04	Respiratory Therapy Device	CDC
CDC 2007-23-08-03	Sink (Environmental)	CDC
CDC 92-03-30	RO Water (Environmental)	CDC
CDC 90-15-60	Tap Water (Environmental)	CDC
ATCC 51331	Patient, CF lung	ATCC
ATCC 49 49130	Patient, Clinical isolate	ATCC
ATCC 13270	Soil (Environmental)	ATCC
ATCC 13637	Patient	ATCC
ATCC 17666	Soil (Environmental)	ATCC
E. coli (DH5alpha)		Invitrogen
B. cenocepacea (K 56-2)		

[y] *B. cenocepacea* and *E. coli* were included as controls

ATCC isolates

These were isolated from different environments. K 279a was isolated from blood culture of a patient suffering from cancer (Crossman *et al*., 2008). "D457 was isolated from bronchial aspirate, ATCC 17666 from contaminated tissue culture, ATCC 700475 from cerebrospinal fluid from an HIV-seropositive Rwandan refugee with primary meningoencephalitis, and ATCC 13637 from Oropharyngeal region of patient with mouth cancer" (www.atcc.org). ATCC 13270 was isolated from soil environment (©Global Catalogue of Microorganisms).

Media and culture conditions

V8 Medium and others

Agar was prepared according to Suckstoff and Berg (2003) [20g agar, 1L distilled water, pH = 6] with the following modifications. Agar was made up of 10g agar (Difco) in 1L of distilled water (1%) and autoclaved. 10ml of agar were dispensed into 15 ml test tubes and stored in a cool dry place until used for canola plant growth. *L. maculans* was grown on V8 medium (200ml V8 juice, 3g $CaCO_3$, 15g Agar and 800ml tap water). Conidia were harvested after 3 days and suspended in sterile distilled water. The suspension was kept on ice until use.

Bacterial cultures

Freezer stocks of bacterial isolates were streaked on Luria Bertani (LB) plates and incubated at 37ºC overnight. A colony from each isolate was inoculated in 5 ml LB broth and incubated at 37 ºC, and 230 rpm for 16 hours. After incubating, bacterial cultures were centrifuged at 5000 x g for 5 min. The supernatant was discarded and the pellets/cells were suspended in 5 ml distilled water to wash and centrifuged at 5000 x g for 5 min. Cells were then suspended in 5ml distilled water.

Germination and treatment of canola seedlings

Canola seeds were placed in a 9 cm sterile glass petri dish with layers of sterile tissue paper soaked with sterile distilled water for 4 days at 25 ºC in the dark for germination. For the non-stress experiment, 10 canola seedlings were placed in sterile petri plate for each isolate. The 5 ml bacterial suspensions, at 10^6cfu/ml as was described above were thoroughly vortexed and added to the seedlings in the various petri dishes. All seedlings were carefully submerged in the bacterial suspension for 4 hours. Thereafter, the seedlings were washed in sterile distilled water and inoculated on agar in test tubes. For the stress experiment, canola seedlings were primed by transferring seedlings into a fresh sterile 9 cm glass petri dish with layers of sterile tissue paper soaked with moderate salinity rate of 6 decisiemens per meter (0.33%) NaCl (www.canolacouncil.org) for 24 hour at 25 ºC. The primed seedlings were rinsed in sterile distilled water and thereafter treated in the same way as the non-stress treatment described above. Growth parameters (number of root branches, root and stem length) were recorded after one week of incubation at 25 ºC and 16/8 photoperiod conditions as described for strawberry by Suckstorff and Berg (2003). Using a pair of forceps, canola plants were carefully removed from test tubes onto a clean paper towel. Root and stem lengths were measured by the use of a ruler and a pair of calipers. Each bacterial isolate was tested in triplicates.

Challenge assays

Based on preliminary results from one run of non-stressed condition, three isolates of *S. malltophilia*, namely, ATCC 13637, ATCC 13270 and CDC 2007-07-01 and the isolates, K279a and D457 which have whole genome sequences were assessed for their suitability to protect against the bacterium *Burkholderia cenocepacia* and the fungus *L. maculans* using the cv. Westar of canola as a host plant. To accomplish this, four day old canola seedlings were

inoculated with 5 ml of each of the five *S. maltophila* isolates for two hours after which 5 mls of either *Burkholderia cenocepacia* or *L. maculans* was applied for an additional two hours. This experiment was carried out under three separate conditions: 1.) four hour exposure period to *Burkholderia cenocepacia* and *L. maculans*; 2.) canola seedlings inoculated with 5 mls *S. maltophilia* initially and subsequently exposed to 5 ml of *Burkholderia cenocepacia* or *L. maculans* and 3.) canola seedlings initially inoculated with 5 ml of *Burkholderia cenocepacia* or *L. maculans* and subsequently exposed to 5 ml of *S. maltophila* isolates for 2 hours. For each *S. maltophilia* isolate, 12 tubes were employed and these were replicated three times. Here again, stem and root length as well as the number of root branches of canola seedlings were the parameters recorded.

gyrB sequencing and analysis

Freezer stocks of bacterial isolates were streaked on Luria Bertani (LB) plates and incubated at 37ºC overnight. A single colony from each isolate plate was inoculated into a 50µl PCR reaction master mix that contained reagents as follows: 5X Go Taq Buffer, 10 mM dNTP, 25 mM $MgCl_2$, XgyrB1F - 5^1- ACG AGT ACA ACC CGG ACA A - 3^1, a threefold XgyrB1R - 5^1- CCC ATC ARG GTG CTG AAG AT - 3^1 (Yamamoto *et al*., 2000), GoTaq Polymerase and distilled water). The reaction was run for 40 cycles under the following thermocycling conditions: denature at 95 ºC for 30 sec., annealing at 55 ºC for1 min., and extension at 72 ºC for 2 min. Amplified PCR products were confirmed by running an agarose gel at 100 volts for 45 minutes. The gel images were visualized by Ultraviolet light using flourchem software (FluorChem™ Q System), Bio-Techne, USA. PCR products were purified using Exosap and incubated at 37^{o}C for 15 minutes and 80^{o}C minutes for 15 minutes and held at 4^{o}C. Products

were diluted and sent to Macrogen USA for sequencing. The products were sequenced by 3730xl DNA analyzer using the Sanger sequencing analysis (capillary gel electrophoresis).

Nucleotide sequences were analyzed using Geneious bioinformatics software 6.1 version (Biomatters Limited, Auckland, New Zealand). The sequences were edited to generate uniform lengths for each isolate for both forward and reverse sequences using the gyrBF and gyrBR primers. Other *S. maltophilia* and closely related genome sequences were searched in National Center for Biotechnology Information (NCBI). The K279a genome was used as a reference sequence. Concatenated isolate sequences were aligned to the reference sequence and edited to obtain the correct sequence length of 725 base pairs. The reference sequence was deleted after alignment. The forward and reverse sequences were aligned to generate each isolate sequence in whole. Related genome sequences were also concatenated to 725 base pairs. Sequences were aligned into a file. Cluster analyses (neighbor-joining) were performed.

Statistical analysis

Data analyses were done using the general linear models procedure (Proc GLM) in statistical analysis software (SAS) version 9.3. The data were analyzed as one-way Analysis of Variance (ANOVA) with the randomized complete block design (RCBD). All inferences were conducted at 5% significance level. Treatments means were compared using the Bonferroni (Dunn)'s and Tukey's tests at $P \leq 0.05$. Before analysis, the difference between the median for each isolate and the "no bacteria" control was found and expressed as percentage change for the canola growth promotion experiment. For canola protection experiments, the raw data was analyzed without any prior manipulations.

RESULTS

The growth promotion potential of *S. maltophilia* isolates on canola seedlings under normal growing condition

In order to determine the potential of *S. maltophilia* isolates to promote growth in canola, 4 day old canola seedlings were inoculated overnight with bacterial cultures suspended in sterile distilled water. Percentage changes of the median in parameters measured were determined using the no bacterial control median as the initial and the isolate medians as the final under both stressed and non-stressed conditions.

No significant differences were observed in number of root branches and root length in canola plants exposed to bacterial isolates relative to the "no bacterial" control (Figs. 1 & 2). Stem length of canola plants, however, was significantly ($P < 0.05$) lower in canola seedlings inoculated with isolate CDC 92-03-30, ATCC 17666, CDC 98-43-10, CDC 2013-11-01 and CDC 2011-09-42 than the "no bacterial" isolate (Fig. 3).

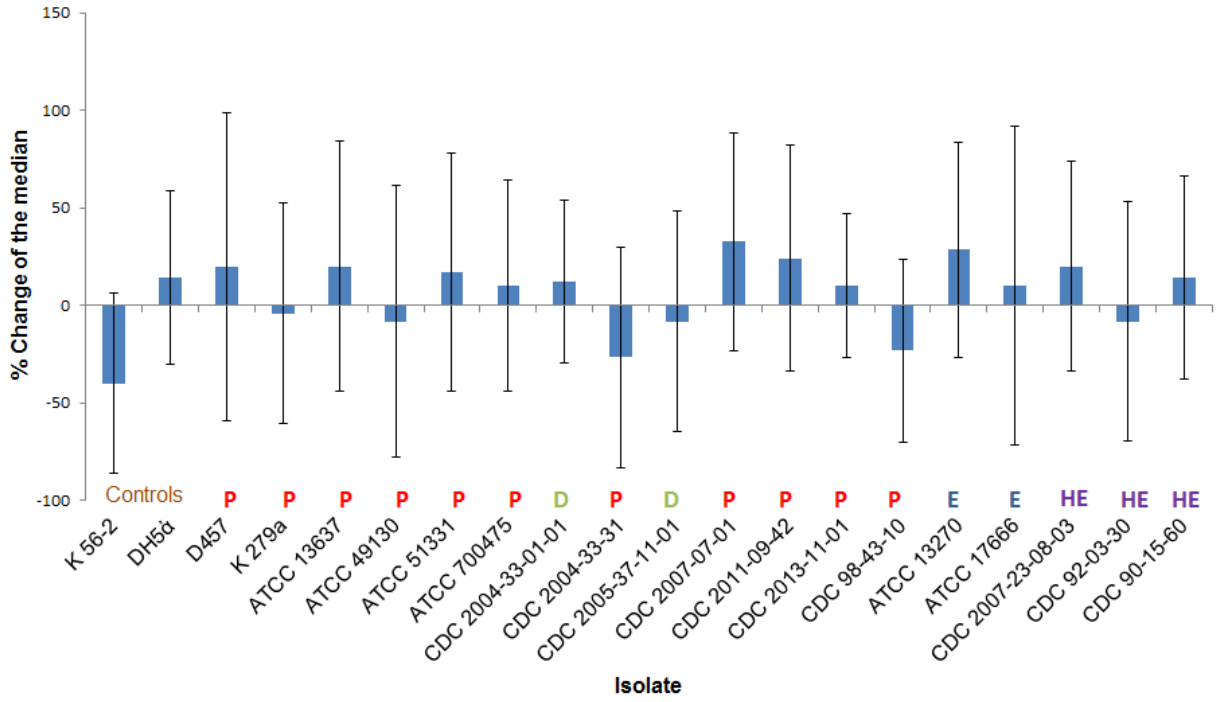

Fig. 1. Percentage change of the median in number of root branches of canola seedlings exposed to different *S. maltophilia* isolates. Vertical bars represent standard deviation. P - Patient; D – Device; E – environmental; HE – hospital environment isolate.

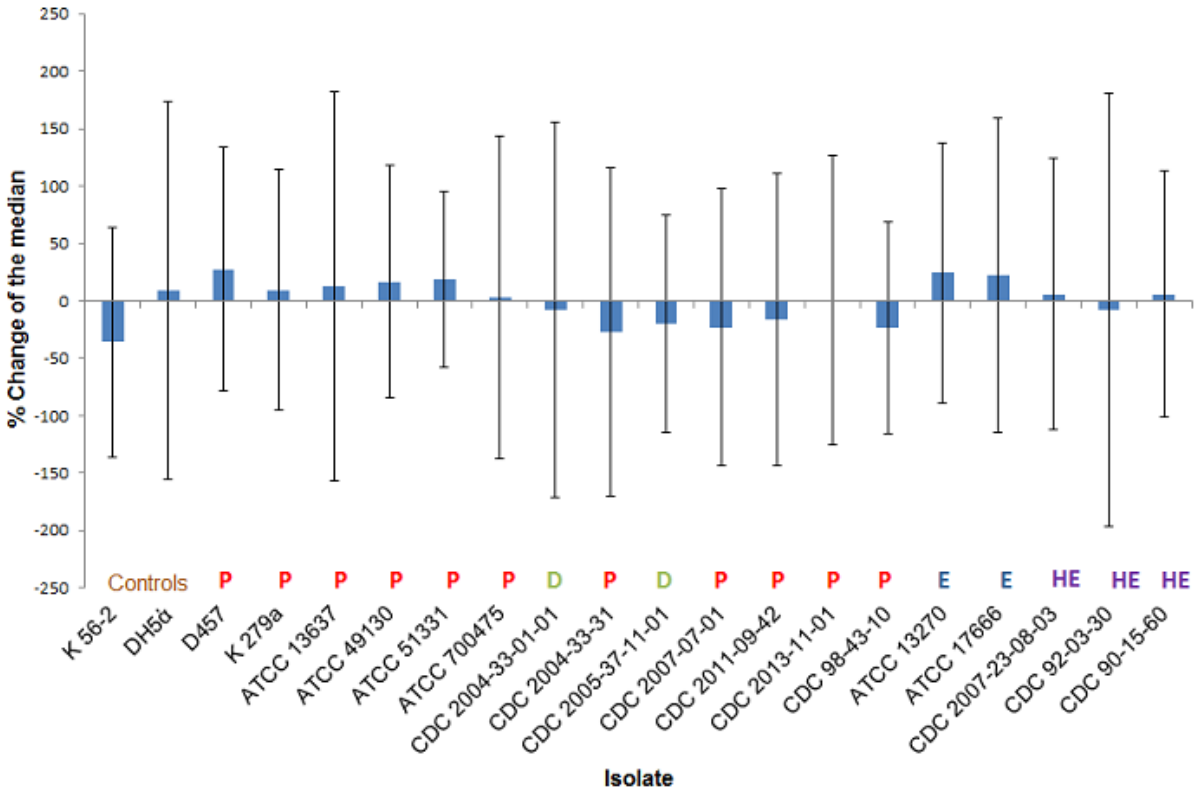

Fig. 2. Percentage change of the median in root length of canola seedlings exposed to different *S. maltophila* isolates. Vertical bars represent standard deviation. P - Patient; D – Device; E – environmental; HE – hospital environment isolate.

