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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Review

Virulence strategies of phytopathogenic bacteria and their role in plant disease pathogenesis

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Phytopathogenic bacteria have evolved several virulence strategies to face hostile environment of the host plant. In this article, we reviewed the recent progress in research on characterization of the virulence factors including secretion system with their protein effectors, toxins production, extracellular polysaccharides, growth regulators, cell wall degrading enzymes, biofilm formation, siderophores and their role in the plant infection and symptom development focusing particularly on a group of bacteria such as *Erwinia amylovora*, *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas compestris* that cause different plant diseases including wilts, spots, blights and cankers. The elucidation of each step in pathogenesis may constitute a key step in any design of new molecules for targeting plant pathogenic bacteria for plant disease control.

Key words: Virulence factors, plant disease, phytopathogenic bacteria, phytotoxins, secretion systems, siderophores.

INTRODUCTION

Most phytopathogens must evolve numerous strategies to survive in different environmental conditions to invade and colonize their hosts known as virulence factors. These factors have the ability to modulate the physiological and biochemical mechanisms to enhance the spread of the pathogen, as well as to facilitate the release of nutrients and water from host cells (Toth et al., 2003).

The plant bacterial pathogens, involve many virulence factors that are secreted in the extracellular environment of the host cells. The most studied factors are: (1) adherence to the host cells, with surface adhesins, (2) production of the degradative enzymes that destroy the plant cells walls, (3) toxins that are in the apoplastic cell, (4) other complex molecules are also deployed including the exopolysaccharide (EPS) and those modulating the

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plant hormone production. The major intensive studies in the field of plant bacteria interactions are the characterization of the pathogen virulence factors and their main roles in the pathogenecity and the specificity of the host. The elucidation of such aspect may lead to the planning and establishment of new strategy in the plant disease control. In the following section, we overviewed the recent development of the function and mechanism in the plant bacterial pathogens.

SECRETION SYSTEM

Plant pathogenic bacteria have evolved numerous sophisticated strategies for selective transport of proteins and nucleoproteins involved in the virulence across cell membranes in both the apoplastic environment and the cytoplasmic of the plant cells. Currently, six major classes of systems implicated in the virulence have been identified and described in plant pathogenic bacteria named from type I to type VI or T1SS to T6SS. The translocation mechanism of effector proteins from the bacterial cytosol to the external bacterial cell is known as secretion (Alfano et al., 2000).

In plant pathogenic Gram-negative bacteria, two major system are described. The single step process, in which secretion proteins are exported across the inner and the outer membrane without any periplasmic step, however, the two steps process namely the Sec and the Tat secretion system are first exported in the periplasmic and then transported across the external membrane to the exterior of bacteria cell. In the following section, we summarized the general features of the six identified secretion system known in the phytoopathogenic Gram-negative bacteria (Alfano et al., 2000; Preston et al., 2001).

T1SS secretion sytem

In phytopathogenic Gram-negative bacteria, the type I secretion system also known as the ATP binding cassette (ABC) transporters is involved in the export of various molecules from the cytosol to the external environment without any periplasmic step (Delepelaire et al., 2004). The type I secretion system consists of three distinct proteins that compose a continuous chanel (Ian et al., 2006). The inner membrane ATP binding cassette (ABC) proteins transporters is a specific outer membrane known as outer membrane protein (OMP) and the so called membrane fusion protein (MFP) which is connected to the inner membrane and spans the periplasmic space and extends to the outer membrane(Ian et al., 2006). Many proteins of great importance in pathogenesis are transferred by the ABC secretion system in plant pathogenic bacteria including proteases, lipases or performing toxins. The T1SS is required for numerous plant pathogenic Gram-negative bacteria including both the *Erwinia amylovora* and *Erwinia chrysanthemi* (Ian et al., 2006; Liu et al., 2008).

T2SS secretion system

In Gram-negative bacteria, the T2SS secretion system known as the sec dependant system translocate folded proteins across the inner membrane either by sec pathway or Tat pathway to the periplasm and then the extracellular environment. The plant pathogenic bacteria, uses such a system to export hydrolytic enzymes involved in degrading different plant substrates including cellulases, xylanases, amylases and proteases. Several plant pathogenic bacteria include *Pseudomonas fluorescens*, *Erwinia carotovora* pv *atroseptica*, *Xanthomonas compestris* pv *compestris* and *X. oryzae* pv *oryzae* (Peabody et al., 2003).

T3SS secretion system

Several plant bacterial pathogens have evolved a strategy of delivering an array of effectors and toxins proteins directly into the cytoplasm of host cells known as the type III secretion systems (Preston et al., 2001; Lindeberg et al. 2012). Theses virulence determinants have the capacity to modulate the physiological functions (Staskawicz et al., 2001; Buttner and Bonas, 2003). The type III secretion apparatus is composed of more than of 20 proteins consisting of basal body spanning both the inner and the outer membrane of the bacterial cells, and an extracellular needle with a tip complex extending into the host cell (Staskawicz et al., 2001). The TTSS in phytopathogenic bacteria is encoded by hypersensitive response and pathogenecity (hrp) gene involved in the transfer of Avr proteins in the host cell inducing both either pathogenecity on sensitive host or hypersensitive reaction on resistant host. The plant pathogens that use the TTSS system include *Xanthomonas* spp., *Erwinia* spp., *Pseudomonas syringae* and *Ralstonia solanacearum* (Birch, 2001; Noel et al., 2002; Angot et al., 2006).

T4SS secretion system

The type IV secretion system (T4SS) is present in both the Gram-negative and positive plant pathogenic bacteria (Wallden et al., 2010). This tranlocation system is an important system that deploy the sec gene to transport

the pathogenecity factors from the inner bacterial cell into the extracellular environment or directly into the plant host cell (Judd et al., 2005). The type IV secretion system is involved in the translocation into the plant cell of either the single stranded DNA (ssDNA), the multi subunit toxins or the monomeric proteins including the permeases. This secretion system is related to a conjugation machines. Among the most representative phytopathogenic bacteria that uses the T4SS secretion system is *Agrobacterium tumefaciens* that target the oncogenic DNA-protein complex in plant cell (Zupan et al., 2000; Juhas et al., 2008; Wallden et al., 2010).

T5SS secretion system

The type V secretion system (T5SS) is widely present among the Gram-negative bacteria (Tseng et al., 2009). This translocation system is considered as one of the simplest secretion pathway (Desvaux, 2004; Tseng et al., 2009). The T5SS translocation system is dedicated to transfer a single specific polypeptide known as the passenger domain in two step process (Moreira et al., 2004). The first step is mediated by a sec translocator across the inner membrane. The second step concerns the own transportation of the passenger through the outer membrane by forming a protected module called a β barrel (Van Sluys et al., 2002). During the translocation of passenger domain, the signal sequence can either remain on the bacterial surface or cleave and then released in the extracellular milieu. The type V secretion system can exist in two subtypes which are the autotransporters (AT) system (Type Va) and the TPS nown as AT-2. In Gram-negative bacteria, the virulence factors associated with T5SS passenger are numerous including biofilm formation, adhesins, toxins, enzymes production and cytotoxic activity (Leo at al., 2012; Jacob-Dubuisson, 2013). Among the plant pathogenic Gram-negative bacteria that involve the T5SS secretion system as pathogenicity determinant include *Xylella factidiosa,* the causative agent of Pierce's disease (Igo et al., 2007), the Xanthomonads (Van Sluys et al., 2002; Moreira et al., 2004) and *E. chrysanthemi* (Tseng et al., 2009).

T6SS secretion system

The type VI secretion system (T6SS) has been recently discovered as new mechanism for effectors transportation across the cell membrane in the Gram-negative bacteria (Bingle et al., 2008, Filloux et al., 2008; Shrivastava and Mande, 2008; Pukatzki et al., 2010). The structure of the T6SS secretion system presents a significant similarity with the bacteriophage tails which inject their effector protein proteins either directly into the host cell or in the extracellular milieu (Tseng et al., 2009; Pukazki et al., 2010). The T6SS secretion system is involved in the translocation of numerous pathogenicity determinants including the biofilm formation, the quorum sensing and antibacterial toxins. This secretion mechanism has been identified in many Gram-negative bacteria including *Agrobacterium tumificiens*, *Pectobacterium atrosepticum* and *Pseudomonas syringae* (Wu et al., 2008; Records and Gross, 2010) and *Xanthomonas oryzae* (Fillouw et al., 2008).

PECTIN DEGRADING ENZYMES

Pectin substrate is a complex polysaccharide presents in all plants in the middle lamella of primary cell wall consisting mainly of galacturonic acid residues linked with an α(1-4) glucosidic bond (Pedrolli et al., 2009; Kothari and Baig, 2013). The acid groups are largely esterified with methyl groups. Plant pathogenic bacteria are known to produce an array of inducible extracellular enzymes that degrade plant cell wall constituents (Collmer and Keen, 1986). These enzymes are thought to play a key role as virulence factors. The most enzymes in bacteria plant pathogen and fungi are those degrading the pectin substances which are also the widely studied as determinants (Collmer et al., 2002). Among the widely pectic enzymes in phytopathogenic bacteria are two important classes namely the pectate lyases (PL) and polygalacturonases (PG) (Collmer and Keen, 1986; Saile et al., 1997). The plant pathogens that secrete complexes of pectic enzymes such as the pectate lyases (PL) (Boch et al., 2002; Collmer et al., 2002) and polygalacturonases (PG) includes the soft rot Erwinias namely *E. carotovora* and *E. chrysanthemi* (Barras et al., 1994; Carpita and McCann, 2000; Collmer et al., 2002).

SIDEROPHORES

Iron is an essential element for nearly all microorganisms including the plant pathogenic bacteria as it participates in numerous process such as redox reactions, oxygen binding and as cofactors for vital enzymes (Buyer and Leong, 1986). To maintain the availability of the free iron at acceptable concentration to limits the growth of invading bacterial pathogen, the host uses two major proteins for the transport and storage of free iron including the transferins and ferritins (Dave and Dube, 2000; Gull and Hafeez, 2012). Many plant pathogenic bacteria secrete molecular weight for ferric ion (Fe^{3+}) chelate and transfer agent known as siderophores from the host then pumped in the bacteria cytosol by specific

membrane receptors (Leong and Neilands, 1981, Williams and Griffiths, 1992). Siderophores have been shown to play a major role as virulence factors for numerous plant pathogenic bacteria in plant disease. Among the compounds secreted include chrysobactin which is a catechol by *E. chrysanthemi* and *E. carotovora* (Perswerk et al., 1989; Alfano and Collmer, 2004) and the hydroxamate which is a siderophore produced by *Agrobacterium tumefaciens* (Leong and Neilands, 1981). Another iron transportation system mediated by specific proteins is known as NRAMP activated by infected plant particularly in response to biotic stress or iron limitation in plant host. The NRAMP are now known to be involved in innate immunity and to be the basic resistance for plant towards the pathogens(Expert et al., 2012; Dellagi et al., 2009).

ANTIMICROBIAL COMPOUNDS DETOXIFICATION

For the defense mechanisms, most plants produce antimicrobial compounds as secondary metabolites in response to pathogen infection (Glazebrook et al., 1997). Phytoalexins are among these antimicrobial substances which are considered as molecules at sufficient concentration that limit and reduce the growth and multiplication of pathogenic microorganisms (Hammond-Kosack and Jones, 1996). Among the major studied and illustrated compounds are pisatins in peas (VanEtten et al., 1975; Van Etten et al., 1989), saponins in oats, isoflavonoids in legumes and terpenoids in Solanaceae (Turbek et al., 1992). On the other hand, different mechanisms were described particularly in fungi which counter these antimicrobial substances. The pisatin are detoxified by cytochrome CP 450 monoxygenase (Matthews and Van Etten, 1983). Similarly, *Fusarium oxysporum f.sp. Lycopersicum* produce an inducible extracellular enzyme known as tomatinase which detoxifies the alpha tomatine. Furthermore, most Xanthomonads detoxify reactives oxygen and superoxyde species using catalases (Qian et al., 2005). Recently, two inducibles enzymes were secreted by *Pseudomonas syringae*are involved in the isothiocyanates detoxification (Fan et al., 2011).

TOXINS (PATHOTOXINS)

Plant pathogenic bacteria are known to produce a wide range of both specific and nonspecific host phytotoxins. Some are polypeptids, glycoproteins others are secondary metabolites that are required as virulence factors in plant disease (Alfano and Collmer, 2004). These toxins acts by using diverse mechanisms from modulating and suppressing plant defense response to alteration and inhibition of normal host cellular metabolic process (Thomas et al., 1983). These toxins act also directly on the expression and development of disease symptoms. Among the most well studied pathotoxins known also as phytotoxins include syringomycins, syringopeptins, tabtoxins, phaseolotoxins and coranotine described particularly in *P. syringae* pathovars(Thomas et al., 1983).

Syringomicins and syringopeptins

Both syringomycins and syringopeptins are a group of polar cyclic peptidide known as lepodepsipeptides toxins which are secreted by several pathovars of *P. syringae* (Lu et al., 2005). These toxins act by disrupting the host cell membrane forming small pores leading to the electrolyte leakage from plant cell cytoplasm inducing necrosis of plant tissue of affected plant (Blender et al., 1999).

Coronatine

Plant pathogenic coronatine is produced by several pathovars of *P. syringae* and contribute as virulence factor. Coronatine consists of two major polyetide components, the coronafacic acid and coronamic acid molecules. Coronatine share similarity in structure with jasmonic acid-isoleucine(JA-Ile) and hence mimic them (Brooks et al., 2005; Katsir et al., 2008). Coronatine plays a key role in early stage of infection by inhibition of the stomatal immune defense leading to the entry of the pathogen. This toxin counteract the pathogen associated molecular patterns (PAMPs) induced stomatal closure in both *P. syringae* and *X. compestris* (Hutchison and Gross, 1997; Gommez-Gomez and Boller, 2002). In fact, PAMPs consists of conserved components motifs that include flagellin and lipolysaccharide (LPS). These molecular patterns are recognized by plant pathogen recognition receptors (PRRs). These perception of PAMPs activates the basal defenses mechanisms in early stages of interacttion of plant pathogen interaction (Bittel and Robatzek, 2007; Melotto et al., 2008; Nurnberger and Kemmerling, 2009). On the other hand, these pathotoxin also contribute to expression of other diseases including the chlorosis symptoms, hypertrophy and lesion formation (Sekai et al., 1979; Brooks et al., 2005). Another well studied class of siderophores, are those synthetized by fluorescent pseudomonads. Pyoverdine play a key role in controlling iron availability in the rhizosphere (Visca et al., 2002; Expert et al., 2012). On the other hand, the pyoverdine was recently identified as virulence factors in *P. syringae* pv.tabaci (Tagushi et al., 2010).

Phaseolotoxins

Phaseolotoxins are synthetized by different pathovars of

Pseudomonas syringae including the pathovars phaseolicola and actinidia. The phaseolotoxin is a tripeptide which is hydrolyzed to produce an octicidine metabolite that is an irreversible inhibitor of ornithine carbonoyl transferase (OCTase) (Arrebola et al., 2003; Melotto and Kunkel, 2013). The OCTase enzyme is considered to play a major role in the urea cycle of the plant (Arrebola et al., 2003).

The tabotoxinine beta lactam (TBL), the active form of the tabotoxine is produced in the host plant after hydrolosis with an aminopeptidase (Moore et al., 1984; Arrebola et al., 2007). The TBL pathotoxin plays a key role as an inhibitor of the glutamic synthetase (Thomas et al., 1983). Both the tabtoxines and phaseolotoxins contribute to the virulence of the *P. syringae* by inhibiting the host response defense at early stage and by inhibiting the photosynthesis process leading to severe chlorosis of the affected plant tissues (Arrebola et al., 2007).

ADHESINS AND EXTRACELLULAR POLYSACCHARIDES

Adhesins are considered as biomolecules such as proteins and glycoproteins that mediate the binding of the bacteria to the host cell (Katzen et al., 1996). The adherence is the first step interaction between the pathogen and the plant host which lead to the attachment and colonization of foliage or root tissues of the host plant (Kao et al., 1992; Alfano and Collmer, 1996). The plant pathogenic bacteria utilize several types of adhesins including a proteinous fimbrial or non fimbrial adhesions. Another group of adhesins which play key role in numerous plant pathogen interaction are the exopolysaccharides (EPS) (Kim et al., 2003; Melotto and Kunkel, 2013). The EPS are carbohydrate compounds secreted and maintained tightly associated with the bacterial capsule or released around the bacterial matrix. The importance of adhesins as virulence factors has been studies in numerous plant pathogens. Hence, *X. compestris* produce a major exopolysaccharide known as Xanthan gum implicated in infection (Denny, 1995; Melotto and Kunkel, 2013). The EPS amylovorin is another example of adhesins produced by *E. amylovora*, the causal agent of fire blight. *P. syringae*, the causal agent of several plant diseases produces different EPS such as alginate, levan (Denny, 1995). Proteinous fimbrial adhesins are also implicated in the infection caused by *P. syringae* (Yu et al., 1999).

QUORUM SENSING AND BIOFILM PRODUCTION

Quorum sensing is a bacterial communication mechanism that regulates the density of microbial population using the gene expression in response to the environmental and chemical sensing system (Kanda et al., 2011; Melotto and Kunkel, 2013). The signal molecules known as autoinducers that are detected by different bound receptors of bacterial cells are produced in coordinate manner at a specific bacterial stage such as disease physiological function including epiphytic growth, competition or colonization and virulence stage (von Bodman et al., 2003; Kanda et al., 2011). Quorum-sensing signal N-acyl homoserine lactones are known to regulate numerous virulence factors including enzymes production and exopolysaccharides in many plant pathogenic bacteria (Teplitski et al., 2000). Among the quorum-sensing regulator detected in *P. syringae* PsrA, is Pel regulator in *E. chrysanthemi.* However, a series of reglators namely MqsR, QseBC and exporter TqsA, could be present in *E. amylovora* (Hugouvieux-Cotte-Pattat et al., 1992).

Biofilm is a complex multilayer cellular structure attached to an inanimate surface or tissues and embedded within an exopolysaccharide material (Welch et al., 2000; Dow et al., 2003). Biofilm provides a protection for bacterial cell from a wide range of hostile and extreme environmental conditions including deshydratation, extreme pH and UV radiation (Welch et al., 2000; Melotto and Kunkel, 2013).

Biofilm also shield bacteria cell from host immune response and antimicrobials compounds (Dow et al., 2003). Several plant pathogenic bacteria have been considered as biofilm producer as virulence factors including *X. compestris* (Dow et al., 2003) and *P. syringae* (Keith et al., 2003).

CONCLUSION

Based on several advances in literature on bacterial disease, it is clear that plant bacteria expresses virulence factors in each specific stage of pathogenesis. The virulence of plant pathogens is a multifactorial phenomenon which involves host-pathogen interactions that must be largely explored. In this review, we summarized the major bacterial virulence determinants that are required for establishing infection and disease development. On the other hand, an efficient strategy for bacterial disease control needs further studies of the virulence factors at the molecular levels in order to know their contribution in the plant pathogen interaction.

Conflict of Interest

The authors have not declared any conflict of interest.

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Review

Alternaria blight of oilseed Brassicas: A comprehensive review

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Oilseed brassicas also known as rapeseed-mustard is an important group of oilseed crop in the world. These crops are susceptible to a number of diseases caused by biotic and mesobiotic pathogens. Among various diseases, Alternaria leaf blight also known as Alternaria dark spot is the most destructive disease of oilseed brassicas species in all the continents. This disease is known to be incited by *Alternaria brassicae*, *Alternaria brassicicola* and *Alternaria raphani* singly or by mixed infection. Alternaria leaf spot pathogens are necrotrophs and produces lesions surrounded by chlorotic areas on leaves, stems and siliquae causing reduction in the photosynthetic areas, defoliation, and early induction of senescence. Alternaria blight causes considerable reduction in quantity and quality of harvested brassica products. The Alternaria leaf blight pathogens are seedborne, soilborne and airborne. The pathogens are greatly influenced by weather with the highest disease incidence reported in wet seasons and in areas with relatively high rainfall. The concentration of conidia, age of the host plants, and wetness period on leaves also influence the severity of the disease. This paper reviews the research and development of Alternaria blight in the oilseed brassicas (rapeseed-mustard) during the past years in relation to pathogen taxonomy, biology, epidemiology, host pathogen interaction and management through chemicals, botanicals, biological, cultural, and biotechnological approaches. The paper also attempts to present future outlook and strategy for Alternaria blight of rapeseed-mustard research.

Key words: Alternaria blight, rapeseed-mustard, symptoms, variability, pathogen, survival, management.

INTRODUCTION

Oilseed brassicas often called rapeseed-mustard is the third most important oilseed commodity in the world after soybean (*Glycine max*) and palm (*Elaeis guineensis* Jacq.) in world agriculture and India is the third largest producer with global contribution of 28.3% acreage and 19.8% production (Shekhawat et al., 2012; Bandopadhyay et al., 2013). Among the oilseed brassicas, mustard (*Brassica juncea*), yellow sarson (*Brassica campestris* var. yellow *sarson*), brown sarson (*Brassica campestris* var. brown *sarson*), toria (*Brassica campestris* var. toria), oilseed rape (*Brassica napus*), and Karan rai (*Brassica carinata*) are grown for edible oil, whereas black mustard

(*Brassica nigra*) is used as a condiment and for pickle making. The leaves of the young plants are used in the human diet as a green vegetable. The oilseed brassicas usually contain 38-57% of erucic acid, 4.7-13% of linolenic acid and 27% of oleic and linoleic acids, which are of high nutritive value required for human health. Oilseed brassicas is gaining importance globally due to its advantage over other oilseeds namely: high yield potential, low moisture requirement, higher return at low cost production, and wider adaptability for various farming conditions which hold promise towards having the next yellow revolution (Kumar, 2012).

Oilseed brassicas are exposed to various pathogens, which infect and disturb the normal physiological functions during growth and development. Among the diseases that hampered the productivity of oilseed brassicas, Alternaria blight is most recognized disease worldwide. The disease is also known as Alternaria black spot or dark spot disease in Europe and Canada (Degenhardt et al., 1974). The Alternaria leaf spot disease incited by *A. brassicae* is more destructive and occurs more frequently than the one caused by other two species namely *Alternaria brassicicola* and *Alternaria raphani*. The disease occurs in Canada (Petrie, 1973, 1974; McDonald, 1959), England (Loof and Appleqvist, 1972; Evans 1983), France (Loof and Appleqvist, 1972), Germany (Borg, 1952), Holand (Flik and Saaltink, 1950), India (Dey, 1949; Mukadam and Despandey, 1977; Kolte and Tiwari, 1978; Vasudeva, 1958), Poland (Francel, 1983), SriLanka (Bond, 1947), Spain (Romero and Jimenez Diaz, 1980), Sweden (Loof and Appleqvist, 1972), Australia (Sivapalan and Browning, 1992), USA (Babadoost and Gabrielson, 1979) and Trinidad (Fajardo and Palo, 1934).

The disease appears as brown or greyish spots on leaves, stems, and on siliquae during ripening stage. Alternaria blight causes substantial yield losses as a result of several factors including reduced photosynthetic potential, early defoliation, flower-bud abortion, premature ripening, siliquae dehiscence, seed shriveling (Seidle et al., 1995), and reduced seed size and impairs seed color and oil content (Kaushik et al., 1984).This review describes the pathogens of Alternaria blight of oilseed brassicas, epidemiology, host pathogen interaction and management through various approaches.

THE PATHOGEN

Alternaria leaf blight of oilseed brassicas is known to be incited by three species namely *Alternaria brassicae* (Berk.) Sacc., *Alternaria brassicicola* (Schw.) Wiltshire., and *Alternaria raphani* Groves and Skolko (Jasalavich et al., 1995; Saharan and Mehta, 2002). The genus Alternaria belongs to the phylum Ascomycota which consists of both saprophytic and pathogenic species. Alternaria belongs to the class Dothideomycetes, order Pleosporales, and family Pleosporaceae. *Alternaria* spp. is characterized by formation of polymorphous conidia either singly or in short or longer chains with longitudinal and transverse septa with long or short beaks. Among these species, *A. brassicae* is the most destructive and occurs more frequently in many parts of world. The *A. brassicicola* is also cosmopolitan in distribution and may cause the infection simultaneously with *A. brassicae* on the same plants. *A. raphani* is less destructive than the other two species but it is the most common in Canada. *A. raphani* has been also reported from Denmark, Egypt, Greece, India, Japan, Netherlands and USA. (Saharan and Mehta, 2002). Khan et al. (1998) reported 26.5% infection by *A. brassicicola* and 22.6% by *A. brassicae* whereas the rest 50.9% is accounted for concomitant infection of *A. brassicae* and *A. brassicicola*. The taxonomy of Alternaria is based primarily on the morphology and development of conidia and conidiophore, and to a lesser degree on host plant association and colony morphology (Elliott, 1917; Wiltshire, 1933; Simmons, 1967). The morphological, cultural and physiological characteristics, genetic diversity and virulence-associated genes of these three species are as follows:

Alternaria brassicae

The mycelium of *A. brassicae* is septate, brown to brownish grey in colour. The conidiophores are dark, septate, arise in fascicles, measuring 14-74 × 4-8 µm. Conidia are brownish black, obclavate, borne singly or sparingly in chains of 2-4, muriform with long beak and the overall conidial size ranges between 148-184 × 17-24 µm with 10-11 transverse and 0-6 longitudinal septa. This species represent slow and rudimentary growth in media and forms chlamydospores in less frequency (Kolte, 1985). Sporulation occurs between the temperatures of 8-24°C but optimum temperatures range between 16-24°C. *A. brassicae* germinates over a wide range of temperature, however, germination occurs most quickly, when the temperature is between 21-28°C. As the temperature decreases, the time period it takes for germination increases (Degenhardt et al., 1982).Sharma et al. (2013) studied the 32 Indian isolates of *A. brassicae* and found that colony of the isolates on PDA varied between light olive gray to olivaceous black whereas mycelia colour varied between brown to golden. Most of the conidia were long obpyriform in shape with long beak and colour was found golden or brown with mostly smooth surface. Sporulation of each isolates on the different

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media was found almost similar. All isolates studied by Sharma et al. (2013) were pathogenic in nature but not directly related to the cultural and the morphological characteristics. Pramila et al. (2014) studied the 10 Indian isolates of *A. brassicae* and grouped the isolates based on cultural characteristics in three groups. Group 1 isolates produces circular white colonies with a fluffy appearance with smooth colony margins. Group 2 isolates have off white colony with a feathery appearance and is circular with all type of margins. Group 3 isolates having light brown colony with cottony appearance and colony are circular in shape with wavy and rough margins. Pramila et al. (2014) also reported that different isolates of *A. brassicae* showed variable pathogenic response on host *B. juncea* cultivar Divya.

A temperature range of 25 to 30°C and 15 to 35°C was found optimum for mycelial growth and sporulation of *A. brassicae*, respectively. Mycelial growth was most favoured by 100% relative humidity with a gradual reduction in growth and sporulation till 70% RH and a decrease in growth and sporulation at 60 and 50% RH (Meena et al., 2012). The most favourable temperature for sporulation of *A. brassicae* had earlier worked out as 23-25°C by Kadian and Saharan (1984) and Ansari et al. (1989) which indicates the adaptability of this fungus to varied environmental conditions (Meena et al., 2012). Polymorphism within an *Alternaria* species by RAPD molecular marker has been described by many workers (Sharma and Tewari, 1995; Sharma and Tewari, 1998; Kumar et al., 2008). Sharma and Tewari (1995) observed polymorphism among *A. brassicae* isolates from different geographical regions of the world. However, Sharma and Tewari (1998) found low intra-regional variation among Indian and Canadian isolates of *A. brassicae* with 75% similarity among them. Sharma et al. (2013) molecularly characterized the 32 Indian isolates by using transcribed spacer region primers ITS1- 5’ TCC GTA GGT GAA CCT GCG 3’ and ITS4- 5’ TCC TCC GCT TAT TCA TAT GC 3’ and found the PCR products of amplicons of ~550 to 600 bp. Analysis of ITS revealed *A. brassicae* isolates were 56% similar to each other and 99% similar to the *A. brassicae* isolates in NCBI database. Pramila et al. (2014) revealed high degree of genetic variability among ten isolates of *A. brassicae* of Pantnagar region of Uttarakhand, India by 26 RAPD primers indicating the existence of different strains of pathogen. Various studies indicates high genetic divergence among isolates of *A. brassicae*. Although, the genus *Alternaria* is known as an imperfect fungus, it shows genetic variability within a species and this variability might be due to the existence of mutation, somatic hybridization, heterokaryosis, uniform host selection, extensive dispersal or of a cryptic sexual stage. This could be the probable possible reason behind extreme and different disease reaction of germplasm at Pantnagar from observations at most of other locations. In order to provide a better picture of the pathogenic as well as genetic divergence among *A. brassicae* populations of India, there is need to conduct similar holistic investigation among higher number of *A. brassicae* isolates which could be helpful to generate resistant material against *Alternaria* blight in oilseed Brassicas (Pramila et al., 2014).