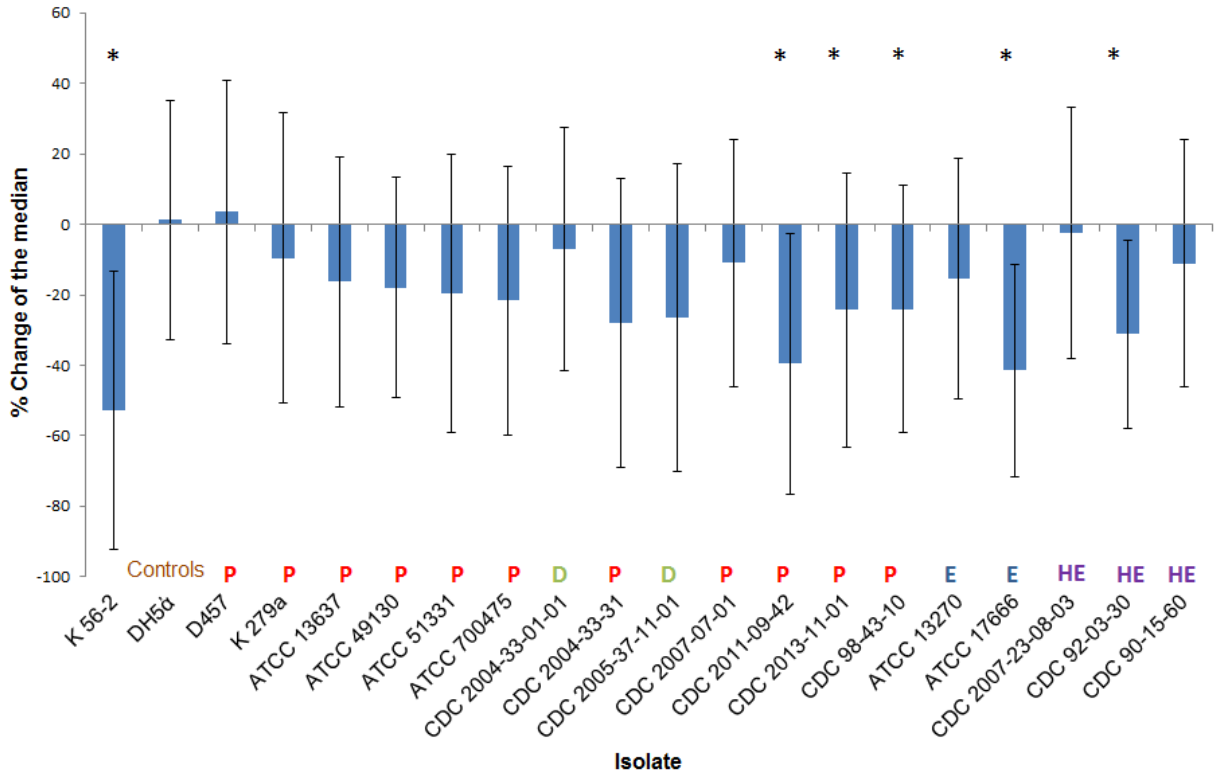

Fig. 3. Percentage change of the median in stem length of canola seedlings exposed to different *S. maltophilia* isolates. Vertical bars represent standard deviation. Isolates were compared using Bonferroni at $P < 0.05$. Isolates with no asterisk are not significantly different from the "no bacteria" control. P - Patient; D – Device; E – environmental; HE – hospital environment isolate. * Isolates differ significantly from the "no bacteria" control.

The growth promotion potential of *S. maltophilia* isolates in canola seedlings grown under salt stress condition

Canola seedlings inoculated with D457 had significantly ($P < 0.05$) higher number of root branches than seedlings which were not inoculated with bacteria (Fig. 4). Seedlings inoculated with *S. maltophilia* isolates, D457, CDC 2004-33-01-01 and CDC 2007-23-08-03 had significantly ($P < 0.05$) longer roots than the "no bacteria" control (Fig. 5). Canola seedlings inoculated with D457, CDC 2005-37-11-04 and CDC 2011-01-42 had the longest stems and

differed significantly ($P < 0.05$) from those of seedlings exposed to the "no bacteria" control (Fig. 6).

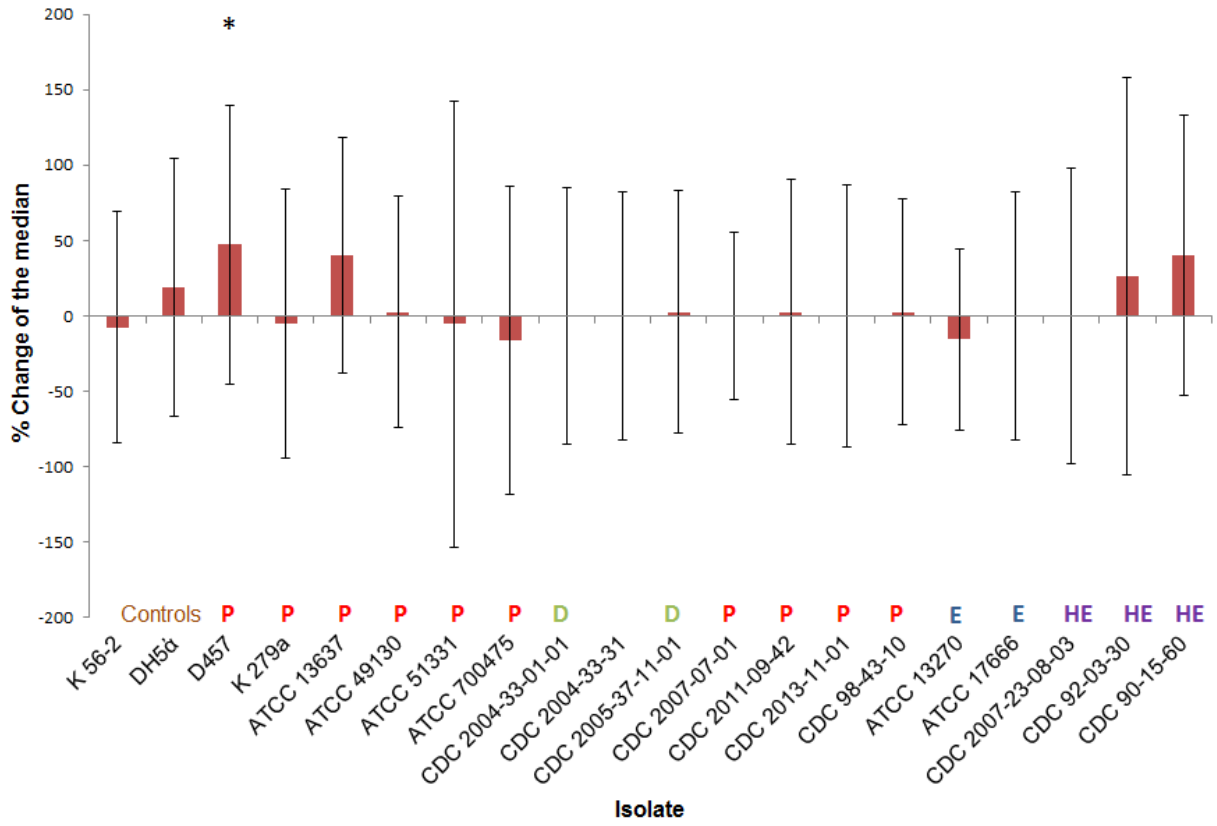

Fig. 4. Percentage change of the median in number of root branches of NaCl stressed canola seedlings exposed to different *S. maltophilia* isolates. Vertical bars represent standard deviation. Isolates were compared using Bonferroni at $P < 0.05$. Isolates with no asterisk do not differ significantly from the no bacteria control. P - Patient; D – Device; E – environmental; HE – hospital environment isolate.
*Isolate differs significantly from the "no bacteria" control.

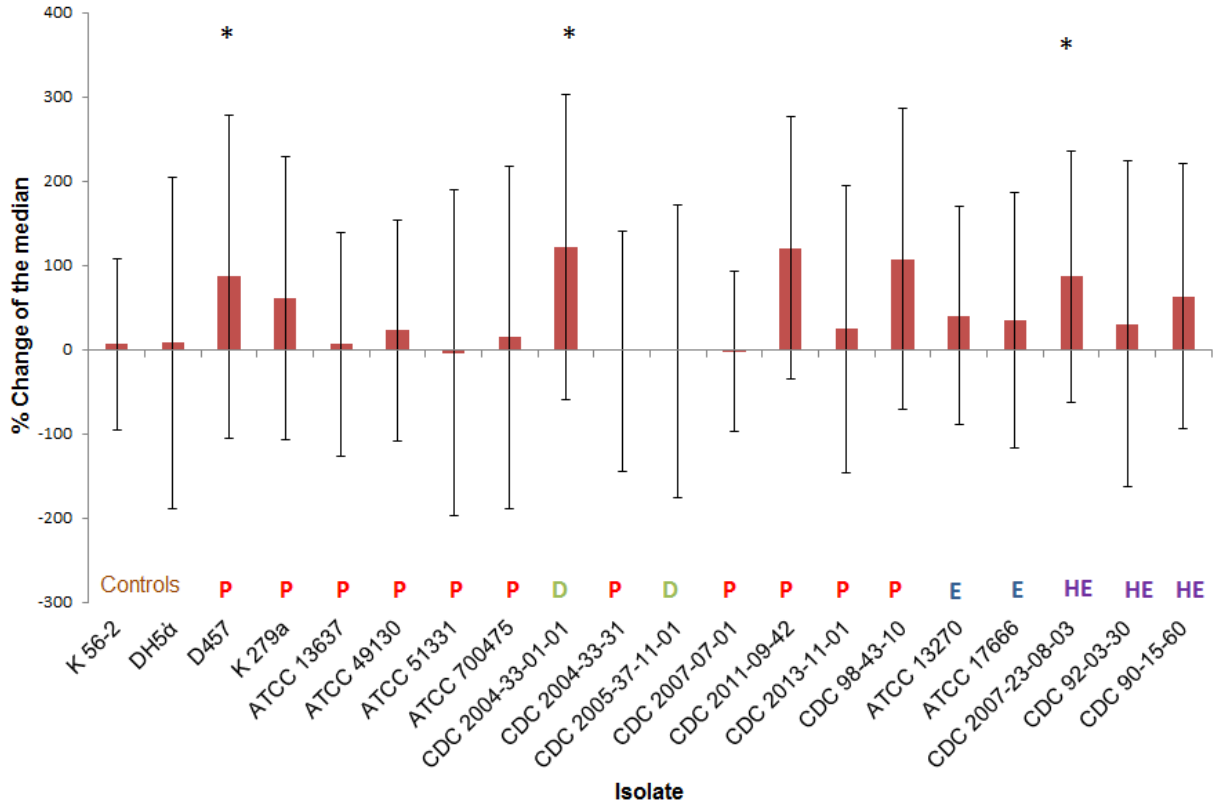

Fig. 5. Percentage change of the median in root length of NaCl stressed canola seedlings exposed to different *S. maltophilia* isolates. Vertical bars represent standard deviation. Isolates were compared using Bonferroni at $P < 0.05$. Isolates with no asterisk are not significantly different from the "no bacteria" control. P - Patient; D – Device; E – environmental; HE – hospital environment isolate.

* Isolate differs significantly from the "no bacteria" control.