A. brassicicola

Mycelium of necrotrophic fungus *A. brassicicola* is septate, olive grey to greyish black in colour. The conidiophores are olivaceous, septate, branched, measuring 35-45 µm in length and 5-8 µm in width. Conidia are dark, cylindrical to oblong, muriform without beak measuring 44-55 µm in length and 11-16 µm in width with 5-8 transverse and 0-4 transverse septa. The fungus grows faster in media with high sporulation and appears as well developed black sooty colony with distinct zonations (Kolte, 1985). *A. brassicicola* sporulates at a temperature range of 8 to 30°C. Optimum temperatures for sporulation is between 18 and 30°C where the average sporulation time is 13 h. *A. brassicicola* germinates at higher temperatures. *A. brassicicola* begins to germinate 98% of its spores at 15°C after 10 h of incubation whereas 98% germination occurs after 3 h at 31°C (Degenhardt et al., 1982). Plants inoculated with *A. brassicicola* develop symptoms most quickly at 25°C, while seedlings from infected seeds develop symptoms most quickly at 30°C. No germination occurs at 3°C for all three pathogens namely *A. brassicicola, A. brassicae and A. raphani* (Bassey and Gabrielson, 1983).

AFLP found reliable tool for identifying *A. brassicicola* because of clear polymorphism both within and between species. The analysis of eighteen isolates of *A. brassocicola* raveled moderate levels of genetic diversity within species. The number of polymorphic loci and the percentage-shared bands indicated genetic differences between species. Within the *A. brassicicola* group, 16.7-27.9% of the loci were polymorphic, while the remainder were monomorphic. The genetic distance between the isolates of *A. brassicicola* ranged from 0.00 -0.04 suggesting that they are closely related (Bock et al., 2002). Teleomorphs of most of the *Alternaria* spp. do not exist or remain unidentified (Simmons, 1978). Many Alternarias are therefore likely to be haploid fungi existing in a vegetative phase, reproducing asexually, and would be expected to have a high level of clonality (Vogler et al., 1991).

During host infection, *A. brassicicola* is exposed to high levels of defense compounds, such as phytoalexins and glucosinolate breakdown products, and the ability to overcome these antimicrobial metabolites is a key factor in determining fungal virulence (Pochon et al., 2013). The *A. brassicicola* genome size is approximately 31.9 Mb. (Ohm et al., 2012). Several genes in *A. brassicicola* linked to the pathogenesis have been reported (Cho et al., 2007, 2009a; Craven et al., 2008; Kim et al., 2007,

2009; Oide et al., 2006; Srivastava et al., 2012). One of the genes of major interest has been a transcription factor, *AbSte 12* that is controlled by *Amk1* in *A. brassicicola* (Cho et al., 2007). The deletion mutant for *AbSte 12* gene showed pleiotropic phenotypes, including the inability to produce mature conidia, slight reduction of vegetative growth rates and complete loss of pathogenicity. Two novel virulence factors encoding a transcription factor *AbPro1* and two component histidine kinase gene *AbNIK1* were discovered. Deletion of *AbPro1* resulted in a 70% reduction in virulence and a 25% reduction in vegetative growth rates *in vitro*. Deletion of *AbNIK1* resulted in a near complete loss of virulence, increased sensitivity to osmotic stress and no changes in vegetative growth rates *in vitro* (Cho et al., 2009b). Cho et al. (2012) reported transcription factor gene, *Amr1*, which negatively regulates a subset of these genes during late-stage pathogenesis and positively regulates melanin biosynthesis during conidiogenesis. *Amr1* is a homolog of *Cmr1*, a transcription factor that regulates melanin biosynthesis in several fungi. The deletion ∆*amr1* mutants used pectin as a carbon source more efficiently than the wild type *A. brassicicola,* where melanin is deficient and more sensitive to UV light and glucanase digestion. RNA-seq analysis revealed that three genes in the melanin biosynthesis pathway, along with the deleted *Amr1* gene, were expressed at low levels in the mutants. In contrast, many hydrolytic enzyme-coding genes were expressed at higher levels in the mutants than in the wild type during pathogenesis. The increase in virulence of the deletion mutants of *amr1* suggested that the loss of the gene was beneficial to pathogenesis. The results of this study suggested that a gene important for survival in nature negatively affected virulence, probably by a less efficient use of plant cell-wall materials. Cho et al*.,* (2012) speculated that the functions of the *Amr1* gene are important for the success of *A. brassicicola* as a competitive saprophyte and plant parasite.

Dehydrins belong to the late embryogenesis-abundant (LEA) protein family believed to play a role in the protection against cold- and dehydration-related stresses. Pochon et al. (2013) studied the role of fungal dyhydrin like proteins in pathogenicity and protection against environmental stresses in *A. brassicicola*. Three dehydrin protein encoding genes called AbDhn1, AbDhn2 and AbDhn3 were identified in the *A. brassicicola* genome. The expression of these dehydrin gene was induced in response to various stresses and found to be regulated by the AbHog1 MAP Kinase pathway. They showed that dehydrin-like proteins have an impact mainly on oxidative stress tolerance and on conidial survival upon exposure to high and freezing temperatures. They also revealed that the double deletion mutant ∆∆*abdhn1-abdhn2* was highly compromised in its pathogenicity. By comparison to the wild-type, this mutant exhibited lower aggressiveness on *Brassica oleracea* leaves. The double mutant was also affected with respect to conidiation, another crucial step in the epidemiology of the disease.

A. raphani

Mycelium is cottony whitish to greenish grey, which become dark olive because of ageing. The conidiophores are septate, olive brown, single or branched, 29-160×4-8 µm in size. Conidia are muriform with poorly developed or no beak, olive brown to dark, obclavate and less uniform in shape in comparison with *A. brassicae* and *A. brassicicola*. Conidia are more or less pointed at each end and appear singly or in chains of upto six spores. The overall length of conidia ranges between 60-83 µm in length and 13-21 µm in width with 6-9 transverse and 3-6 longitudinal septa. The fungus appears as cottony colonies on the media with less sporulation. The fungus produces olive brownish chlamydospores in culture as well as on the partially decayed affected plant part (Kolte, 1985). Conidia of *A. raphani* germinate at a temperature range of 7 to 31°C. The optimum temperature for *A. raphani* is 23°C or greater, where, 98% of the spores germinate after 6 h of incubation. The lowest temperature where 98% of the spores germinate is at 13°C, which requires 10 h of incubation for germination (Degenhardt et al., 1982).

Jasalavich et al. (1995) studied the nuclear 18s rRNA, 5.8s rRNA and the internal transcribed spacers (ITS1 and ITS2) sequence of *A. raphani, A. brassicae*, *A. brassicicola, A. alternata* and *Pleospora herbarum*. The 5.8s rDNA sequences from the *A. raphani, A. brassicae*, *A. brassicicola, A. alternata* were identical and differed at only one base pair from that of *P. herbarum*. The internal transcribed spacer sequences, especially ITS1, were very variable in both base composition and length. The 18s rDNA sequences were highly conserved, but enough variability was present to distinguish genera clearly. Phylogenetic analysis of the sequence data sets by both parsimony and maximum likelihood methods clearly separated genera and species. All the *Alternaria* species were closely related. Pleospora also appeared to be more closely related to Alternaria than to leptosphaeria. Based on the taxonomy, Wiltshire (1947) considered *A. raphani* and *A. brassicae* to be closely related. Jasalavich et al. (1995) found that *A. brassicicola* is actually more closely related to *A. raphani* than to *A.brassicae* based on the rDNA sequences data. *A. alternata*, *A. brassicae*, *A. brassicicola* and *A. raphani* formed clade of very closely related sister taxa. The 18s rDNA resolved two subclades of species within Alternaria at a level of confidence of 98%, based on five informative sites contained within the 300 bp at the 3'-end of the sequences alignment. Complete resolution of the species of Alternaria was achieved with the ITS sequence data which were much variable and contained more phylogenetically informative sites than the 18s rDNA. The study indicates that *A. raphani, A. brassicae*, *A. brassicicola,*

A. alternate encompasses less genetic variability.

Chou and Wu (2002) studied the phylogenetic analysis of internal transcribed spacer regions (ITS 1and ITS 2) of different fungi and they got positioned filament beaked Alternaria as a monophyletic group discrete from the other members of genus Alternaria. Filament beaked *Alternaria* spp. formed a well supported group. The second group consisted of small spored *A. brassicicola and A. raphani* and the large spored *A. brassicae*. This study also indicates less genetic diversity in Alternaria species causing Alternaria leaf blight on rapeseed-mustard.

HISTORY OF *ALTERNARIA* AND ALTERNARIA LEAF SPOT

Nees first described the genus *Alternaria* in 1817 with type species *Alternaria tenuis*, which was later renamed as *A. alternata*. Berkeley (1836) noticed fungal infection on plant belonging to the family Brassicaceae and identified this fungus as *Macrosporium brassicae* Berk., which was later renamed as *A. brassicae* (Berk.) Sacc by Saccardo (1886). In India, the Alternaria blight was first observed on sarson from Tirhoot in 1901 (Butler, 1918) but the fungus was thought to be new and described as *Sporodochium brassicae* Mass. Later, Mason (1928) first observed the *Alternaria* spp. from a herbarium material of sarson from Pusa (Bihar) India. Elliott (1917) and Wiltshire (1933) studied the taxonomy of *Alternaria* in detail. Later, Neergaard (1945) made a detailed study of the taxonomy, parasitism and economic significance of *Alternaria* genus. Joly (1959) described the morphological variations in *Alternaria* spp. and later he divided these into three sections and gave a key for identification of most common species of *Alternaria* genus (Joly, 1964). Subramanian (1971) described the Indian species of *Alternaria* in detail. The morphological characteristics of various *Alternaria* spp. are described in Dematiaceous Hyphomycetes (Ellis, 1971) and in more Dematiaceous Hyphomycetes (Ellis, 1976). Simmons (2007) compiled his lifetime work in his book *Alternaria*: An Identification Manual in which he described the taxonomy, nomenclature and classification to facilitate accurate identification of species of *Alternaria*.

YIELD LOSSES

Alternaria blight occurs every year in all the rapeseed-mustard growing areas of the world. This disease causes an average yield loss of 46-47% in yellow *sarson* and 35-38% in mustard (Kolte, 1985a, b; Kolte et al., 1987, 2002; Chattopadhyay, 2008) and even up to 70% in *Brassicas* species. In Canada, 20 to 30% yield losses were recorded due to this disease (McDonald, 1959; Conn et al., 1990). In India, losses of 15 to 71% were reported by different workers (Kadian and Saharan, 1984; Singh and Bhowmik, 1985; Kumar, 1986; Ram and Chauhan, 1998). Kolte et al. (1987) reported that the disease causes losses in 1000-seed weight (g) of yellow sarson and mustard of 23 and 24%, respectively. In addition to quantitative loss, seed quality in terms of seed size, seed color and oil contents are also reduced due to the fungus infection (Kaushik et al., 1984; Kumar, 1997). Reduction in oil content up to 4.8% have been reported by Degenhardt et al.(1974) but Ansari et al.(1988) reported the reduction in oil content of rapeseed cultivars between 14.58 and 35.97% and 14.12 - 29.07% in mustard cultivars in India. Rotem (1994) stated that Alternaria black spot could be a devastating disease resulting in 25-50% yield reductions in crops such as canola or rape.

DISEASE SYMPTOM

A. brassicae, *A. brassicicola*, and *A. raphani* cause more or less similar symptoms on leaves, stem and siliquae of oilseed brassicas. Spots produced by *A. brassicae* appear to be usually grey in color when compared with black sooty velvety spots produced by *A. brassicicola*. Spots induced in response to *A. raphni* showed distinct yellow hallow around them. However, the symptoms may vary with the host and environment (Meena et al., 2010). Symptoms are first visible with appearance of black points. Later, these spots enlarge and develop in to prominent round spots with concentric rings showing target board characteristic of the spot. Many spots coalesce to form large patches and causing blighting and defoliation of the leaves. In some Brassicas species, formation of constricting rings in the lesion and zone of yellow halo around the lesions are very prominent (Saharan and Mehta, 2002). Disease symptoms often occurs on the older leaves, since they are closer to the soil and are more readily infected as a consequence of windblown or rain-splashed spores. At the later stage of plant growth, symptom of the disease also develops elongated spots without concentric rings on stem and siliquaeas. Deep lesions on the siliquae cause infection in the seeds. Alternaria spot on leaves and siliquae reduces the photosynthetic area drastically and cause the formation of the small, discoloured and shrivelled seeds. Alternaria blight adversely affects the oil content in seed and quality of the seed (Meena et al., 2010) (Figure 1).

HOST AND PATHOGEN INTERACTION

Fungi infect their hosts in a sequence of events including adhesion to the host surface, followed by penetration and growth into the host tissue. Alternaria leaf spots start to initiate after landing conidia of *A. brassicae*, *A. brassicicola* and *A. raphani* on the host surface. After adhesion on the host surface, conidium germinates in the presence of moisture readily by giving rise to a germ tube

Figure 1. a: Symptoms of leaf spot caused by *Alternaria brassicae* on mustard; b: Symptoms of leaf spot caused by *Alternaria brassicicola* on mustard; c: Symptoms of leaf spot caused by *Alternaria raphani*on mustard; d: Large necrotic patches on mustard leaf after merging of spots; e: Symptoms of *Alternaria* spp. on the pods of *Brassica juncea*; f: Symptoms of *Alternaria* spp. on the pods of *B. campestris* var. yellow *sarson*; g: Concentric ring type of spot of *Alternaria* spp. on pod of *B. campestris* var. yellow *sarson;* h: Dark black symptoms of *Alternaria* leaf blight pathogens on stem of mustard; i: Concentric ring type of symptoms of Alternaria leaf spot on the stem of *B. campestris* var. yellow *sarson*; j: Conidia of *Alternaria brassicae* (600×); k: Conidia of *Alternaria brassicicola* (1050×); l: Conidia of *Alternaria raphani* (1000×).

and appressorium develops from the end of a fungal germ tube and grow into the underlying epidermal cell using a penetration peg. Pathogens undergo morphological changes during the initial stages of pathogenesis leading to the formation of infection structures accompanied by physiological changes to support the

penetration process (Cho et al., 2009). Morphogenesis and physiological alteration are often triggered by signals provided by the host plant (Yang et al., 2005). After growth of the fungal hyphae in the host plant, a typical necrotic lesion develops on the surface of the host leaves. Eventually, aerial conidiophores differentiate from hyphae within the lesions, releasing thousands of new conidia. Since, Alternaria leaf spot is a polycyclic plant disease, the conidia servers as secondary inoculum for the next infection cycles. Alternaria lesions often occurs on the older leaves, since they are closer to the soil and are more readily infected as consequence of rain splash or windblown rain. Since *Alternaria* is a necrotroph, it must kill the host plant's cell to survive. This is accomplished by secretion of toxins consisting of secondary metabolites and proteins. These toxins are host specific and non-host specific in nature which imparts the ability to the pathogen to infect wide range of hosts (Kohmato et al., 1995; Nishimura and Kohmoto, 1983; Agrios, 2005).

TOXINS OF *A. BRASSICAE* AND *A. BRASSICICOLA*

Host-pathogen factors play a crucial role in the initiation and severity of the diseases. Toxins released by the pathogens during pathogenesis are one of such factors. Many pathogenic species of *Alternaria* are prolific toxin producers which facilitate their necrotrophic life style as two step process: Killing host cell directly (Necrosis) or inducing programmed cell death with toxins, then decomposing host tissue with cell wall degrading enzymes (Cho et al., 2012). *A. brassicae* is known to produce four phytotoxins, which are low molecular weight cyclodepsipeptides named destruxins (Agarwal et al., 1994; Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987; Buchwaldt and Jensen, 1991; Tewari and Bains, 1997). Destruxin B is the major phytotoxin produced by the pathogen in liquid culture, and other three namely homodestruxin B, destruxin B_2, and desmethyl destruxin are produced in less amount (Tewari and Bains, 1997). Bains and Tewari (1987) reported that destruxin B is toxic to Brassica but not to non-host and they classified destruxin B as host specific toxin. In contrast, Buchwaldt and Green (1992) observed the non-hostspecific toxicity of destruxin B. Parada et al. (2007) studied the host specificity of destruxin B and indicated its non-host specific nature supporting the result of Buchwaldt and Green (1992). Bains et al. (1993) worked on the host specificity of homodestruxin B and observed the symptom of different severities in leaves of various non-host plants and suggested that homodestruxin B is non-host specific toxin. Host selective toxins are toxic only to the host plant, and have an important role in pathogenesis as primary determinants of virulence or pathogenicity. The majority of the host specific toxins are low molecular weight secondary metabolites belonging to various classes of chemical compounds and have been extracted from liquid cultures. However, production of toxins by germinating spores on host plants suggests the very early participation of toxins at the site of initial contact of pathogen on plant surface (Nishimura and Kohmoto, 1979, 1983). In search of host specificity of *Alternaria* toxin on brassicas plants, Otani et al. (1998) reported the proteinaceous AB toxin in spore germination fluids of *A. brassicicola* on brassica plants. In addition to AB toxin, *A. brassicicola* produces other toxic substances including despipeptides and fucicoccin like compounds (Cooke et al., 1997; MacKinnon et al., 1999; Mckenzie et al., 1988). Parada et al. (2008) observed that *A. brassicae* produces a new host specific toxin named ABR toxin in spore germination fluids on host plants and induce water soaked symptoms followed by chlorosis only in Brassica leaves. Unlike other toxins reported to be produced by *A. brassicae*, ABR toxin appeared to be a protein. ABR toxin at the concentration of 0.5 to 1.0 µg/ml produced water soaked symptoms followed by chlorosis on brassicas leaves while non- host leaves were not affected even at 50 µg/ml indicating the host specific toxicity of ABR toxin. These results indicated the ABR toxin from *A. brassicae* fits the criteria of host specific toxin and plays a key role as a disease determinant of *A. brassicae*. *A. brassicicola* and *A.brassicae* pathogenic to brassicas have a similar host range and AB and ABR toxin have similar host specificity but these toxins are different in their molecular weight. In recent studies, Brassicicolin A emerged as most selective phytotoxic metabolite produced in liquid culture of *A. brassicicola* (Pedras et al., 2009). Toxic metabolites released by the *A. brassicae* and *A. brassicicola* are most important factor in interaction between host and pathogen. Homodestruxin B and Destruxin B caused chlorosis and necrosis similar to that caused by *A. brassicae* and phytotoxicity of these toxins are similar (Buchwaldt and Green, 1992). Homodestruxin B may play a minor role in black spot disease when compared with that of destruxin B, because it is produced in much smaller quantities (Ayer and Pena-Rodriguez, 1987; Buchwaldt and Jensen, 1991). AB and ABR toxin cause water soaked symptoms followed by chlorosis only in Brassicas leaves indicating the importance of these toxins. The role of the host specific toxins in initial colonization is not to kill host cells prior to penetration but to predispose the host cell beneath germinating spores to accept penetration. Toxin also induces accessibility of the host plant to fungal invasion (Parada et al., 2008). There is no report so far on toxin production by *A. raphani.*

ENVIRONMENTAL INFLUENCE ON THE PATHOGEN BIOLOGY AND ALTERNARIA LEAF SPOT

The ideal weather condition required for adhesion, germination, penetration and establishment of pathogen in host plays a major role in the development of disease. Other factors such as density of airborne conidia (Humpherson-Jones and Ainsworth, 1982), plant age

(Awasthi and Kolte, 1994), temperature, humidity and wetness period (Hong and Fitt, 1995) are reported to influence the severity of Alternaria leaf blight. The greater density of pathogen inoculum within or near fields of host plants reaches the hosts and increases the chances of an epidemic greatly. Moisture not only promotes new succulent and susceptible growth in the host, but, more importantly, it increases sporulation of pathogenic fungi. The most common effect of temperature on epidemics, however, is its effect on the pathogen during the different stages of pathogenesis, that is, spore germination, host penetration, pathogen growth or reproduction, invasion of the host, and sporulation. Plants change in their reaction to disease with age. The change of resistance with age is known as ontogenic resistance (Agrios, 2005). The susceptibility in oilseed brassicas increase with the age of the host plant (Saharan and Mehta, 2002)

For infection, a minimum period of 4 h of leaf wetness is required to cause infection in the host plant. Increase in the period of leaf wetness at 25°C increases infection and spread of the disease rapidly (Saharan and Mehta, 2002). Degenhardt et al. (1982) reported that *A. brassicae* germinates more quickly at 21-28°C. Saharan and Mehta (2002) reported congenial factor for germination of Alternaria spores are darkness or low light intensity, 25°C temperature and more than 90% relative humidity. *A. raphani* and *A. brassicicola* germinate at a range of 7-31°C but the optimum temperature for *A. raphnai* is 23°C or greater. At 13°C, 98% conidia germinate after 10 h of incubation. *A. brassicicola* conidia begin to germinate 98% at 15°C after 10 h of incubation whereas the conidia take only 3 h for germination at 31°C. Conidia of all three species are unable to germinate at 35°C. Kadian and Saharan (1984) reported that darkness or low light intensity with 25°C temperature and 90% humidity is optimum for conidial germination of *A. brassicae*. Continuous moisture of 24 h or longer practically guarantees infection (Chupp and Sherf, 1960; Rangel, 1945). Spread of the disease is mainly by the rain and wind dislodged spores. Optimum conditions for sporulation and infection include a minimum wet period of 13 h and ambient temperatures between 20-30°C (Humpherson-Jones and Phelps, 1989; Rotem,1994). Relative humidity of 91.5% (at 20°C) or higher will result in the production of large number of mature spores in 24 h. Sporulation of *A. brassicae* has been reported to be favoured by darkness. Moisture in the presence of rain, dew, or high humidity is essential for infection, and a minimum of 9 -18 h is required for *A. brassicae* and *A.brassicicola* in oil rape and cabbage (Humpherson-Jones and Phelps, 1989).

SOURCES OF INOCULUM

Seed

Siliquae are also infected by the Alternaria blight pathogens at late stage of plant growth. Hence, infected seeds with spores on their coat or mycelium under their seed coats could be the main source of transport for these pathogens (Shrestha et al., 2000). *A. brassicicola* survives as dormant mycelium or conidia in or out of the seed coat (Richardson, 1970; Petrie, 1974; Neergaard, 1977; Knox-Davies, 1979). Seed borne inoculum plays a role in disease cycle in temperate climate whereas it fails to survive in tropical regions (Awasthi and Kolte, 1994). *A. brassicae* predominantly found in the seed coat and rarely in embryos of rapeseed-mustard and causes lesion developments on the cotyledonary leaves and then in the first true leaves (Shrestha et al., 2000). Shrestha et al. (2003) reported the survival of *A. brassicae* in seeds stored in room temperatures (11-25°C) for 10 and 6 months at 30°C. Some workers reported that the inoculum present on the seed get eliminated in hot period and do not serve as a primary source of inoculum in plains. Shrestha et al. (2003) studied the importance of survival of the fungus in the seeds at low temperature and concluded that infected seeds act as a source of primary inoculum in Nepal.

Crop residue

Infected crops left on the soil after harvest also serves as a source of inoculum for *A. brassicae* and *A. brassicicola.* Humpherson-Jones (1989) observed that infected leaves of oilseed rape placed outdoors on soil produced viable spores for up to 8 weeks, as leaf tissues remained intact. On leaves exposed in November and January, spore concentration decreased with the time but on leaves exposed between April and June, spores concentrations increased up to 9 fold in the first 4-6 weeks and then declined. On stem section of seeds plants of oilseed rape and cabbage similarly placed on the soil, the fungi produced viable spores for up to 23 weeks with spore concentration increasing up to 11 fold in the first 6-8 weeks after harvest. Humpherson-Jones (1989) concluded that infected debris of brassicas crop remaining in the soil after harvest may provide a source of inoculum for Alternaria leaf spot infection which may implicate the spread of the disease within and between crops.

Weed host and other alternative host

The fungus can survive in susceptible weeds or perennial crops (Chupp and Sherf, 1960; Rangel, 1945; Maude and Humpherson-Jones, 1980a, b) and these weed host plants help the Alternaria blight pathogen to propagate the infective entities which serve as the source of inoculum for the oilseed Brassicas. The weed hosts of Alternaria blight pathogens infecting oilseed brassicas are namely: *Convolvulus arvensis, Camelina sativa, Crambe maritime, Chenopodium album, Crambe abyssinica, Anagallis arvensis.*

MEASUREMENT OF THE DISEASE SEVERITY

An appropriate method of disease assessment is a prerequisite for the identification of resistance to Alternaria blight in oilseed brassicas. The appearance of disease is observed both on leaves and siliquae, which is responsible for yield losses. Therefore, disease is assessed both on leaves as well as on siliquae based upon visual assessment. A key for assessment of Alternaria blight on rapeseed and mustard has been proposed by Conn et al. (1990). The infected leaves defoliates after some time which poses some difficulty in assessment of Alternaria blight in mustard. The study of Alternaria blight progression in mustard indicated that the resistance is of slow disease development. Recently, AUDPC is being used to assess the level of Alternaria blight in mustard (Meena et al., 2011b). They also reported that development of the disease was influenced by the growth stages of the crop and delay in sowing results in an increase in disease severity and reduction in yield was observed (Howlider et al., 1989). Sowing of rapeseed–mustard between 30 September to 15 October was found most favourable for reducing leaf spot incidence and increasing yield (Sinha et al., 1992) in India.

MANAGEMENT OF ALTERNARIA BLIGHT

Structural and biochemical defense

Oilseed brassicas are continually exposed to a number of pathogens and, as a result, they have evolved intricate defense mechanisms to recognize and defend themselves against a wide array of these pathogens by structural defense (Meena et al., 2010) and by inducing a set of defense responses that can defeat the invading pathogens (Vishwanath et al., 1999). Structural defense against Alternaria blight is found to be associated with factors that discourages conidial retention on host surface like high deposits of epicuticular wax (Meena et al., 2010) that form a physical barrier as a hydrophobic coating to reduce deposition of water borne inoculum, reduce rate of conidial germination, and germ tube formation (Skoropad and Tiwari, 1977; Saharan,1992). The species *B. napus, B. carinata* and *B. alba* have relatively more epicuticular wax than *B. rapa* and *B. juncea,* and tend to be less sensitive to infection of Alternaria blight pathogens (Conn et al*.,* 1984; Tewari, 1986). Biochemical defense against Alternaria leaf blight in mustard has been found to be associated with leaf enzymes associated with the phenolic pathway and higher leaf sugar contents (Singh et al., 1999). Resistance in *Camelina sativa* (wild allies of Brassicas also known as false flax) against *A. brassicae* has been found to be associated with the presence of chemical compounds, camalexins, somewhat similar to a fungicide available in the market (Browne et al., 1991). Resistance in *Camelina sativa* against *A. brassicae* due to production of the Phytoalexin camalexin has been also reported by Jejelowo et al. (1991) and Thomma et al. (1999). Camalexin also contributes to Alternaria resistance in an indirect way, as camalexin was found to inhibit production of the toxin destruxin B in *A. brassicae* (Pedras et al., 1998).