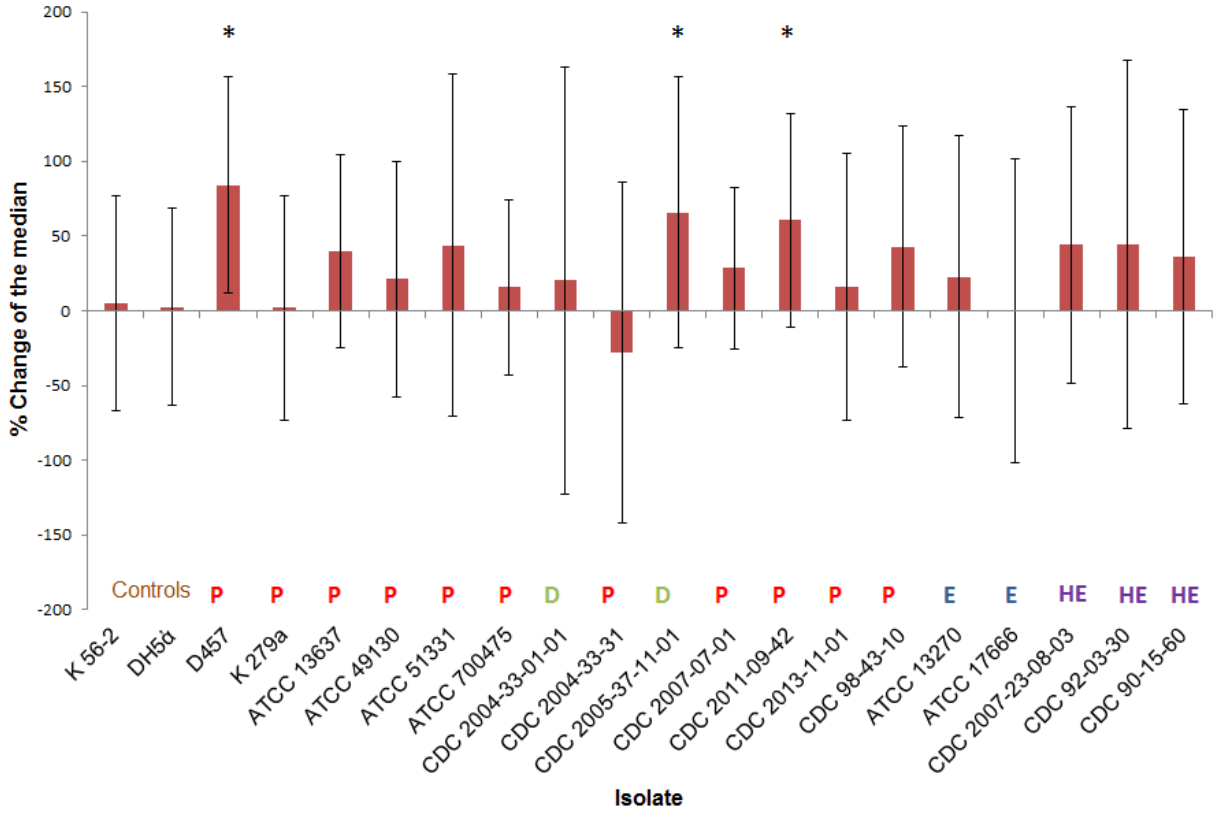

Fig. 6. Percentage change of the median in stem length of NaCl stressed canola seedlings exposed to different *S. maltophilia* isolates. Vertical bars represent standard deviation. Isolates were compared using Bonferroni at $P < 0.05$. Isolates with no asterisk are not significantly different from the "no bacteria" control. P - Patient; D – Device; E – environmental; HE – hospital environment isolate.
* Isolate differs significantly from the "no bacteria" control.

The ability of *S. maltophilia* isolates to protect canola seedlings against *L. maculans*

Inoculation of *S. maltophilia* and *L. maculans* on canola was done in three ways:

i. Canola seedlings were first inoculated with *L. maculans* and then with *S. maltophilia*,

ii. Canola seedlings were inoculated with *S. maltophilia* and *L. maculans* simultaneously

iii. Canola seedlings were first inoculated with *S. maltophilia* and then with *L. maculans*

Canola seedlings exposed to *L. maculans* and then with *S. maltophilia*

Significant differences ($P < 0.0001$) were found among isolates in all three parameters (number of root branches, root and stem length) investigated. Isolates D457, ATCC 13270, ATCC 13637, and ATCC 2007-07-01 protected canola seedlings from *L. maculans*-induced decrease in root branching. The mean number of root branches ranged from 1.3 in the *L. maculans* alone treatment to 5.1 in canola seedlings which were not inoculated with either pathogen (none) (Fig. 7). The longest roots (average of 1.9 cm) were observed in seedlings exposed to isolate ATCC 13637 and this differed significantly ($P < 0.05$) from seedlings exposed to the isolate K279a (average of 0.9 cm) and the *L. maculans* alone (average of 0.46 cm) treatment (Fig. 8). Canola seedlings inoculated with ATCC 13637, CDC 2007-07-01, ATCC 13270 and D457 had significantly ($P < 0.05$) higher root length than the *L. maculans* alone treatment (Fig. 8). The stems of canola seedlings exposed to *S. maltophilia* isolate ATCC 13270 (average of 2.76 cm) were the longest and this differed significantly from those of seedlings inoculated with the *L. maculans* alone (average of 0.89 cm) (Fig. 9). Canola seedlings inoculated with D457, CDC 2007-07-01, K279a, ATCC13637 and ATCC 13270 had significantly ($P < 0.05$) higher stem length than the *L. maculans* alone treatment (Fig. 9).

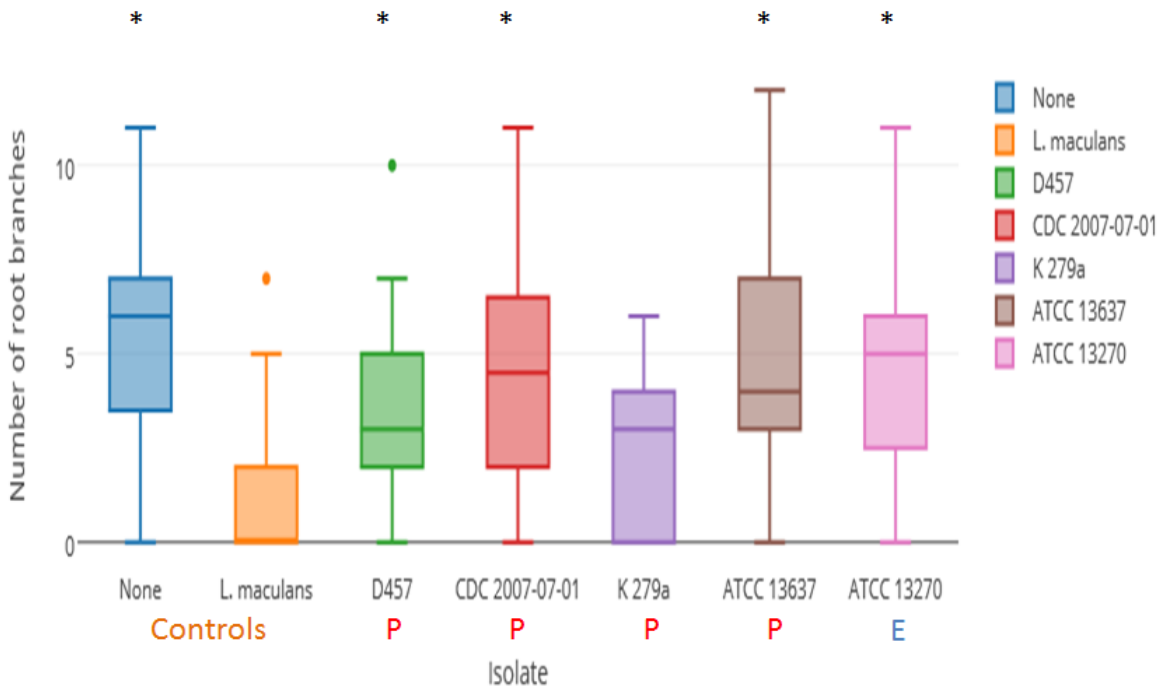

Fig. 7. Mean number of root branches of canola seedlings inoculated with *L. maculans*, then to *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from the "*L. maculans* alone" treatment.

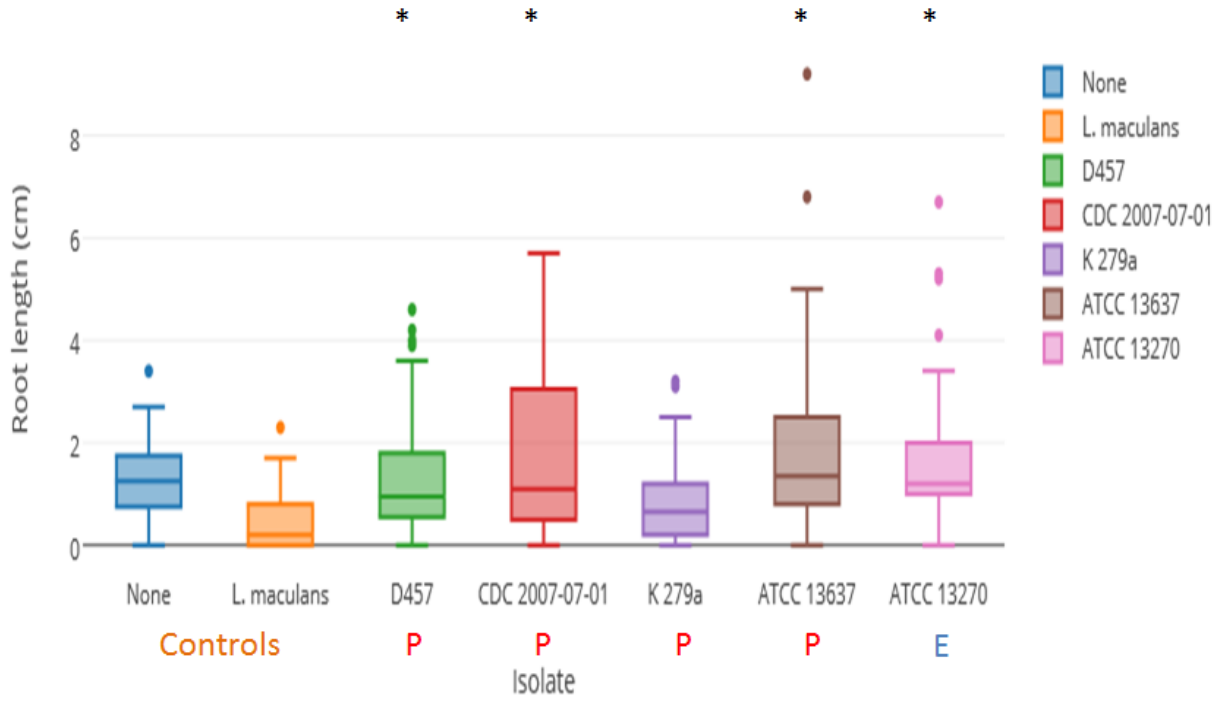

Fig. 8. Mean root length of canola seedlings inoculated with *L. maculans*, then *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate. *Isolates differ significantly from the "*L. maculans* alone" treatment.

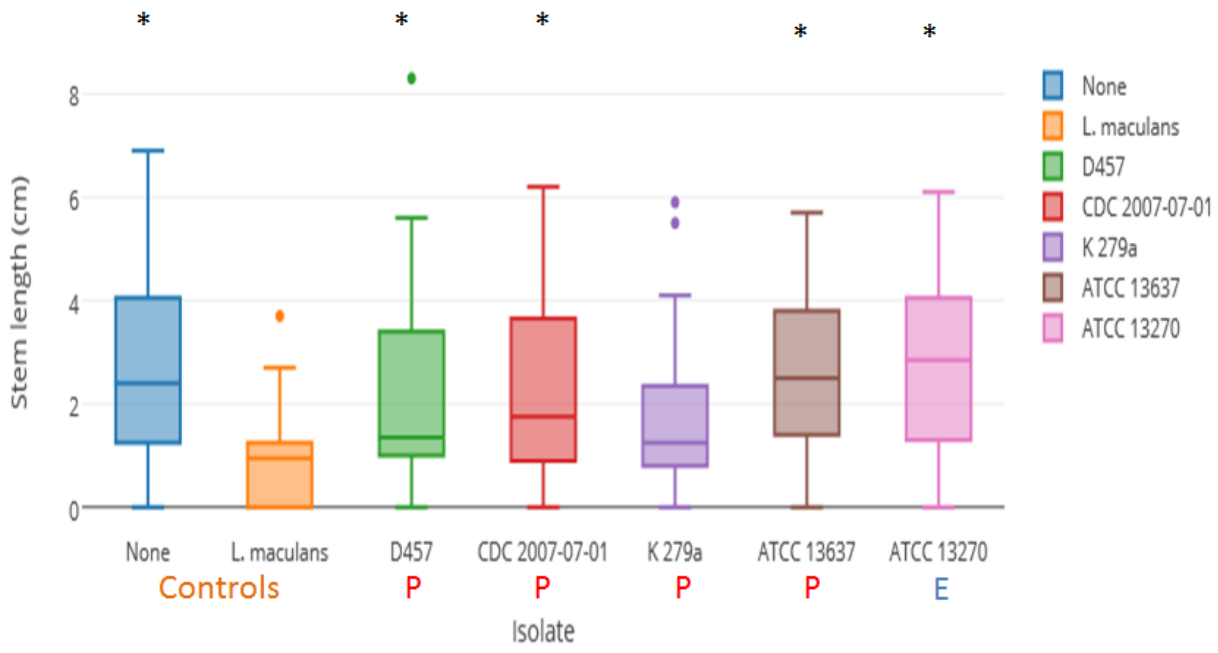

Fig. 9. Mean stem length of canola seedlings inoculated with *L. maculans*, then *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from the "*L. maculans* alone" treatment.