Search for resistant genotypes

Due to severe losses caused by Alternaria blight in oilseed brassicas, the objective of oilseed breeders is the development of resistant lines against Alternaria blight. Several attempts have been made in past to find out the sources of resistance against Alternaria blight, but resistant sources have not been reported in any cultivated *Brassica* species, but a high degree of resistance against the Alternaria blight has been reported in *B. alba* (Conn et al., 1988), *Eruca sativa* (Conn and Tewari, 1986; Tewari, 1991), and *Sinapis alba* (Kolte, 1985a; Brun et al., 1987; Ripley et al., 1992; Sharma and Singh, 1992; Hansen and Earle, 1995, 1997). The highest degree of resistance to *A. brassicae* was found in the wild relatives of Brassica outside the tribe Brassicaceae. These are false flax (*Camelina sativa*), Shepherd's purse (*Capsella bursa-pastoris*) (Conn et al., 1988), and *Neslia paniculata* (Tewari and Conn, 1993). In search of resistance sources for transfer of resistant genes, Sharma et al. (2002) evaluated thirty-eight species belonging to nine genera, including cultivated and wild allies of the genus *Brassica* under epiphytotic conditions for two years. Eight species namely *B. desnottesii, C. sativa, Coincya pseuderucastrum, Diplotaxis berthautii, Diplotaxis catholica, Diplotaxis cretacea, Diplotaxis erucoides,* and *Erucastrum gallicum* were found completely resistant, whereas others were classified as moderately resistant, susceptible and highly susceptible. A wide range of variation was also observed within the species of genus *Diplotaxis* but the genus *Diplotaxis* was found to be more resistant than the genus *Brassicas*. Sharma et al. (2002) concluded that source of resistance to *A. brassicae* are available within coeno species of *Brassicas* (Tribe) from which genes for resistance can be introgressed into cultivated *Brassica* species. The seven species other than *C. sativa* were identified as completely resistant to Alternaria leaf spot, belong to coeno species of *Brassica* (tribe Brassicaceae). Since resistance is unavailable within the cultivated species, these eight resistant wild species could be used as donor parents for introgressing resistance to leaf spot disease in Indian mustard.

Development of transgenics

Unavailability of resistance gene within the crossable germplasm of *Brassica* necessitates use of genetic

engineering strategies to develop genetic resistance against this pathogen. Mondal et al. (2007) got the integration and expression of the class 1 basic glucanase gene in mustard transgenic and observed that the transgenics arrested hyphal growth of *A. brassicae* by 15-54%.

Gene transfer for resistance in *Brassica* against Alternaria blight

Tuan and Garg (2001) did the gene transformation in *Brassica* sp. using particle bombardment. Cotyledon and hypocotyls of different species of *Brassica* have been used as target explants. Transient expression of uidA gene has been obtained when either been constructed with CaMV35S or Actin promoters. The uid4 gene encodes the 1, Beta-glucuronidase (GUS) enzyme. Its a reporter genesystem, particularly useful in plant molecular biology and microbiology. The highest expression was recorded between 10 to 15 h after bombardment. Plasmids pBI121, pBI221 and pDM803 were used to carry uidA gene. Further transformation events should be carried out to obtain highest transformation efficiency.

Use of fungicides

In the absence of resistant cultivars, fungicides provide the most reliable means of disease control (Vyas, 1993). Multiple applications of fungicides are required to achieve economic yield and acceptable quality in infected crops. Khan et al. (2007) sprayed three systemic fungicides Thiophanate methyl, Ridomil MZ (Mancozeb, 64%+ Metalaxyl, 8%), and Carbendazim alone and in combination with four non systemic fungicides Captan, Mancozeb, Zineb, and Thiram in the field at 0.2% a.i.L^{-1}. Ridomil MZ was most effective followed by the combination of Carbendazim + Captan. Singh and Singh (2006) reported that three consecutive sprays of Mancozeb resulted in maximum control of Alternaria leaf blight intensity followed by schedule with two consecutive sprays of Mancozeb (0.2%) and third of Rodomil MZ (0.25%). Foliar sprays of Mancozeb have been found most effective in disease management (Meena et al., 2004, 2011; Mondal et al., 2008).

Use of botanicals

Spray of Eucalyptus leaf extracts significantly reduced the number of spots/leaf, minimum size of spots, minimum disease index and highest yield followed by *Calotropis*, *Ocimum* and *Polyanthai* extracts spray (Patni et al., 2005; Patni and Kolte, 2006). Foliar sprays of aqueous bulb extract of *Allium sativum* (garlic) and *Eucalyptus globulus* (Eucalyptus) have been reported to effectively manage the Alternaria blight on leaves and pods and could be ecofriendly substitute for chemical fungicide mancozeb in management of mustard diseases (Meena et al., 2008, 2011a; Yadav, 2009).

Biological management

Some research findings indicate possibility of biological management of Alternaria blight of *Brassicas*. Foliar application of soil inhabitants isolates of *T. harzianum* (Patni et al., 2005; Meena et al., 2004, 2008, 2011a) and *P. fluorescens* (Patni et al., 2005; Meena et al., 2011a) were found effective in management of Alternaria blight.

Cross protection

Resistance in susceptible mustard cv. PR-15 against the highly and moderately virulent isolates of *A. brassicae* was induced using an avirulent *A. brassicae* isolate. The induction of resistance due to avirulent isolate against highly virulent and moderately virulent isolate of *A. brassicae* resulted in significant reduction in disease severity (Vishwanath et al., 1999).

Integrated management

There are many methods which are presently being used to manage Alternaria blight of *Brassicas*, that is, chemical, cultural, modification of nutrient, and biological. Due to increased awareness on the risks involved in use of fungicides, much attention is being focused on the integrated approach of pathogen management. Burning of crop debris of previous year, timely sowing, use of healthy certified seed, timely weeding, use of balance dose of nutrient, maintenance of optimum plant population, avoidance of irrigation at susceptible stage of crop (45 and 75 DAS) may help to minimize the disease incidence. Application of potash at 40 kg/ha (Sharma and Kolte, 1994; Godika et al., 2001), and soil application of minerals like sulphur, borax, potash and zinc are found effective in the management of Alternaria blight of mustard (Meena et al., 2011). These minerals were found to increase resistance in plants. Kumar and Kumar (2006) found minimum disease severity at 45 cm row spacing in comparison with broadcast method of sowing and found less disease severity in early sown, weeded crops.

FUTURE WORK STRATEGY

The following issues need to be addressed for Alternaria blight in oilseed *Brassicas*.

1. Relative dominance of *A. brassicae, A. brassicicola* and *A. raphani* during different stages of growth of oilseed

brassicas.

2. Development of molecular markers for identification of Alternaria leaf spot pathogens infecting oilseed brassicas.
3. Phylogeny of Alternaria blight pathogens of oilseed brassicas.
4. Gene expression in Alternaria bilght pathogens and host during infection of susceptible, tolerant and resistant varieties.
5. Search of resistant and moderately resistance sources against Alternaria blight in oilseed brassicas and wild relatives.
6. Search for alternative control methods of Alternaria leaf spot of oilseed brassicas.

CONCLUSION

There is no doubt that Alternaria leaf blight is the most destructive disease of oilseed brassicas across the world because of the cosmopolitan and damaging nature of pathogenic *Alternaria* species. Alternaria blight causes considerable reduction in the quality and quantity of harvested products of oilseed brassicas, and no proven source of resistance has been identified among cultivated oilseed brassica crop species. However, high level of resistance to *A. brassicae* have been reported in coenospecies (wild species) of *Brassicas* viz., *B. desnottesii, C. sativa, C. pseuderucastrum, D. berthautii, D. catholica, D. cretacea, D. erucoides* and *E. gallicum* which were found completely resistant. Due to lack of resistance in cultivated *Brassicas* against Alternaria leaf blight, other methods could be used for management of this disease.

One of the most commonly used methods is the use of fungicides. In spite of tremendous use of fungicides against pathogens, these fungicides cause serious health hazards to human beings and also they cause environmental pollution. Hence, nowadays more emphasis is made on other methods of disease management like growing moderately resistant varieties, use of plant and natural products, and bio-control agents and alterations in agronomic practices because they are more economical, eco-friendly and safe.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Partial biochemical characterization of a thermostable chitinase produced by *Streptomyces owasiensis* isolated from lichens of the Amazonian region

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The current study aims to identify *Streptomyces* spp., isolated in the Amazon region and capable of producing chitinase as well as to partially characterize the enzyme. Optimum temperature and pH, thermal and pH stabilities and the behavior of chitinase were determined as compared to other substances. *Streptomyces owasiensis* was the best chitinase producer in submerged culture fermentation using chitin 1% (w/v) at 140 rpm for 96 h at 34°C. The enzyme showed optimal activity at pH 7.0 and stability at the assessed pH variations. It also showed optimal temperature of 80°C and 180 min of thermostability between 30 and 90°C. The enzymatic activity was potentiated in the presence of various ions, especially Fe^{2+}, the same occurred in the presence of the anionic surfactant sodium dodecyl sulfate (SDS), but it suffered inhibition influence by EDTA. The chitinase produced by *S. owasiensis* showed characteristics of industrial relevance and it highlights the first report on enzyme production from the species isolated in the Amazon.

Key words: N-acetyl-D-glucosamine, chitin, chitinase, actinomycetes, *Streptomyces owasiensis.*

INTRODUCTION

The Amazonian region has an immense biodiversity, however, much of its species and their phylogenetic relationships are unknown, mainly the microbiological diversity and its interactions (Souza et al., 2004). Micro-organisms present a wide biochemical diversity which, along with the possibility of using genetic engineering

to modify its genetic makeup, which results in an excellent biological source of biomolecules producer for both industrial and pharmaceutical interest including enzymes (Neves et al., 2006).

Streptomyces spp. is a genus that stands out in secondary metabolites production. In accordance with González et al. (2005), they are a group of Gram positive bacteria characterized by the formation of aerial mycelium on solid medium and by spore formation. These microorganisms may be found in different environments such as water, plants and in association with lichens. The biochemical characteristics of the chitinase enzyme produced by the genus *Streptomyces* spp. are of great industrial interest, especially for the use in the environmental field and in biocontrol (Bon et al., 2008).

Chitin is a linear polymer of β-1,4 linked N-acetyl-D-glucosamine (GlcNAc), considered as the second most abundant polysaccharide in nature. It is commonly found in the structure of fungal cell walls and in the exo-skeleton of arthropods, crustaceans and nematodes (Dahiya et al., 2006; Rattanakit et al., 2007). Chitinases are enzymes that can hydrolyze chitin by catalyzing the cleavage of linkages β-1,4 between GlcNAc residues (Alcazar-Fuoli et al., 2011).

Chitinolytic enzymes have several applications, among which, the preparation of chito-oligosaccharides and N-acetyl-D-glucosamine are of pharmaceutical importance. These enzymes are also used in protoplast isolation from fungi and yeast, fungi biocontrol, in the treatment of waste containing chitin and in the control of malaria transmission (Dahiya et al., 2006).

However, chitinase obtainment through microbiological pathways has shown to be promising in recent years. *Chitiolyticbacter meiyuanensis* SYBC-H1 (Hao et al., 2012); *Serratia marcescens* (Zarei et al., 2011); *Streptomyces* sp. TH-11 (Hoang et al., 2011) and *Micrococcus* sp. AG84 (Annamalai et al., 2010) are among the microorganisms used for the production of the enzyme.

The present study aimed to select and identify a strain of *Streptomyces* spp. producing chitinase as well as to partially characterize the enzyme.

MATERIAL AND METHODS

Microorganisms

The current study assessed thirty strains of *Streptomyces* spp. stored in the culture collection of the Department of Parasitology, Federal University of Amazonas (DPUA), Brazil. For the activation and maintenance of microorganisms, we employed the ISP-2 culture medium, and the inoculum was standardized to a concentration of 10^8 colony forming units (CFU) mL^{-1} by spectrophotometry (Libra S22, Biochrom, Cambridge - England).

Screening the best producer of chitinase

The medium used for screening and producing the enzyme was composed by 1% chitin powder (Sigma, St. Louis, EUA), 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ (Wang et al., 2010), incubated in an orbital shaker at 140 rpm for 96 h at 34°C. The liquid resulting from the fermentation was filtered by means of qualitative filter paper, thus the crude enzymatic extract was obtained and used for all analytical determinations described in the present study.

Identification of the microorganism

The identification at the level specie of the isolate that showed the best production of the enzyme chitinase was carried out by polymerase chain reaction (PCR). The DNA of strain was extracted using the purification kit of genomic DNA (Promega Corporation, Madison, EUA) according to the manufacturer's instructions. Amplification of 16S ribosomal DNA gene was performed by PCR using universal primers for eubacteria FD1 (5'-AGAGTTTGATCCTGGCTCAG3') and RD1 (5'AGGAGGTGATCCAGCC-3') (Weisburg et al., 1991). The reaction mixture consisted of 10 to 50 ng of DNA, 5 pmol of each primer: 200 mM dNTP, 1.5 mM $MgCl_2$, 1X buffer, 1 U Taq DNA polymerase Platinum (Invitrogen Life Technologies) to a final volume of 25 ul. The reaction was performed with 5 min of denaturation at 94°C, 25 cycles were performed for 1 min at 94°C, 30 min at 52°C and 2 min at 72°C. These cycles were followed by a final elongation period of 10 min at 72°C. The amplification product was sequenced and the resulting sequence was compared with all sequences available in GenBank using BLAST software of the National Center for Biotechnology Information (NCBI).

Assay of chitinolytic activity and determination of total protein

The colloidal chitin is used as substrate in the detection of enzyme activity in accordance with the protocol by Harighi et al. (2007). The determination of the chitinase activity was performed according to the method by Waghmare and Ghosh (2010), although with some changes. The reaction mixture was composed of 1 mL of colloidal chitin 1% (w/v), 0.5 mL of sodium phosphate buffer 25 mM, pH 7.4 and 0.5 mL of the crude enzyme extract incubated at 37°C for 30 min. The reducing sugars were detected by applying the method described by Miller (1959). The calibration curve was performed by using N-acetylglucosamine, as per the standard, at concentrations of 0 to 5 mg mL^{-1}. One chitinase activity unit was defined as the proper amount of enzyme in order to release 1 µmol of N-acetylglucosamine per minute.