Canola seedlings exposed to *S. maltophilia* and *L. maculans* simultaneously

Significant ($P < 0.0001$) differences were found among *S. maltophilia* isolates in number of number of root branches, root length and stem length of canola seedlings. Canola seedlings which were not inoculated (none) had the highest number of root branches (average of 5.06) and this differed significantly from those of seedlings exposed to DH5α (average of 3.03) as well as those which were exposed to *L. maculans* alone (average of 1.33) (Fig. 10). Apart from DH5α, all isolates protected canola plants against *L. maculans*-induced decrease in root branching relative to the *L. maculans* alone treatment (Fig. 10). For root length, canola seedlings exposed to ATCC 13637 (average of 1.17 cm) and DH5α (average of 0.62 cm) were not protected against the effects of *L. maculans* (Fig. 11). However, seedlings inoculated with D457, CDC 2007-07-01, K27a and ATCC 13270 had significantly higher root length than the *L. maculans* alone treatment (Fig. 11). The stems of canola seedlings exposed to ATCC 13637 (average of 1.86 cm)

and DH5α (average of 1.59 cm) did not differ statistically from those of *L. maculans* alone treatment (average of 0.89 cm) (Fig. 12). Seedlings inoculated with K279a, D457, CDC 2007-07-01, and ATCC 13270 had significantly higher stems lengths than seedlings inoculated with *L. maculans* alone.

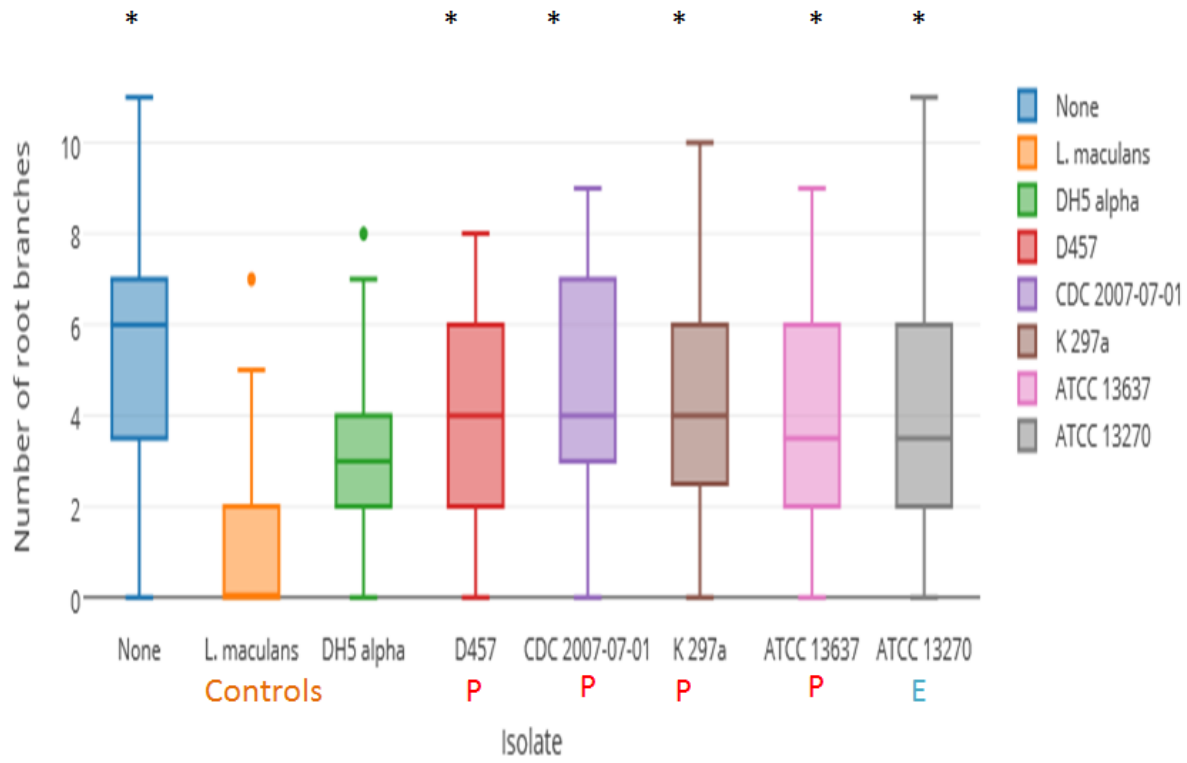

Fig. 10. Mean number of root branches of canola seedlings inoculated with *L. maculans* + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from the "*L. maculans* alone" treatment.

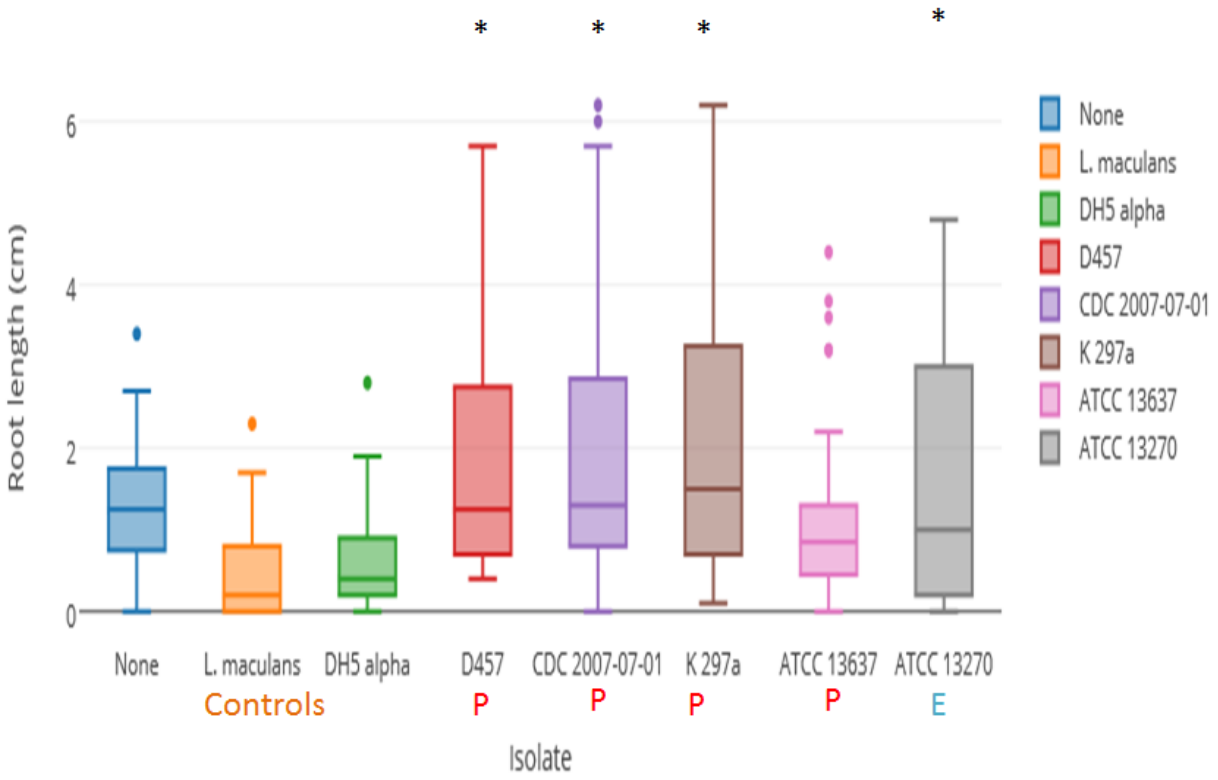

Fig. 11. Mean root length of canola seedlings inoculated with *L. maculans* + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from the *L. maculans* alone treatment.

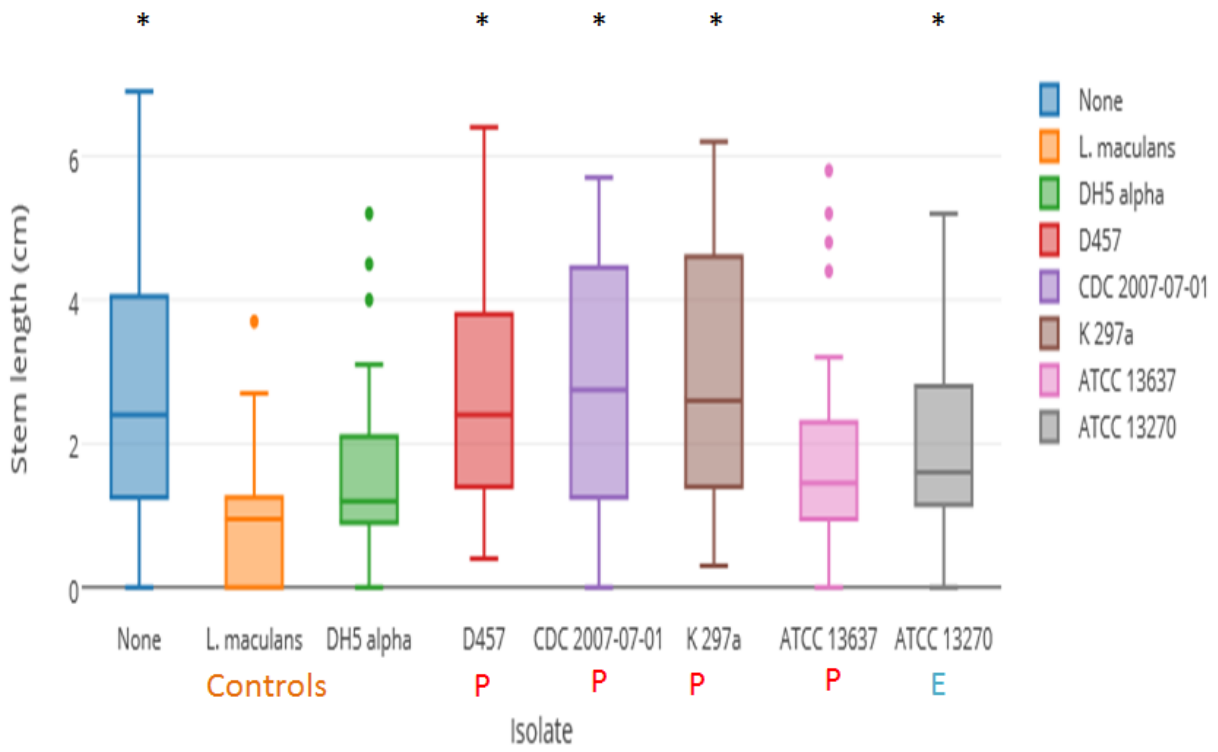

Fig. 12. Mean stem lengths of canola seedlings inoculated with *L. maculans* + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from the "*L. maculans* alone" treatment.

Canola seedlings first inoculated with *S. maltophilia* and then with *L. maculans*

Significant ($P < 0.0001$) differences were observed in all three parameters investigated (number of root branches, root and stem length). Seedlings inoculated with D457, ATCC 13637, ATTC 13270 and K279a were protected against inhibition of root branching caused by *L. maculans* (Fig. 13). The number of root branches found in seedlings inoculated with CDC 2007-07-01 was not statistically different from those found in the *L. maculans* alone treatment (Fig.13).

The root length of canola seedlings exposed to isolates ATCC 13270 (average of 1.84 cm), K279a (average of 1.66 cm), CDC 2007-07-01 (average of 1.49 cm) and D547 (average of 1.46 cm) were significantly higher than those seedlings exposed to *L. maculans* alone (average of 0.46 cm) (Fig. 14). Seedlings inoculated with ATCC 13637 had statistically similar root length

as the *L. maculans* alone treatment (Fig. 1.14). Like root length, stem length of canola seedlings inoculated with ATCC 13637 was statistically similar to that of the *L. maculans* alone treatment (Fig. 1.15). However, canola seedlings received protection against *L. maculans* when they were inoculated with D457, CDC 2007-07-01, K279a and ATCC 13270 (Fig. 15).

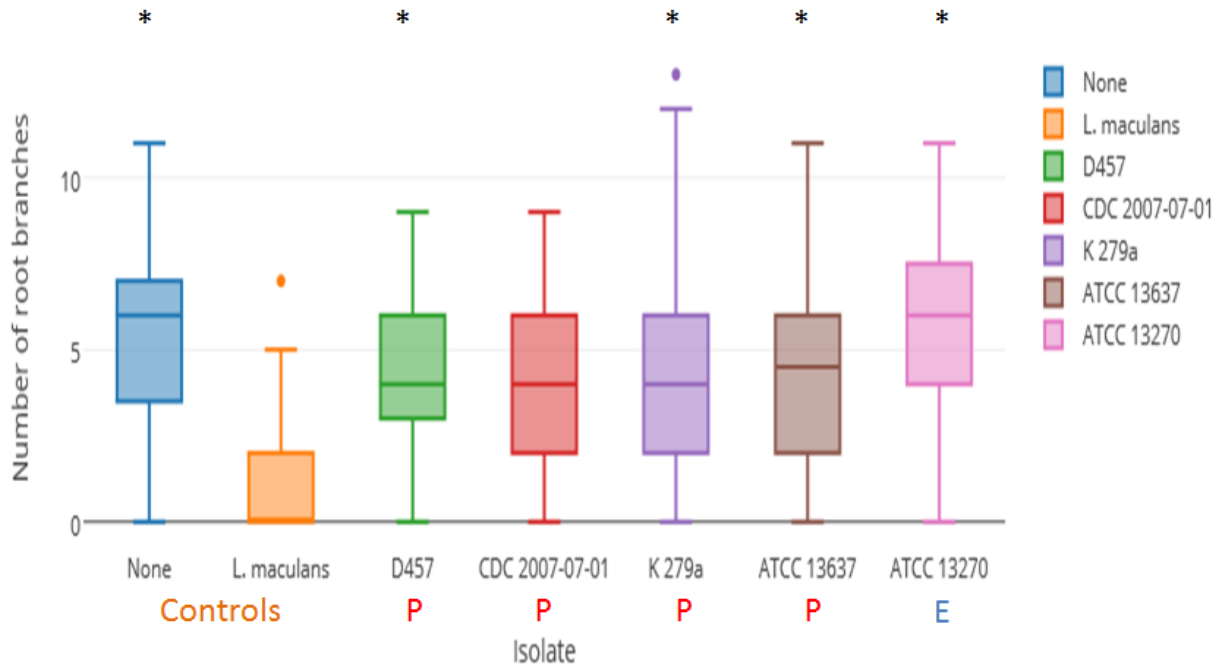

Fig. 13. Mean number of root branches of canola seedlings inoculated with *S.maltophilia* isolates, then *L. maculans.* Mean separation was by Tukey's at $P < 0.05$. P – patient;
E – environmental isolate.
*Isolates differ significantly from *L. maculans* alone treatment.

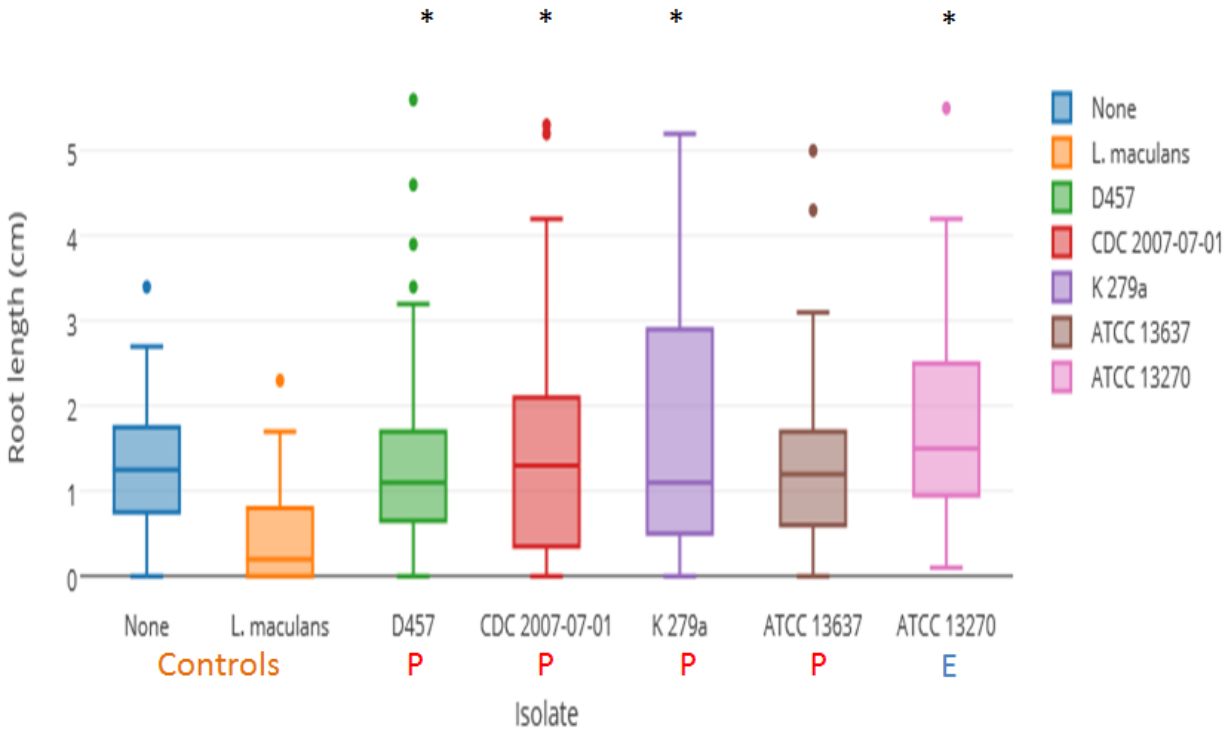

Fig. 14. Mean root lengths of canola seedlings inoculated with *S. maltophilia* isolates, then *L. maculans.* Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from *L. maculans* alone treatment.

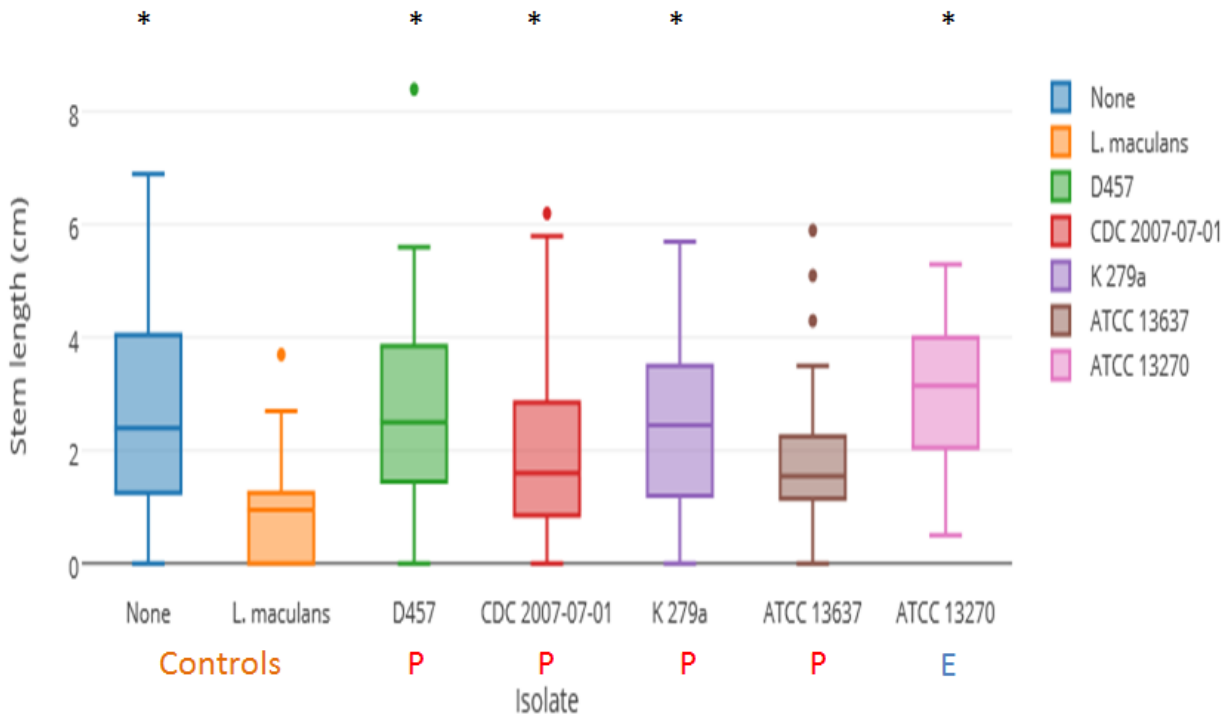

Fig. 15. Mean stem lengths of canola seedlings inoculated with *S. maltophilia* isolates, then *L. maculans.* Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from *L. maculans* alone treatment.

Protective capabilities of *S. maltophilia* against the effect of K 56-2

The inoculation of *S. maltophilia* and K 56-2 was done in three ways:

i. Canola seedlings were first inoculated with K 565-2 and then with *S. maltophilia*

ii. Canola seedlings were inoculated with *S. maltophilia* and K 56-2 simultaneously

iii. Canola seedlings were first inoculated with *S. maltophilia* and then with K 56-2

Canola seedlings first inoculated with K 56-2 and then with *S. maltophilia*

Significant differences were found in the number of root branches ($P < 0.0015$), root length ($P < 0.0020$) and stem length ($P < 0.0057$) of canola seedlings. The number of root branches that developed on seedlings inoculated with isolates ATCC 13637 and K279a as well as "none" treatment was significantly higher than the K 56-2 alone treatment (Fig 16). Seedlings

inoculated with D457, CDC 2007-07-01 and ATCC 13270 had statistically similar root branching as the K 56-2 alone treatment (Fig. 16). Canola seedlings which were not exposed to any bacterium (none) and those exposed K279a had significantly longer roots than those of seedlings exposed to K56-2 only (Fig. 17). The rest of the isolates had statistically similar root lengths as the K 56-2 alone treatment (Fig. 17). Canola seedlings inoculated with isolate K279a had the longest stems (average of 2.11 cm) and this significantly differed from those of seedlings exposed to the K56-2 alone treatment (average of 0.98 cm) (Fig. 18). Stems lengths of seedlings inoculated with the remaining isolates did not differ from those of the K 56-2 alone treatment (Fig. 18).

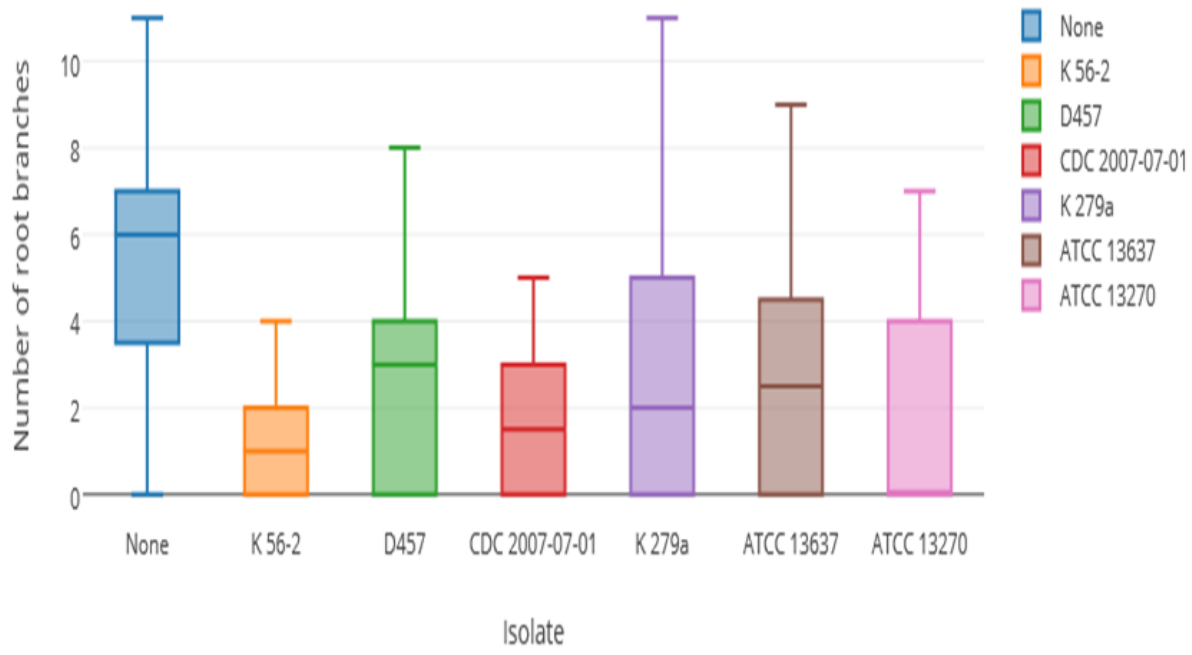

Fig. 16. Mean number of root branches of canola seedlings inoculated with K 56-2, then *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
*Isolates differ significantly from "K 56-2 alone" treatment.

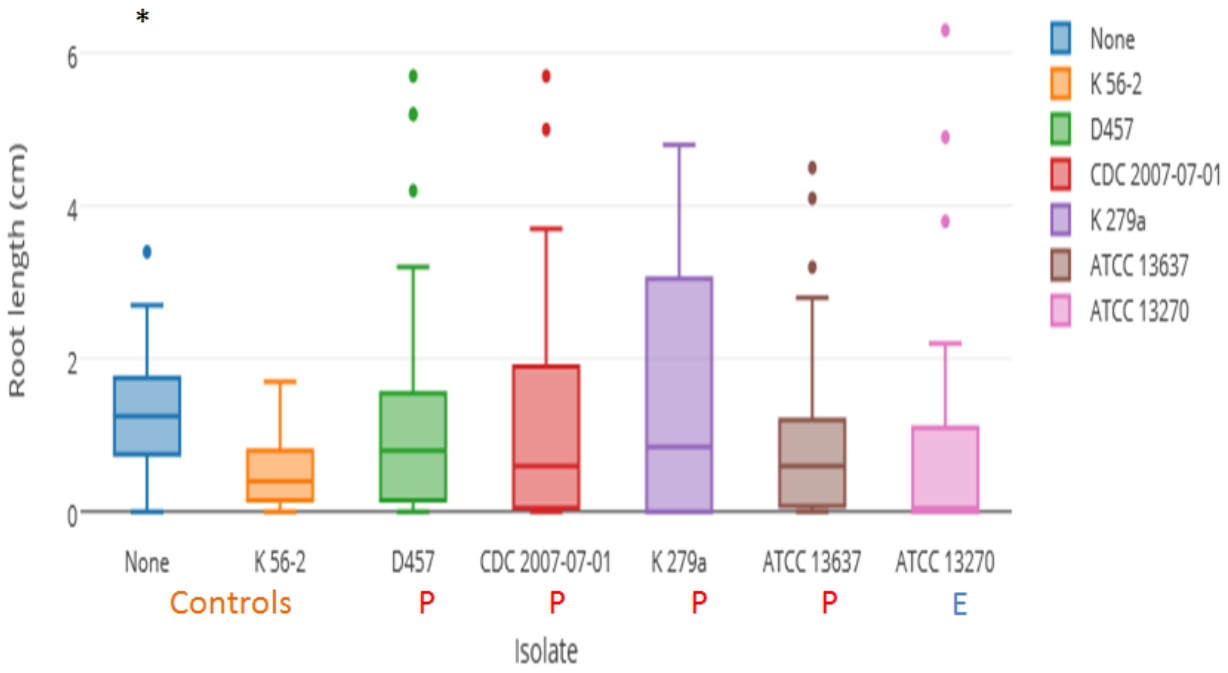

Fig. 17. Mean root length of canola seedlings inoculated with K56-2, then *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