A commercial kit that employs the bicinchoninic acid (Pierce, Rockford, USA) using bovine serum albumin as standard in different concentrations was applied to determine total protein. The measurements of protein concentration were expressed in mg mL^{-1}.

Optimum temperature and pH, thermal and pH stabilities

Incubation temperatures of 30, 40, 50, 60, 70, 80 and 90°C were

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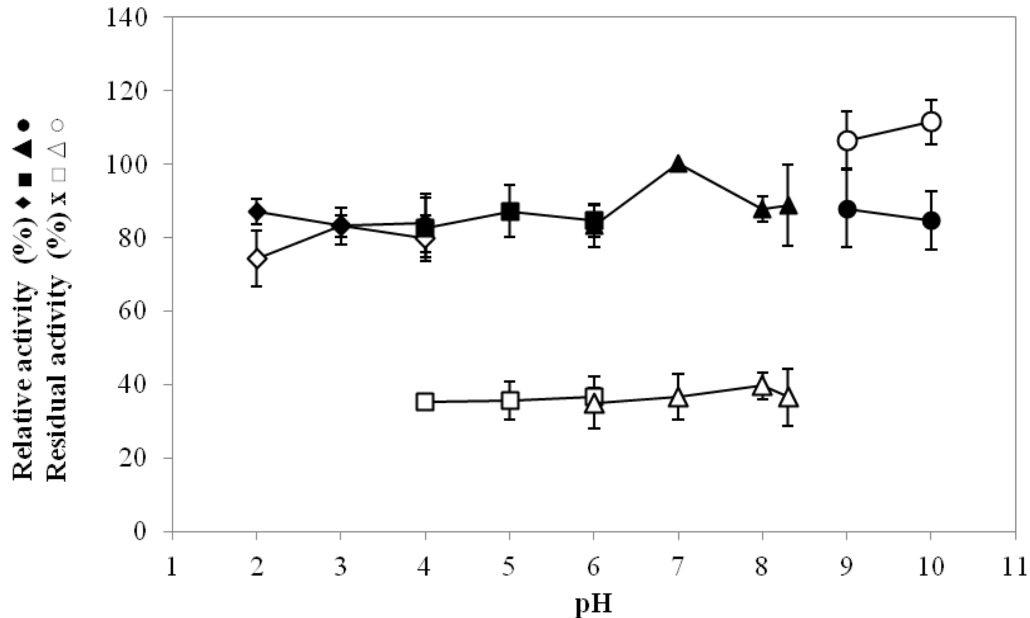

Figure 1. Optimum pH (closed symbols) and pH stability (open symbols), after 180 min of chitinase produced by *S. owasiensis* in the buffers: Glycine-HCl (♦ ◊) (pH 2.0, 3.0, 4.0); acetate (■ □) (pH 4.0; 5.0; 6.0); phosphate (▲ Δ) (pH 6.0; 7.0; 8.0; 8.3); carbonate-bicarbonate (● ○) (pH 9.0, 10.0).

used to analyze the optimum temperature of chitinolytic activity. The enzymatic activities were expressed as relative activity (%). The thermal stability was measured by incubating the enzyme extract at the indicated temperatures, over a total of 180 min. The enzymatic activity was expressed as residual activity (%). To determine the optimum pH of the enzyme activity, we used the following buffers: at 100 mM glycine-HCl (pH: 2.0; 3.0; 4.0), acetate (pH: 4.0; 5.0; 6.0), sodium phosphate (pH: 6.0; 7.0; 8.0; 8.3) and carbonate-bicarbonate (pH: 9.0; 10.0), and the enzyme activities were expressed as relative activity (%). The pH stability of the enzyme was determined by using the same buffers applied in the investigation of the optimum pH, aliquots were taken every 30 min, totaling 180 min. The enzymatic activity was expressed as residual activity (%).

Effect of ions and inhibitors

The effect of different metal ions and other substances on the chitinolytic activity of the enzyme was measured by subjecting the enzymatic extract to solutions containing substances and ions (5 mM), and this mixture was maintained at room temperature (±25°C) for 60 min, and then, chitinase activity was measured. The following ions were evaluated: Zn^{2+}; Mg^{2+}; Mn^{2+}; Fe^{2+}; K^{+}; Cu^{2+}; Ca^{2+}; Ni^{2+}; Ba^{2+}; Pb^{2+}. Ethylenediaminetetraacetic acid (EDTA), iodoacetic acid and sodium dodecyl sulfate (SDS) were also tested substances. The results were expressed as relative activity (%).

RESULTS AND DISCUSSION

Identification of the microorganism and producer of chitinase

The best microorganism producer of chitinase was identified as *Streptomyces owasiensis* by 16S DNA gene sequencing. The specific activity of chitinase produced by this strain was 2.923 U mg^{-1} and a total protein of 0.065 mg mL^{-1}. The chitinase production evidenced in the present study stands out as the first report regarding the production of extracellular enzymes from the species *S. owasiensis.*

Effect of pH on enzyme activity and stability

The chitinase showed optimal activity in neutral pH range and stability in all the assessed conditions. Thus, the optimal chitinolytic activity occurred at pH 7.0 (sodium phosphate buffer), proving to be stable up to pH 10.00, showing a linear behavior after 180 min of testing (Figure 1).

Han et al. (2009) evaluated a chitinase enzyme produced by *Streptomyces* sp. DA11 and found pH 8.0 as optimum for enzyme catalytic activity, although the enzymatic stability was maintained up to pH 11.0, suggesting a very similar behavior to that observed by the current study. Thus, the chitinase enzyme isolated from *S. owasiensis,* in the present study, has shown better enzymatic activity in neutral and moderately basic pH range versus the chitinase produced by *Streptomyces* sp. DA11. According to Yuli et al. (2004), neutral chitinase can be used for many purposes in the pharmaceutical industry, especially in the production of chitooligosaccharides. Taking into account the biochemical characteristics exhibed by chitinase of *S.*

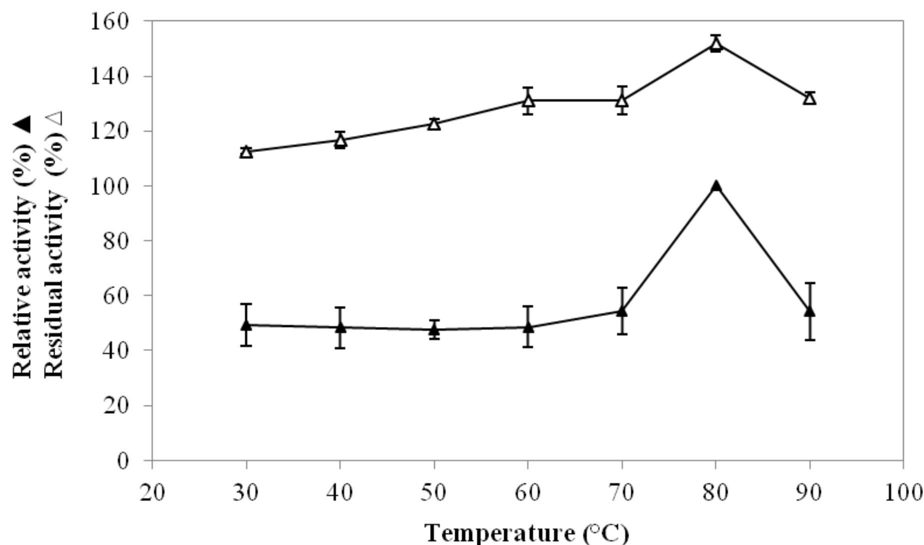

Figure 2. Optimum temperature (■) and temperature stability (●), after 180 min on the activity of enzymatic extract containing chitinase produced by *S. owasiensis.*

owasiensis, it seems to be attractive to the industrial production of this enzyme.

Optimum temperature and thermal stability

As shown in Figure 2, the optimum temperature of chitinase characterized in this study was 80°C. Furthermore, the enzyme was able to keep residual activity above 100% when it was submitted to variable temperatures as well as to exposure time of 180 min (Figure 2). It was observed that the optimum temperature for the enzyme catalysis is different from the vast majority of common enzymes. The optimum of the chitinase occurred at 80°C and the enzyme was stable throughout the period of study at different temperatures, such temperature tolerance features are unusual and of great industrial interest. Regarding a thermostable chitinase obtained by *Bacillus* sp. 13.26, Yuli et al. (2004), have found that the optimum temperature of the enzyme was 60°C, keeping the stability for 300 min at 70°C. According to Bruins et al. (2001), the thermostability can be considered as the outcome of an evolutionary strategy that requires both intrinsic factors that are directly associated with the structure of the molecule (which provide the rigidity and flexibility of the molecule) and the extrinsic factors that contribute to the stabilization of proteins in a particular medium, including some solutes, molecular chaperones, and the binding of substrate. In this respect, the discovery and study of thermostable enzymes is a promising research work with a wide commercial scope, which interest is additionally increased because of the fact that the elaboration of many industrial products is commonly developed under high temperatures in order to reduce the risk of contamination, especially microbial.

Effect of ions and inhibitors in the chitinolytic activity of *S. owasiensis*

As can be seen in Table 1, the presence of some substances in the reaction mixture can suppress or enhance the enzyme activity. The majority of the ions evaluated in the current study stimulated the catalytic activity of chitinase produced by *S. owasiensis* with significant prevalence in the addition of Fe^{2+}. This Fe^{2+} enhancing characteristic was also observed in chitinase produced by *Micrococus* sp. AG84 (Annamalai et al., 2010). Taken together, the results presented in both studies suggest that the increased enzymatic activity in the presence of Fe^{2+} or Cu^{2+} may be related to the residues of aspartic and glutamic acid found in the primary sequence of these chitinases (Annamalai et al., 2010), since the glutamic acid residues are structurally involved in the catalytic process of this enzyme.

An enzyme inhibitor, according to Marques and Yamanaka (2008), is a substance that is capable of specifically interfering with an enzymatic reaction by reducing or even slowing down the catalytic process. The influence of several ions and substances on the chitinase enzymatic activity was assayed. Table 1 shows that the chitinolytic activity underwent inhibition in the presence of the chelating agent EDTA. Another substance which enhanced the chitinolytic activity was the anionic surfactant SDS, thus an increase of 59% was noticed in relative activity as compared to the control sample (Table 1). Intriguingly, this result is opposite to results

Table 1. Effect of different ions and other substances on the activity of an enzymatic extract containing chitinase produced by *S. owasiensis.*

Ions	Relative activity(%)
Control	100±0
Zn^{2+}	148±12
Mg^{2+}	107±2
Mn^{2+}	171±5
Fe^{2+}	234±5
K^{+}	129±9
Cu^{2+}	141±9
Ca^{2+}	164±2
Ni^{2+}	120±8
Ba^{2+}	117±6
Pb^{2+}	99±2
Substances	
EDTA	62±6
Iodoacetic acid	100±10
SDS	159±5

that had been previously reported by Wang et al. (2009), Han et al. (2009) and Kim et al. (2003) because in these cases, the presence of SDS in the reaction mixture inhibited the chitinolytic activity. On the other hand, the simultaneous addition of chelating agent EDTA negatively affected the chitinolytic activity, showing a 38% decrease in the relative activity as compared to the control (Table 1). Similar results of inhibition of chitinase in the presence of EDTA were obtained by Wang et al. (2009) with a decrease of 43% (Wang et al., 2008), 75% (Han et al., 2009) and with 57% reduction in relative activity, respectively.

Conclusion

In summary, the *S. owasiensis* selected in the current study demonstrated the ability to produce an extracellular chitinase with properties which showed thermostability and increased efficiency in the neutral pH range, which makes this chitinase an enzyme of interest for both scientific study and pharmaceutical industry.

Conflict of Interests

The authors have not declared any conflict of interests.

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Effect of fungicides, plant extracts / botanicals and bioagents against damping off in brinjal

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Brinjal damping off incited by *Pythium ultimum* Trow. is one of the most important and destructive disease of brinjal, which can cause mortality upto 90% (pre -emergence seed rot and post emergence seedling mortality). Being soil borne, *P. ultimum* is very difficult to manage with fungicides alone and also uneconomical. Therefore, the present *in vitro* studies were undertaken to test bioefficacy of the nine fungicides (each at 500, 1000 and 1500 ppm), ten plant extracts / botanicals (each at 10, 15 and 20 %) and seven bioagents against *P. ultimum.* The experiments were designed with CRD and all the treatments replicated thrice. Results reveal that all the fungicides, botanicals and bioagents tested were found effective and were fungistatic against the test pathogen and significantly inhibited its growth over untreated control. Of the fungicides tested, Metalaxyl was found most effective and recorded 84.22% mean growth inhibition of the test pathogen. The second and third best fungicides found were Captan + metalaxyl and carbendazim + Mancozeb with mean growth inhibition of 82.42 and 62.88%, respectively. The rest of the fungicides tested recorded mean growth inhibition in the range of 24.50 to 52.79%. Of the botanicals evaluated, garlic was found most effective and recorded significantly the highest mean mycelial growth inhibition (94.83%). The second and third best botanicals found effective were Adulsa (75.53 %) and Datura (60.65 %). The rest of the botanicals tested recorded mean growth inhibition in the range of 20.82 to 56.83%. Of the bio-agents evaluated, *Trichoderma viride* was found most effective and recorded significantly highest mean mycelial growth inhibition (69.44%). The second and third best bioagents found effective were *Trichoderma koningii* (67.32%) and *Trichoderma hamatum* (63.99%); the rest of the bioagents also recorded significant inhibition of the test pathogen. Results reveals that seed treatment of captan (at 1.5 g/kg) + metalaxyl (at 3g/kg seed)+ garlic extract (at 100ml/kg soil) +soil application of *T. viride* (at 25g/kg soil) was the most effective treatment which could be practiced on large scale for management of damping off disease in brinjal and other solanaceous vegetable crops.