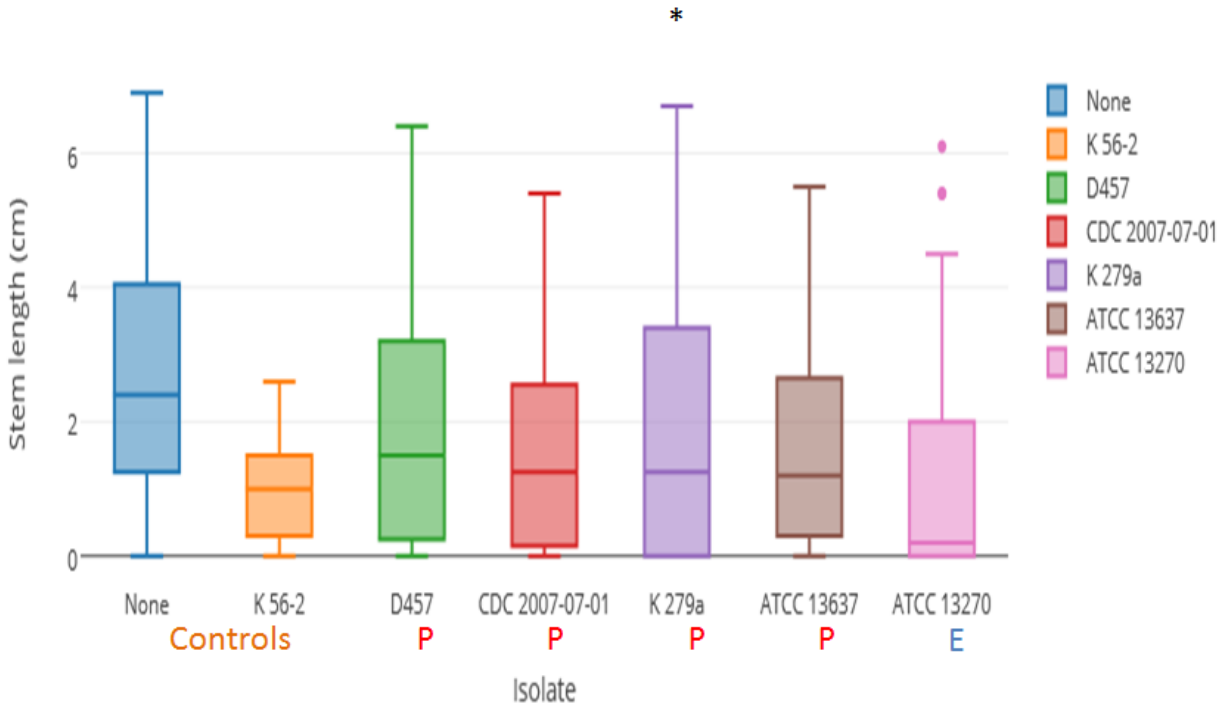

Fig. 18. Mean root length of canola seedlings inoculated with K56-2, then *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

Canola seedlings inoculated with *S. maltophilia* and K 56-2 simultaneously

Significant ($P < 0.0001$) were found in all three parameters investigated (number of root branches, root and stem length). Simultaneous inoculation of K56-2 and CDC 2007-07-01, DH5α, D547, K279a or ATCC 13637 resulted in significantly higher number of root branches than seedlings exposed to K 56-2 alone treatment (Fig. 19). Root branching among canola seedlings inoculated with ATCC 13270 did not differ statistically from the K 56-2 alone treatment (Fig 19). Seedlings which were inoculated with CDC 2007-07-01 and the "none" control had significantly higher root length than those seedlings exposed to K 56-2 alone (Fig. 20). Seedlings inoculated with the rest of the isolates did not differ in root length from those of K 56-2 alone (Fig. 20). Like root length, canola seedlings inoculated with CDC 2007-07-01 and

"none" had the longest stems which differed significantly from stem lengths of seedlings inoculated with K56-2 only (Fig. 21).

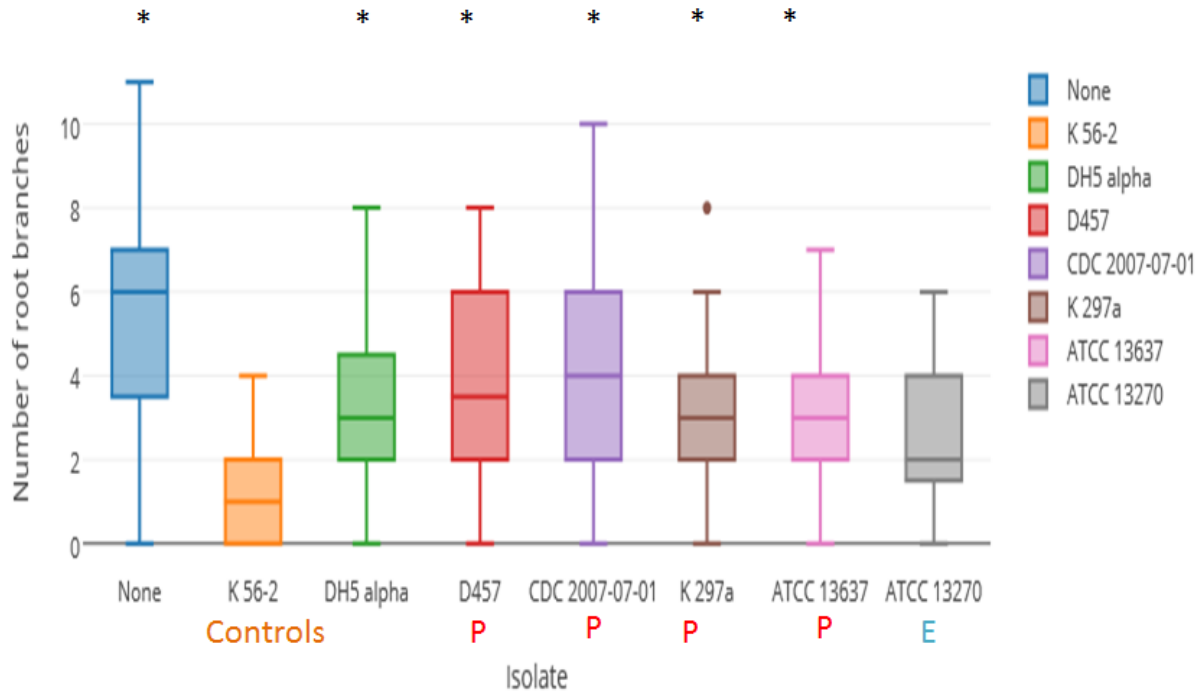

Fig. 19. Mean number of root branches of canola seedling inoculated with K 56-2 + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

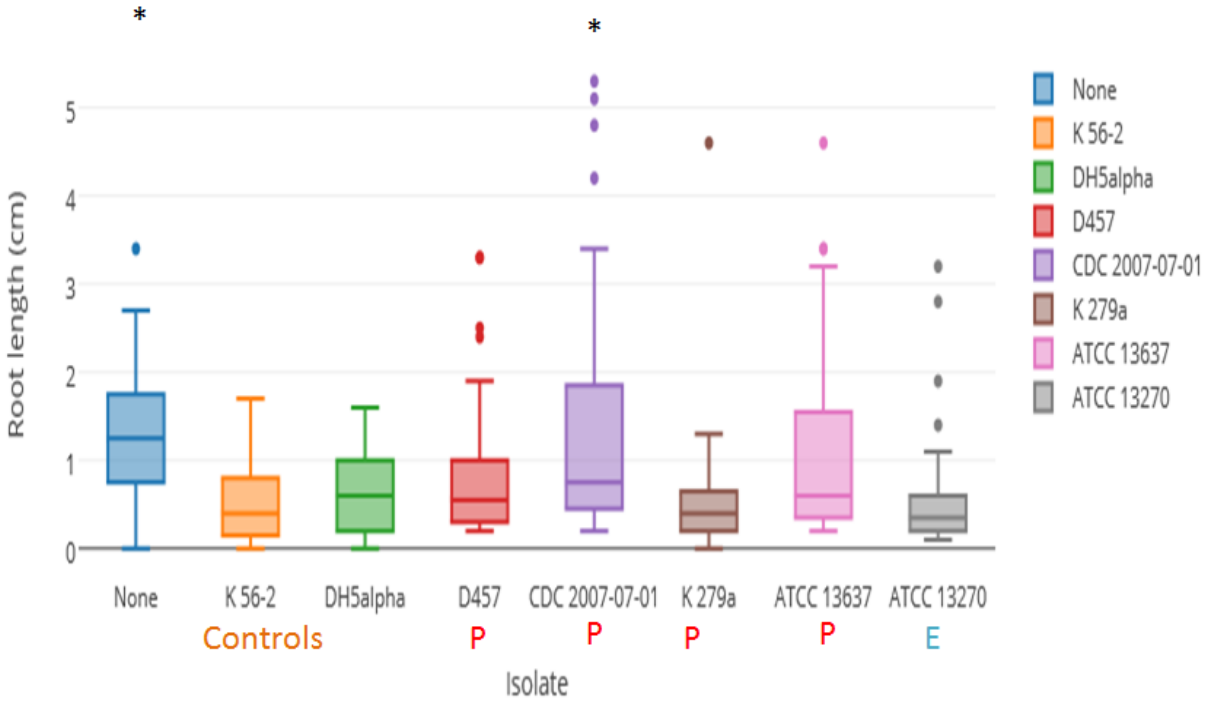

Fig. 20. Mean root length of canola seedlings inoculated with K 56-2 + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

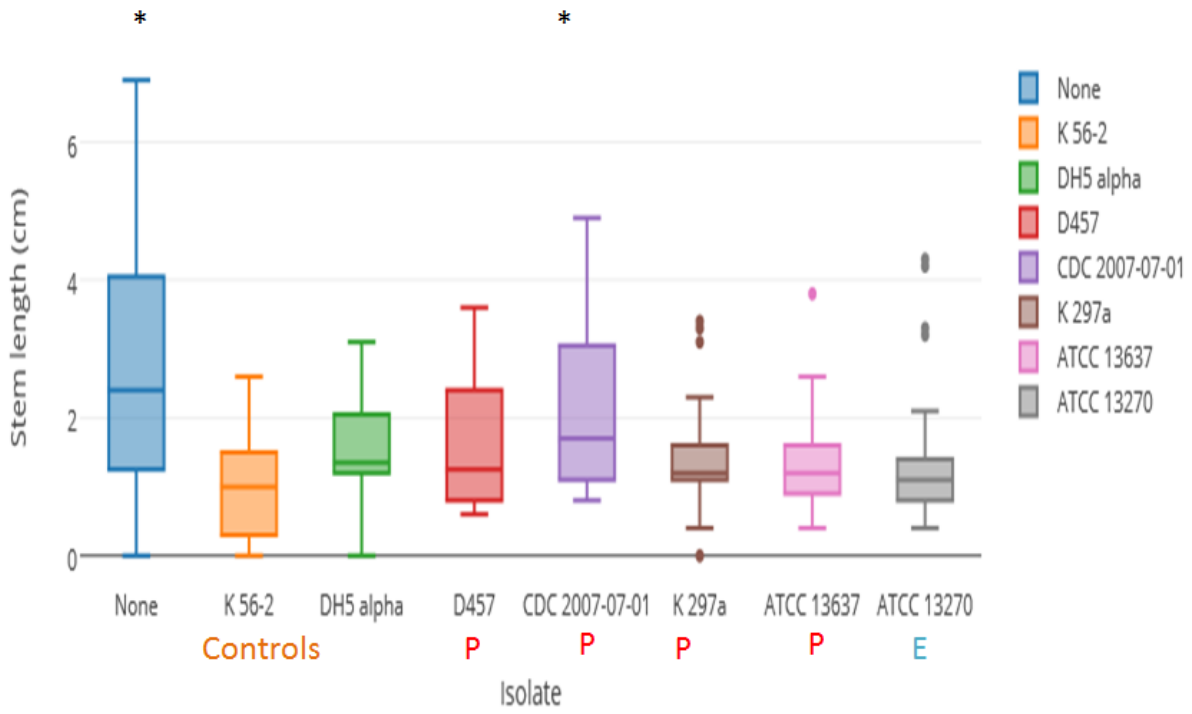

Fig. 21. Mean stem length of canola seedlings inoculated with K 56-2 + *S. maltophilia* isolates. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

Canola seedlings were first inoculated with *S. maltophilia* and then with K 56-2

Significant differences were found in the number of root branches ($P < 0.0001$), root length ($P < 0.0026$) and stem length ($P < 0.0013$). Exposure of seedlings to ATCC 13637 resulted in the highest number of canola root branches which significantly differed from those of seedlings exposed to K56-2 alone (Fig. 22). Seedlings exposed to the other isolates did not have significantly different number of root branches than the K 56-2 alone (Fig 22). Seedlings which were not inoculated with either of the pathogens had the longest roots and this was the only one that differed significantly from the K56-2 alone treatment (Fig. 23). For stem length, only seedlings which were inoculated with ATCC 13270 were significantly higher than seedlings inoculated with K56-2 alone (Fig. 24).

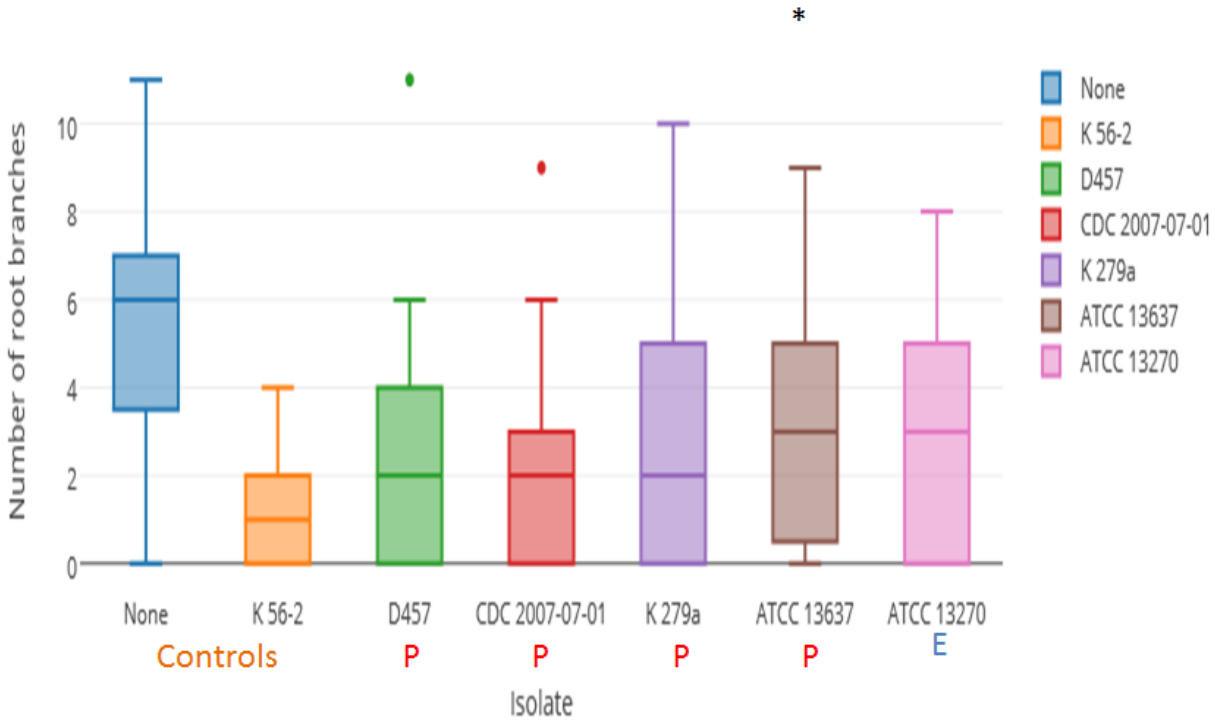

Fig. 22. Mean number of root branches of canola seedlings inoculated with *S. maltophilia* isolates, then K 56-2. Mean separation was by Tukey's at $P < 0.05$. P – patient;
E – environmental isolate.
* Isolates differ significantly from K 56-2 alone treatment.

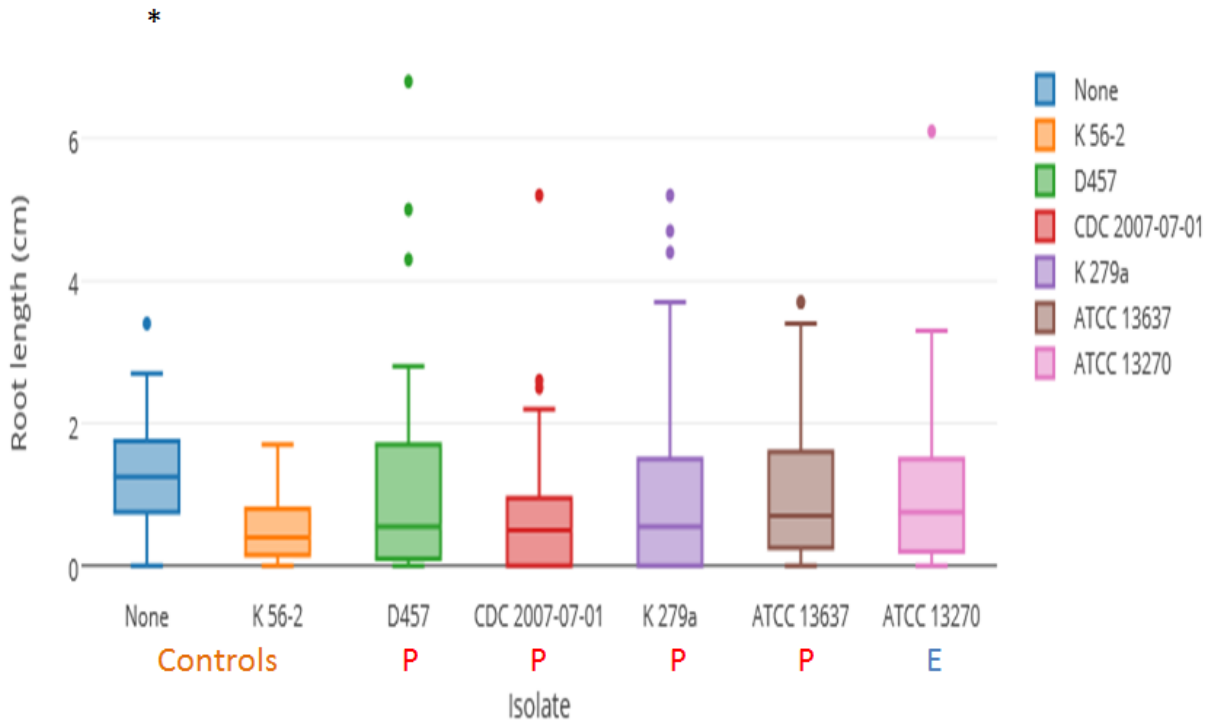

Fig. 23. Mean root length of canola seedlings inoculated with *S. maltophilia* isolates, then K 56-2. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate. * Isolates differ significantly from K 56-2 alone treatment.

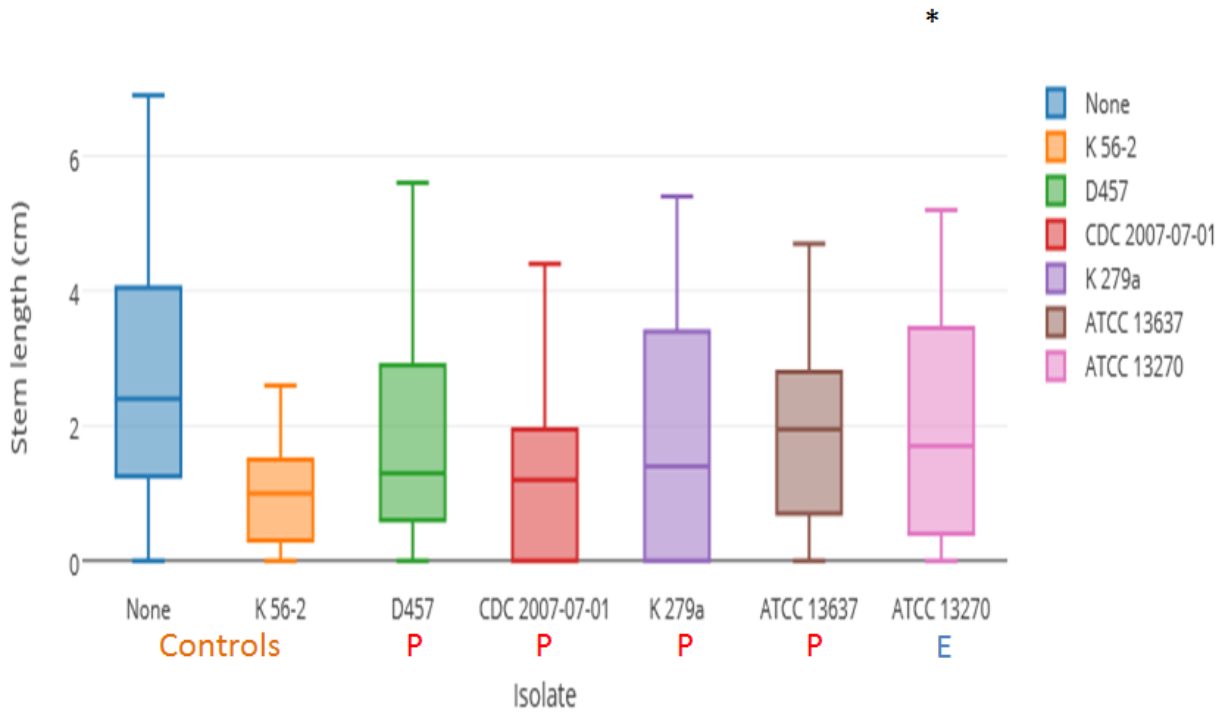

Fig. 24. Mean stem length of canola seedlings inoculated with *S. maltophilia* isolates, then K56-2. Mean separation was by Tukey's at $P < 0.05$. P – patient; E – environmental isolate. * Isolates differ significantly from K56-2 alone treatment.

gyrB sequencing

The aim of this part of the project was to determine the relatedness of the *S. maltophilia* isolates used in this study through phylogenetic analysis. This was done through *gyrB* primers as designed for *Pseudomonas* (Yamamoto *et al*., 2000). The *gyrB* gene is a housekeeping gene that encodes the DNA gyrase B-subunit. It is involved in negative supercoiling catalysis of DNA (Huang, 1996). Out of 18 isolates, 10 complete sequences were obtained and used to draw the phylogenetic tree. Efforts to get complete sequences for the other 8 isolates were unsuccessful after three attempts. Two separate clusters were observed (Fig. 26). The isolates written in bold are the *S. maltophilia* isolates used to build the phylogenetic tree. The isolates were evenly distributed in both clusters. Only two isolates were found to be closer to each other, *S. maltophilia* isolates, CDC 2007-07-01 and D457. In this study, D457 consistently promoted

canola growth grown under salt stress conditions in all parameters investigated. The isolate D457 and CDC 2007-07-01 had similar results in canola protection experiments. The gyrB primers used had threefold degeneracy in the reverse primer.

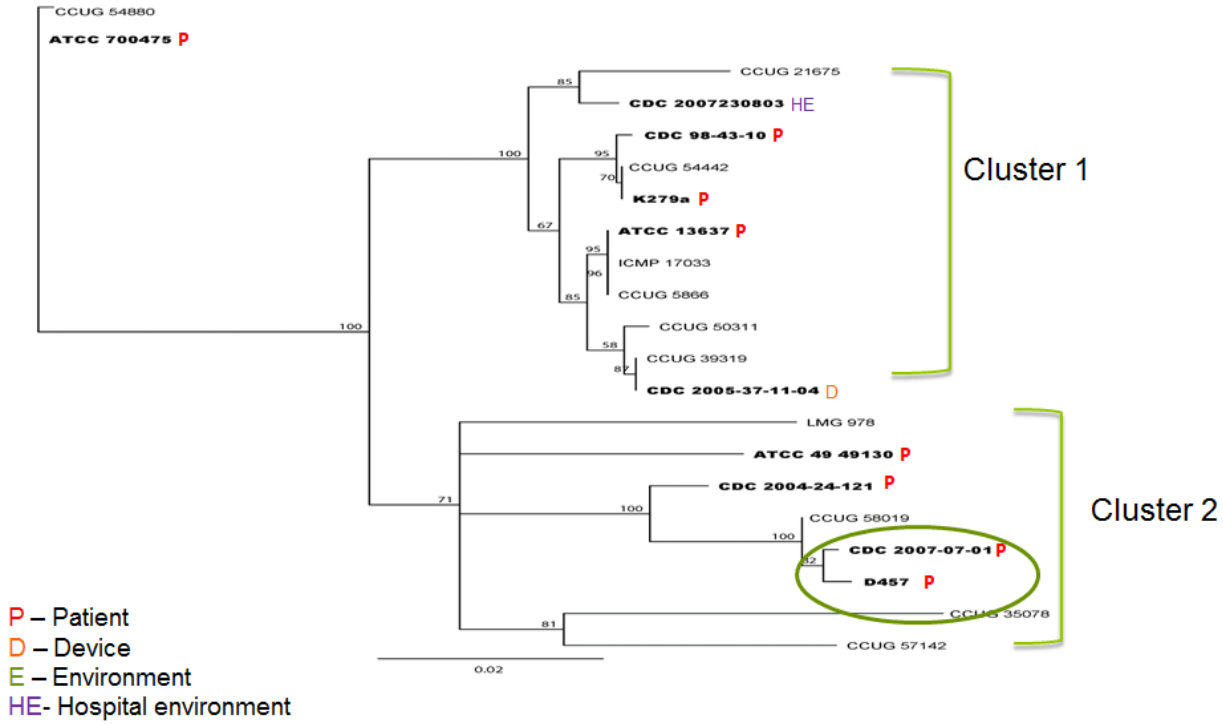

Fig. 25. A phylogenetic tree showing the relatedness of *S. maltophilia* isolates. The phylogenetic analysis was done via gryB sequencing.