Key words: *Pythium ultimum,* plant extracts/ botanicals, bioagents, fungicides, Brinjal.

INTRODUCTION

Brinjal or egg plant (*Solanum melongena* L.) is an important Solanaceous vegetable crop of sub tropics and tropics, and supposed to have originated in India. Brinjal occupies second position among the vegetable. Brinjal is

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Plate 1. Brinjal seedlings showing damping off (*P. ultimum).*

known to have ayurvedic medicinal properties and good for diabetic patients. It has also been recommended as an excellent remedy for those suffering from liver complaints (Shukla and Naik, 1993). For brinjal, the following diseases have been reported this includes fungal diseases *viz.,* damping off, Phomopsis, blight and wilt, viral diseases *viz.,* mosaic and mottled dwarf and mycoplasma of leaf. Many fungi prevalent in soils can cause damping-off. *Fursarium* spp., *Pythium* spp., and *Phytophthora* spp. are most active in cool, wet soils whereas, *Cylindrocladium* spp. and *Rhizoctonia* spp. are more common in warm, wet soils.

The infected tissue become soft and water soaked, the collar portion rots and the seedlings ultimately collapse and die. The guaranteed supply of quality seedlings in required quantities is a major pre requisite for stabilized production of Brinjal. While raising seedlings in beds, the farmers face major problem of damping off incited by *Pythium* spp.

The damping off in brinjal is caused by *Pythium* spp., including *P. aphanidermatum, P.irregulare* and *P. ultimum*Trow, which can cause pre -emergence damping off and results in seed rot before the plants emerge out of the soil. The post emergence damping off phase is characterized by infection of the young tissues of the collar at the ground level. The pathogen is a soil borne with wide host range and almost worldwide distribution. The disease seems very difficult to control by conventional chemical means, due to its wide host range. Further, the use of chemicals/fungicides alone for the control of P. *ultimum* has been found to be impracticable and uneconomical. Therefore, an integrated disease management approach that encompass the use of chemicals, biocontrol agent, and plant extract could be the most economical and effective strategy for controlling the damping off and other soil borne plant diseases. Considering economic importance of the crop and losses caused by disease damping off in Brinjal, the present investigation was undertaken.

MATERIALS AND METHODS

The pathogen was isolated from the damping-off brinjal plants collected in nursery beds in *Kharif*, from the Department of Horticulture, VNMKV, Parbhani, Maharashtra, India in 2011. The Brinjal seedlings on nursery beds showing the symptoms of damping off (Plate 1) were collected in the polythene bags, labelled and brought to the laboratory. These samples were processed after surface sterilization (0.1% $HgCl_2$) for isolation of *P. ultimum* Trow. The isolate of the test pathogen were purified, numbered and maintained on potato dextrose agar (PDA) slants and stored at 8 to 10°C in a refrigerator.

Efficacy of fungicides

The efficacy of fungicides against *P. ultimum*Trow. was evaluated in three concentrations (500,1000 and1500 ppm) *in vitro* by applying poisoned food technique (Nene and Thapliyal, 1993) and using PDA as basal medium. The experiment was conducted by Completely Randomized Design with ten treatments and three replications.

100 ml PDA medium was poured in 250 ml capacity sterile glass conical flask and sterilized at 15 lbs pressure for 15 min. Required quantity of test fungicides for 500, 1000 and 1500 ppm was calculated and added in the sterilized PDA medium separately and mixed thoroughly.

This fungicide amended PDA medium with different concentrations of the test fungicides was poured (20 ml/plate) in sterilized glass Petri dish (90 mm.dia) and allowed to solidify at room temperature. The plates were inoculated by pure culture of *P. ultimum* Trow. For this purpose, 5 mm disc of one week old culture was cut with a sterilized cork borer. The disc was lifted and transferred aseptically in the centre of Petri plates containing the medium with test fungicides. Three plates per treatment per replication were maintained. The PDA plates without fungicides were also inoculated with the test pathogen and maintained as a uninoculated control. All the plates were incubated at 26 + 2°C.

The observations on colony diameter were recorded after a week of incubation. Per cent inhibition of the test pathogen was calculated by using the formula of Vincent (1927) and the data was statistically analysed:

$$PI = [(C-T)/C] \times 100$$

Where, PI= percent of inhibition, C= growth in control plates, T= growth in plates treated with fungicides.

Efficacy of plant extracts

Ten botanicals were evaluated *in vitro* at 10, 15 and 20% each for their fungistatic against *P. ultimum* Trow. by poisoned food technique (Nene and Thapliyal, 1993). The experiment was conducted by completely randomized design with 11 treatments and three replications.

Leaves/ rhizomes of the test botanicals were washed first in tap water, then in distilled water. Then 100 g of plant tissues + 100 ml distilled water were crushed (1:1 w/v) in mortar and pestle. The extract was filtered through double layered muslin cloth. The filtrate thus obtained was centrifuged at 5000 rpm for 15 min. The supernatant was collected and pellet was discarded. The supernatant obtained was strained through whatman No.1 filter paper and filtrate thus obtained was used as stock solution (100% conc.).

Aqueous plant extract (100%) were poured at 10, 15 and 20 ml each and separated into 100 ml autoclaved and cooled PDA in conical flask. The plant extract amended PDA was poured (each 20

Plate 2. Mass multiplication of *P. ultimum* (B) on sand : maize medium.

ml/plate) in sterile glass Petri plates (90 mm dia.) and allowed to cool. Five mm disc of *P. ultimum* Trow. was placed on the center of the solidified PDA plate under aseptic conditions. The PDA plates without plant extract and inoculated with the test pathogen served as untreated control.

These Petri plates were incubated at 26±2°C till the growth of the test pathogen in control plate was fully covered. The radial mycelial growth in all the plates was recorded and percent inhibition of mycelial growth over control was calculated by applying the formula (Vicent, 1927):

$$\text{Percent Inhibition (I)} = \frac{C - T \times 100}{C}$$

Where, C= growth of test fungus in control plates and T= growth of test fungus in treatment plates.

Efficacy of bioagents

The antagonistic potential of seven bioagents *viz*, *T. viride, T. harzianum, T. koningii, T. hamatum, Gliocladium virens, Bacilius subtilis* and *Pseudomonas fluorescence* against *P. ultimum* Trow. was evaluated *in vitro* by Dual culture technique (Stack et al., 1986) on PDA medium. The experiment was conducted by completely randomized design with eight treatments and three replications.

Autoclaved and cooled PDA medium was poured at 20 ml/plate in Petri plates (90 mm) and allowed to solidify. The plates were inoculated with 5 mm disc of 7 days old culture of biocontrol agents as well as 5 mm disc of 7 days old culture of *P. ultimum* Trow. at equidistance and exactly opposite with each other on PDA in plates. For bacteria, antagonist were streaked with the help of sterilized inoculating needle at one end of the PDA Petri plate. After 24 h of incubation, just opposite to the bacterial streak, a 5 mm disc of the test pathogen was placed. The PDA plates inoculated in center with the disc of the culture of the test pathogen only served as control. A triplicate set of inoculated PDA plates per treatment per replication was maintained and all the treatments were replicated thrice.

All these plates were incubated at 26±2° C in incubator. Observations on radial mycelial growth of the fungal pathogen and biocontrol agents was measured and per cent inhibition of the test fungus (*Pythium ultimum* Trow.) was calculated by applying formula given by Arora and Upaddhyay (1978) as follows:

$$\text{Percent Inhibition (PI)} = \frac{\text{Colony growth in Control Plate - Colony growth in intersecting plate}}{\text{Colony growth in control plate}} \times 100$$

Integrated evaluation of fungicides, botanicals, and bioagents in pot culture

Pot culture experiment was conducted to integrate the effective fungicides, bioagents and botanicals for integrated management of damping off (*Pythium ultimum*) disease in brinjal Cv.Hadgaon local. The experiment was conducted by Completely Randomized Design with eleven treatments and three replications.

Pathogen multiplied on sand: maize medium (Plate 2) was mixed with autoclaved (30 lbs for 30 min) potting mixture soil: sand: FYM (2:1:1) (at 25 g / kg potting mixture) and filled into pots, (disinfected with 5 % solution of copper sulphate) watered lightly and incubated at room temperature for two weeks in screen house. Within this period test pathogen multiplied in the pots. Those fungicides, plant extract /botanicals and bioagents found effective in *in vitro* studies were used for integrated disease management (IDM) experiment. The effective test fungicides *viz*., Metalaxyl (alone) and Captan (in combinations) were applied as seed treatment as detailed above. The 20% aqueous crude extract of garlic bulk was applied (alone and in combination) as soil drenching (100 ml/kg soil). The carrier based preparation of bioagent *T. viride* was applied (alone and in combination) in the soil at 25 g/kg soil, as detailed above. The fungicide treated seed of brinjal Cv. Hadgaon local were sown (6 seeds /pot) as per the treatment details. For T3, T4 and control treatments, the surface sterilized (0.1%$HgCl_2$) seed of brinjal Cv. Hadgaon local were sown (6 seeds/pot).The suitable untreated control with soil and surface sterilized seed sown of brinjal Cv. Hadgaon local was maintained. Three pots per treatments per replication were maintained and all the treatments replicated thrice. All these pots were watered regularly and maintained in screen house at the Department of Plant Pathology, Parbhani.

Observations on pre-emergence seed rot were recorded at one week after sowing and on post -emergence seedling mortality recorded at interval of 7 days and counted till 35 DAS and averaged finally. Observations were recorded for pre-emergence seed rot, post-emergence seedling mortality and per cent pre-emergence seed rot and per cent post-emergence seedling mortality were calculated by formula devised by Kataria and Grover (1967).

$$\%PESR = \frac{\text{Number of seeds rot per pot}}{\text{Total number of seeds per pot}} \times 100$$

$$\%POESM = \frac{\text{Number of seedlings affected per pot}}{\text{Total number of seeds per pot}} \times 100$$

PESR is pre emergence seedling mortality and POESM is post emergence seedling mortality.

RESULTS AND DISCUSSION

The efficacy of fungicides under *in vitro* tests

The results obtained on *in vitro* bio-efficacy of the nine fungicides (alone and combination) *viz.,* Captan, Thiram, Carbendazim,, Mancozeb, Carbendazim + Mancozeb, Metalaxyl, Benomyl, Carbendazim + Thiram, Captan + Metalaxyl against *P. ultimum* of the present study are presented in Table 1, Plates 3, 4 and 5 and Figure 1.

Radial mycelial growth

Result (Table 1) revealed that all the fungicides tested

Table 1. Efficacy of the fungicides against *Pythium ultimum* Trow.

Treatment	Fungicides	Colony diameter (mm)*			Average Mean col. dia. (mm)	Inhibition %			Average Inhibition (%)
		500 ppm	1000 ppm	1500 ppm		500 ppm	1000 ppm	1500 ppm	
T_1	Captan	71.86	40.06	35.20	49.04	20.14 (26.63)	55.48 (48.14)	60.88 (51.28)	45.50
T_2	Thiram	63.26	56.13	49.60	56.33	29.69 (33.01)	37.62 (37.82)	44.88 (42.05)	37.39
T_3	Carbendazim	60.30	58.70	52.21	57.07	32.96 (35.03)	34.77 (36.12)	41.98 (40.38)	36.57
T_4	Mancozeb	71.86	70.03	61.93	67.94	20.14 (26.63)	22.18 (28.07)	31.18 (33.86)	24.50
T_5	Carbendazim + Mancozeb	35.20	33.56	31.43	33.33	60.88 (51.28)	62.70 (52.35)	65.07 (53.76)	62.88
T_6	Metalaxyl	31.33	11.26	00.00	14.19	65.18 (53.83)	87.48 (69.28)	100.00 (89.98)	84.22
T_7	Benomyl	45.06	42.90	39.46	42.47	49.92 (44.95)	52.32 (46.32)	56.14 (48.52)	52.79
T_8	Carbendazim + Thiram	66.99	62.30	57.20	62.16	25.66 (30.42)	30.77 (33.68)	36.44 (37.12)	30.95
T_9	Captan + Metalaxyl	27.83	10.43	09.16	15.80	69.07 (56.20)	88.40 (70.08)	89.81 (71.38)	82.42
T_{10}	Control	90.00	90.00	90.00	90.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
	SE±	0.83	0.88	1.12		0.58	0.61	0.77	
	CD	2.44	2.59	3.32		1.71	1.79	2.29	
	CV %	2.68	3.21	4.58		2.66	2.50	2.88	

*Mean of three replications; figure in parenthesis are angular transformed values.