DISCUSSION

Stenotrophomonas maltophilia can be found in different environments such as soil, plant rhizosphere, surface water, food, drinking water, contaminated medical fluids or devices (Looney *et al*., 2009). *S. maltophilia* is unique in that on one side, it acts a nosocomial multidrug-resistant pathogen associated with immuno-suppressed patients (Denton & Kerr, 1998) and on the other side, it is non-pathogenic to plants (Wolf *et al.*, 2002) and has properties that enhance plant growth and development (Taghavi *et al*., 2009). *S. maltophilia* has been reported to be associated with a variety of plant species including canola (Berg *et al.*, 1996).

The overall purpose of this study was to determine the phenotypic differences between clinical and environmental isolates of *S. maltophilia*. As a first step, laboratory experiments were conducted to determine if clinical *S. maltophilia* isolates retain their plant-growth promoting properties or alternatively, if they lose them while under selection in the clinic. A total of 13 of the isolates were obtained from clinic sources while five environmental isolates were included. The results of *gyrB* phylogenetic analysis of the isolates revealed that two of them, CDC 2007-07-01 and D457 are closely related. It was also noticed that one of the environmental isolates, ATCC 13637 was placed in the same cluster as clinical isolates, indicating that clustering did not depend on the source of isolates or that clinical and environmental isolates may be closely related. In a study, Adamek *et al*. (2011) also found through *gyrB* analysis that some clusters contained only clinical isolates while others contained environmental isolates but most clusters contained both. In our study, only one environmental isolate was included in the phylogenetic analysis due to a challenge in getting complete gene sequence for the second isolate. Out of all 18 isolates, complete sequences were generated for 10 isolates. The inability to obtain complete sequences for the remaining eight isolates may have been caused by primer degeneracy in our

reverse primer. Primer degeneracy has been shown to cause problems during PCR amplification (Souvenir *et al*., 2003).

In order to investigate whether clinical isolates of *S. maltophilia* promote plant growth, canola seedlings were inoculated with the isolates under normal and NaCl stress conditions. Soil salinity constitute a serious problem in crop production worldwide (Flowers, 2004), especially in production systems where irrigation is applied (Cicek and Cakirlar, 2002). High concentration of salt in the soil reduces plant growth and yield and also reduces/delays germination in seedlings through inhibition of water uptake or by affecting the balance of specific ions such as Na^+, Ca^{2+} and K^+ (Yildirim *et al*., 2006). Finding adaptation and mitigating strategies that are inexpensive and easy to apply is therefore very crucial. This made the inclusion of salt stress in the plant growth experiment worthwhile. Our results show that under normal conditions, inoculation of canola seedlings with the isolates of *S. maltophilia* did not result in enhanced root growth or root branching. Canola stem length was negatively affected by some *S. maltophilia* isolates (CDC 92-03-30, ATCC 17666, CDC 98-43-10, CDC 2013-11-01 and CDC 2011-09-42). Under NaCl stress conditions however, enhanced root and stem growth as well as root branching were observed across most of the isolates. Isolate D457 showed consistency in promoting growth in salt stressed canola seedlings. In a study on strawberry in Germany, it was reported that *Stenotrophomonas* strains promoted root growth and root hair development under normal growing conditions but a number of strains had a negative influence on stem length (Suckstorff and Berg, 2003). It is obvious that both similarities and inconsistencies exist between the results presented in the current study and those of Suckstorff and Berg (2003). It worth noting that the data reported in this study were taken one week after inoculation of canola seedlings with *S. maltophilia* isolates while Suckstorff and Berg (2003) recorded their data 5 weeks post

inoculation. The difference in the period during which strains were in contact with the plants differed between the two studies and this, coupled with the fact the different plants and strains were investigated could explain the disagreements in results. *S. maltophilia* promotes plant growth through a variety of mechanisms such as the production of IAA, nitrogen fixation (Park *et al*., 2005) and ability to produce anti-fungal metabolites (Minkwitz & Berg, 2001). Suckstorff and Berg (2003) found a positive correlation between plant growth promotion and the production of IAA and concluded that regardless of origin, *Stenonotrophomonas* stains can produce IAA in vitro and subsequently influence plant growth. Parameters such as stem and root length and root branching have been used in investigations as plant growth indicators (Suckstorff and Berg, 2003). Results of plant growth promotion by *S. maltophilia* could potentially be influenced by factors such as proximity of bacterial isolates to the host plant. Root colonization after bacterial inoculation is therefore essential for growth promotion. The lack of growth promotion under normal (non-stress) condition could have been caused by low or lack of root colonization by the isolates.

Our results suggest that *S. maltophilia* isolates might have played a role in plant recovery from salt stress. This is consistent with results of other previously published studies (Berg *et al*., 2010, Roder *et al*., 2005; Mayak *et al*., 2004). Berg *et al* (2010) noted that the plant-promoting effect of Stenotrophomonas is much enhanced under salt stress conditions than in non-saline soil. *S. maltophilia* has been reported to exhibit tolerance to external osmolarity by the accumulation of certain osmoprotective compounds and can grow well under NaCl stress (Miller & Wood, 1996). These compounds are produced in response to salt stress and protect biological structures like membranes and proteins. *Achromobacter piechaudii*, a plant growth promoting bacterium, has been reported to significantly increase the fresh and dry weights of tomato seedlings grown

in the presence of up to 172 mM NaCl salt, slightly increased the uptake of phosphorous and potassium and increased water use efficiency (Mayak *et al*., 2004). These activities may have in part, contributed to activation of processes involved in the alleviation of the effect of salt on the plants. Salinity has been reported to cause an increase in the rate of ethylene biosynthesis and this stress-induced ethylene inhibits root growth. Any factor capable of curbing the accelerated ethylene production can result in improvement in the growth of plants under salt stress. It has been reported that bacteria capable of producing 1-aminocyclopropane-1-carboxylic acid (ACC) can hydrolyze endogenous levels of ACC, an ethylene precursor into α-keto butyrate and ammonia (Hontzeas *et al*., 2004). The removal of ACC reduces the harmful effects of ethylene and this aids plants to recover from stress (Shah *et al*., 1997, Glick *et al*., 2007), leading to enhanced growth. The production of siderophores by *S. maltophilia* has also been reported to play a role in plant recovery from salt stress (Tank and Saraf, 2010). The actual mechanism (s) through which growth promotion in salt stressed canola seedlings occurred in this study was not investigated and this will be an interesting subject for future studies.

In order to investigate the potential of *S. maltophilia* isolates to protect canola plants against the harmful effect of the fungus *L. maculans*, the causal pathogen of blackleg, a series of experiments were conducted with variable sequence of pathogen inoculations. The results show that growth of canola seedlings exposed to *L. maculans* was enhanced only when *S. maltophilia* isolates were present. On its own, *L. maculans* reduced canola root/stem length as well as root branching. The sequence of inoculation did not affect the protective ability of *S. maltophila* against *L. maculans*. The protective ability of *S. maltophilia* isolates reported here was based on the comparison of growth parameters of canola seedlings inoculated with *S. maltophilia* isolates and those of the non-treated control. Assessments of parameters such as conidia production and

mycelial growth of *L. maculans* with and without *S. maltophilia* isolates would have afforded a more direct inference to be made but this was outside the scope of this study. In future studies, it will be worthwhile to investigate these direct indicators of fungal growth inhibition. Mechanisms by which *S. maltophilia* acts as a biocontrol agent include antibiotic production (Jakobi and Winkelmann**,** 1996, O'Brien and Davies, 1982), extracellular enzyme activities such as protease and chitinase (Dunne *et al*., 1997, Kobayashi *et al*., 1995) and rhizosphere colonization potential (Juhnke *et al.*, 1987, Dunne *et al*., 1997). Antifungal antibiotics maltophilin and xanthobaccins which are strain-specific have recently been reported (Jacobi *et al*., 1996, Nakayama *et al*., 1999). *Stenotrophomonas* strains have also been shown to produce volatile organic compounds which can inhibit the growth of pathogenic fungi (Wheatley, 2002). It is likely that *S. maltophilia* isolates like K279a and D457 which demonstrated high antifungal activity possess genes which code for effectors capable of inhibiting fungal growth.

In this study, we also investigated the protective capability of *S. maltophilia* when challenged with the plant pathogenic bacterium *Burkholderia cenocepacia* (K 56-2). The results show that when canola seedlings were first inoculated with K 56-2 and later challenged with *S. maltophilia* isolates, K279a showed the greatest plant protection potential. Simultaneous inoculation of *S. maltpohilia* and K 56-2 had the greatest impact on the number of root branches as seedlings inoculated with D457, CDC 2007-07-01, K279a, ATCC 13637 and DH5α all had significantly higher root branching than the non-treated control. A possible explanation of this result could be that the simultaneous inoculation of the two bacteria might have caused the plant to be heavily stressed, resulting in more root branching in an effort to increase uptake of water and nutrients. This result should therefore be interpreted with caution. Unlike *L. maculans*,

inhibition of K 56-2 by *S. maltophilia* isolates seemed to be influenced by the sequence of inoculation.

The results of this study did not provide evidence of clear differences between clinical and environmental isolates based on the growth data. The inclusion of more environmental isolates could have afforded a better comparison. The results demonstrate that isolates of *S. maltophilia* (clinical and environmental) promote plant growth but only plants were exposed to sodium chloride stress before bacteria inoculation. Under normal conditions, some *S. maltophilia* isolates caused reduction in the stem length of canola seedlings. Canola seedlings were protected by *S. maltophilia* isolates against the deleterious effects of *L. maculans* and this occurred irrespective of the sequence of pathogen inoculation. Similarly, *S. maltophilia* isolates demonstrated protective capability against growth inhibition effects of the plant pathogenic bacterium K 56-2, however, this seemed to be influenced by the sequence of bacteria inoculation.

The results contribute vital information on *S. maltophilia*-canola interactions. With the use of irrigation in crop production increasing nowadays, the problem of soil salinity is expected to become more important. The discovery of plant growth promotion potential of *S. maltophilia* isolates under salt stress conditions in this study can therefore bring relief to crop growers who have salinity issues. The protective capability of *S. maltophilia* isolates against *L. maculans* and K56-2 makes them suitable candidates for use in biotechnology. Since some strains of *S. maltophilia* can cause disease in humans, the possible commercial application of this bacterium in crop production need to be authorized by appropriate regulatory agencies.

Future perspectives

With the above findings, it would be relevant to conduct further experiments that will answer some of the questions raised. For example, quantifying the levels of IAA produced by *S. maltophila* isolates upon inoculation to canola seedlings and performing correlation analyses between IAA levels and growth promotion will be useful in explaining the mechanism of underlying canola growth promotion by S. *maltophilia*. IAA can be quantified using the colorimetric method described by Gordon & Weber (1951). This involves growing *S. maltophilia isolates* into cultures for five days, reading the supernatant at 530nm and using a standard curve. Under salt stress conditions, some bacteria are known to produce 1-aminocyclopropane-1-carboxylic acid (ACC) which causes the depletion of stress-induced ethylene, thereby enhancing plant growth. ACC can be assayed as described by Bulens *et al.* (2011). Here again, correlation analyses could be carried out to establish the association between ACC production by *S. maltophilia* isolates (if any) and plant recovery from salt stress.

Siderophores have also been reported to play a role in plant recovery from salt stress (Tank and Saraf, 2010). To determine the presence of siderophores, Blue Agar CAS Assay (blue Fe (III)) dye complex of CAS (chrome azurol S) and HDTMA (hexadecyltrimethylammonium bromide) for detection of gram-negative bacteria as described by Schwyn and Neilands (1987) can be used. This test involves testing for chelation change on CASS agar plates. An orange halo production causes the removal of Fe (III) from an Fe (III)–CAS–HDTMA complex, resulting in the dye turning blue. *S. maltophilia* isolates grown on the agar plate will show siderophore production by forming orange halos around bacterial colonies whiles those without orange halos lack siderophore production.

It would also be important to conduct an assessment of parameters such as changes in the ability of *L. maculans* to grow and reproduce in the presence of *S. maltophilia* by measuring mycelium and spore production. This would enable a more direct inference to be made on the ability of *S. maltophilia* to control *L. maculans*. In addition, parameters such as lesion development on stems and leaves of canola seedlings caused by *L. maculans* could be measured.

Canola experiments can be carried out in the greenhouse or growth chamber with growing conditions of 20-30ºC, 16/8 hour period and humidity around 70%. The plants take approximately 3-4 months to mature when grown in soil (Kandel and Knodel, 2011). Where necessary, seeds can be stressed with an appropriate concentration of NaCl before planting in pots of soil. Parameters such as stem and root length of canola plants can be measured using a pair of calipers as well as the number of root branches counted. Other parameters that can be considered are fresh and dry weight. These weights will be useful in quantifying the amount of chlorophyll produced plants by exposed to *S. maltophilia* isolates. To reduce variation among replications or runs, all technical replicates, where possible, should be inoculated on the same day and data collections must also be done on the same day.

Another thing to consider is repeating the canola protection experiment. The experiment was carried out in three set-ups: before, mix and after. However, data for the controls was collected for only the mix set-up due to time constraints. As a result of this, 2-way ANOVA analysis using timing and strain as factors could not be performed with the resultant inability to determine if interaction occurred between the factors. It will be worthwhile setting up experiments which will have all controls in each of the set-up timings in order to determine this interaction.

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