Plate 3. *In vitro* efficacy of fungicides at 500 ppm concentration on radial growth of *P. ultimum.*

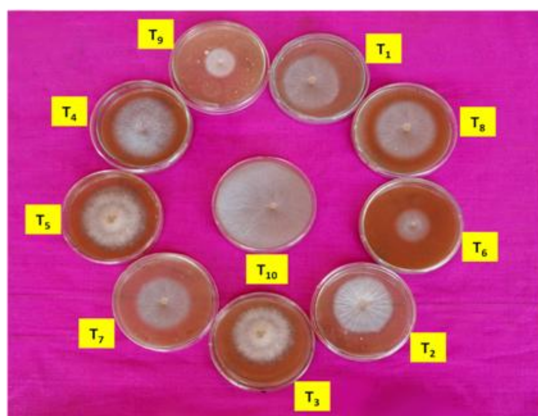


Plate 4. *In vitro* efficacy of fungicides at 1000 ppm concentration on radial growth of *P. ultimum.*

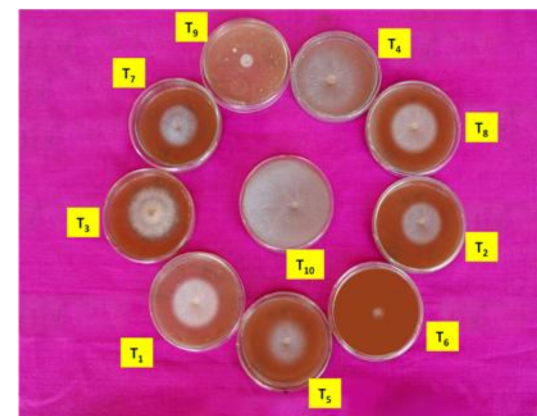
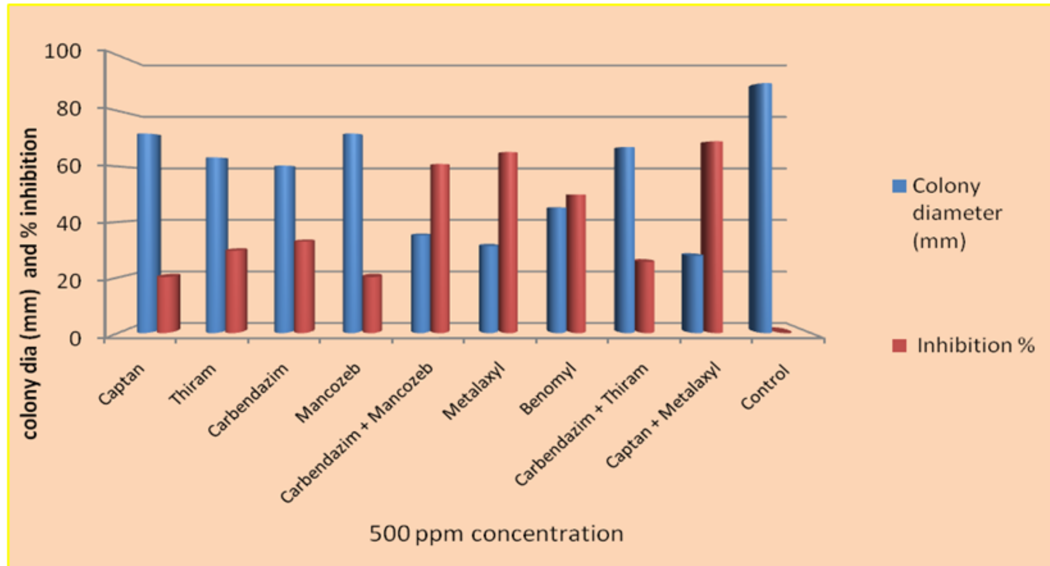
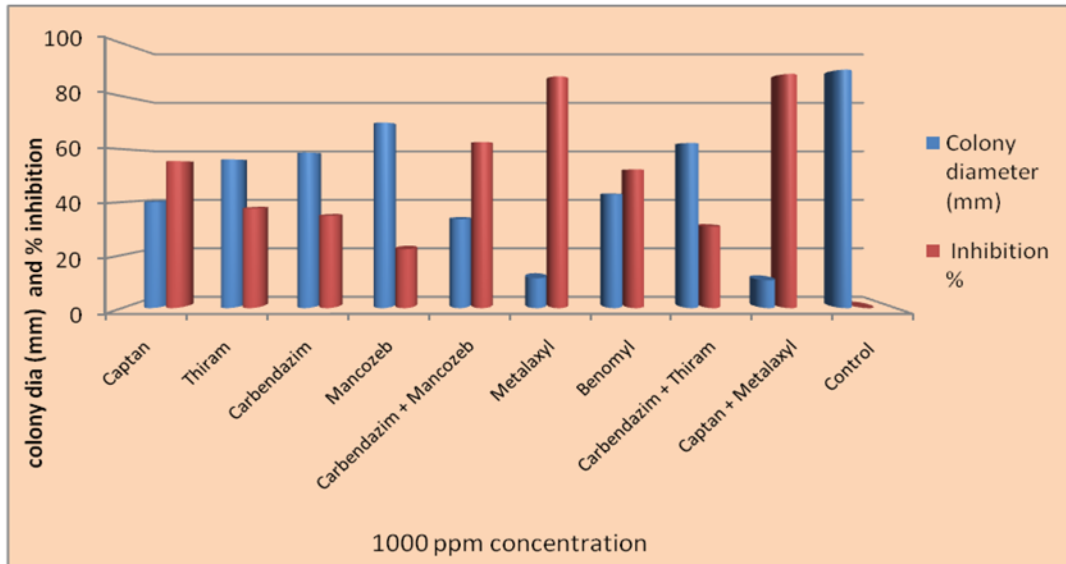


Plate 5. *In vitro* efficacy of fungicides at 1000 ppm concentration on radial growth of *P. ultimum.*





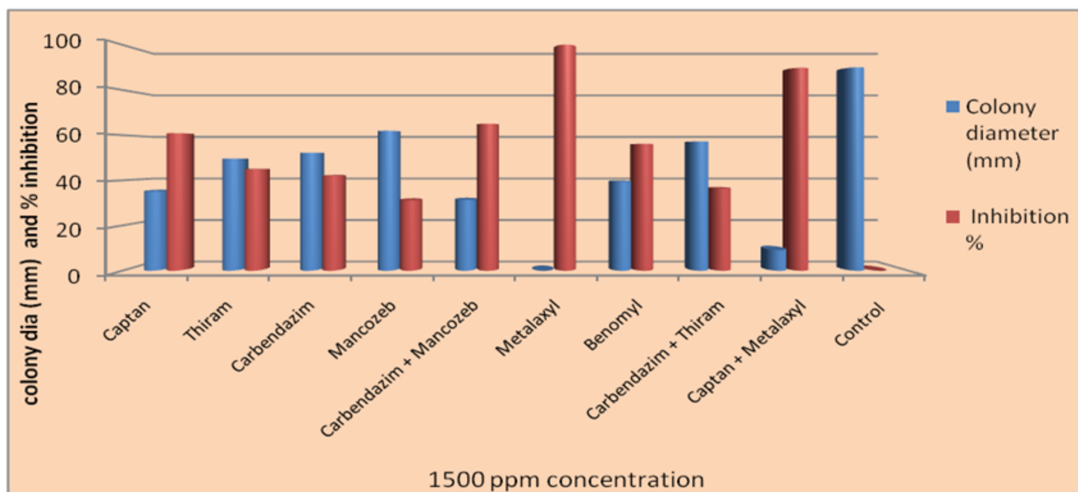


Figure 1. *In vitro* efficacy of fungicides at 500, 1000, 1500 ppm concentrations on radial growth of *P. ultimum* Trow.

recorded a wide range of radial mycelial growth of the test pathogen and it was varied with concentrations used of the test fungicides.

At 500 ppm, concentration (Plate 3) and Figure 1. radial Mycelial growth of the test pathogen was ranged from 27.83 (Captan + Metalaxyl) to 71.86 mm (Captan) and (Mancozeb). However, it was maximum with Captan (71.86 mm) and Mancozeb (71.86 mm) each both of which were on par. This was followed by Carbendazim + Thiram (66.99 mm), Thiram (63.26), and Carbendazim (60.30mm).Significantlyleast mycelial growth was recorded with the treatment Captan + Metalaxyl (27.83mm); followed by Metalaxyl (31.33 mm), Carbendazim + Mancozeb (35.20 mm), Metalaxyl (31.33 mm), captan + metalaxyl (27.83 mm) and Benomyl (45.06 mm).

At 1000 ppm concentration, (Plate 4 and Figure 1) radial mycelial growth of the test pathogen was ranged from 10.43 (captan + metalaxyl) to 70.03 mm (mancozeb). All the fungicide tested exhibited similar trend of radial mycelial growth as that of 500 ppm. However, maximum radial mycelial growth was recorded with mancozeb (70.03 mm) and was followed by carbendazim + thiram (62.30 mm), carbendazim (58.70 mm) and thiram (56.13 mm). Significantly least mycelial growth was recorded with Captan + Metalaxyl (10.43 mm); followed by Metalaxyl (11.26 mm), carbendazim + mancozeb (33.56 mm) captan (40.06 mm) and benomyl (42.90 mm).

At 1500 ppm concentration (Plate 5 and Figure 1), radial mycelial growth ranged from 00.00 (metalaxyl) to 61.93 (mancozeb). However, it was maximum with, mancozeb (61.93 mm) and this followed by carbendazim + thiam (57.20 mm) carbendazim (52.21 mm) and thiram (49.60 mm). Significantly least mycelial growth was recorded with captan + metalaxyl (09.16 mm), followed by carbendazim+mancozeb (31.43 mm), captan (35.20 mm), benomyl (39.46 mm) and fungicide metalaxyl recorded nil (00.00) growth of the test pathogen.

Average radial mycelial growth recorded with all the fungicides tested (at 500, 100, 1500 ppm) ranged from 14.19 (Metalaxyl) to 67.94 mm (mancozeb). However, highest mean radial mycelial growth was recorded with mancozeb (67.94 mm) which was followed by Carbendazim + Thiram (62.16 mm), Carbendazim (57.07 mm), Thiram (56.33 mm), and Captan (49.04 mm). Significantly least mean mycelial growth was recorded with metalaxyl (14.19 mm), followed by Captan + Metalaxyl (15.80 mm), Carbendazim + Mancozeb (33.3 mm) and Benomyl (42.47 mm).

Mycelial inhibition

Results (Table 1) revealed that all the fungicides at 500, 1000 and 1500 ppm significantly inhibited mycelial growth of the test fungus over untreated control. Further, it was found that per cent mycelial inhibition was increased with the increase in the fungicides concentrations.

At 500 ppm concentration, (Table 1) per cent mycelial growth inhibition ranged from 20.14 (Mancozeb) to 69.07% (Captan + Metalaxyl). However highest mycelial inhibition was recorded with Captan + Metalaxyl (69.07%). This was followed by the fungicides, Metalaxyl (65.18%), Carbendazim + Mancozeb (60.88%), Benomyl (49.92%), Carbendazim (32.96%), Carbendazim + Thiram (25.66%), Captan (20.14%) and Mancozeb (20.14%).

At 1000 ppm concentration, (Table 1) similar trend of mycelial growth inhibition with the test fungicides was recorded and it ranged from 22.18 (Mancozeb) to 88.40% (Captan + Metalaxyl). However, highest percentage mycelial inhibition was recorded with Captan + Metalaxyl (88.40%), this was followed by Metalaxyl (87.48%), Carbendazim + Mancozeb (62.70%), Captan (55.48%), Benomyl (52.32%), Thiram (37.62%), Carbendazim (34.77%), Carbendazim + Thiram (30.77%), and Mancozeb (22.18%).

At 1500 ppm concentration, (Table 1) the percentage of mycelial inhibition ranged from 31.18 (Mancozeb) to 100% (Metalaxyl). However, percent mycelial inhibition (100%) was recorded with Metalaxyl. This was followed by the fungicides, Captan + Metalaxyl (89.81%), Carbendazim + Mancozeb (65.07%), Captan (60.88%), Benomyl (56.14%), Thiram (44.88%), Carbendazim (41.98%), Carbendazim + Thiram (36.44%), and Mancozeb (31.18%).

Mean percentage mycelial inhibition of all the fungicides at 500, 1000 and 1500 ppm ranged from 24.50 (Mancozeb) to 84.22% (Metalaxyl). However, Metalaxyl was found to be most fungistatic with significantly highest mean mycelial inhibition of 84.22%. This was followed by Captan + Metalaxyl (82.42%), Carbendazim + Mancozeb (62.88%), Benomyl (52.79%), and the fungicides *viz.,* Mancozeb, Carbendazim + Thiram were found least effective against the test pathogen with the mean mycelial inhibition 24.50, 30.95 and 37.39%,respectively. Similar *in vitro* fungistatic effects of the test fungicides against *P. ultimum* infecting brinjal and other *Pythium* spp. infecting many other crops were reported earlier by several workers (Satija and Hooda, 1987; Sawant and Mukhopadhyay, 1990; Nene and Thapliyal, 1993; Ayub et al., 1998; Taylor et al., 2002; Jiskani et al., 2007).

In vitro bioefficacy of plant extracts/botanicals

Bioefficacy of ten botanicals/plant extracts *viz.,* Mehandi (*Lawsonia innermis*), Ginger (*Zingiber afficinale*), Datura (*Datura metal*), Tulsi (*Oscimum sanctum*), Parthenium (*Parthenium hysteriphorus*), Neem (*Azardirachta indica*), Garlic (*Allium sativum*), Turmeric (*Curcuma longa*), Satawari (*Asparugus racemosus*) was evaluated at 10, 15, and 20% *in vitro* against *Pythium ultimum* applying Poisoned food technique and using PDA as a basal medium, and the results obtained are presented in the Table 2 and depicted in Plates 6, 7, 8 and Figure 2.

Table 2. Efficacy of plant extracts/botanicals against *Pythium ultimum* Trow.

Botanicals	Colony diameter mm)*			Average Col. Diameter	Per cent inhibition* at			Average Inhibition (%)
	10%	15%	20%		10	15	20	
Mehandi	35.56	38.61	35.13	36.43	60.47 (51.04)	49.06 (57.08)	60.96 (51.32)	56.83
Ginger	47.46	44.69	41.52	44.55	47.25 (43.42)	50.33 (45.18)	53.85 (47.20)	50.47
Datura	37.43	35.43	33.34	35.40	58.40 (49.83)	60.62 (51.12)	62.94 (52.49)	60.65
Tulsi	67.90	64.36	61.37	64.54	24.55 (29.55)	38.47 (38.10)	31.80 (34.32)	31.60
Parthenium	47.50	44.43	41.44	44.45	47.22 (43.40)	50.62 (45.35)	53.95 (47.26)	50.59
Neem	66.06	63.73	60.46	63.41	26.58 (31.03)	29.18 (32.69)	32.81 (34.94)	29.52
Garlic	13.93	00.00	00.00	04.64	84.51 (66.81)	100.00 (89.98)	100.00 (89.98)	94.83
Turmeric	73.86	71.56	68.33	71.25	17.92 (25.04)	20.47 (26.89)	24.07 (29.37)	20.82
Adulsa	25.30	23.06	17.66	22.00	71.88 (57.97)	74.36 (59.57)	80.36 (63.69)	75.53
Shatawari	66.96	63.73	58.63	63.10	25.58 (30.37)	29.18 (32.68)	34.84 (36.16)	29.86
Control	90	90	90	90	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
SE±	1.23	0.47	0.58		0.88	1.88	0.39	
CD	3.60	1.37	1.70		2.59	5.51	1.16	

* Mean of three replications. (Figure in parenthesis are angular transformed values).

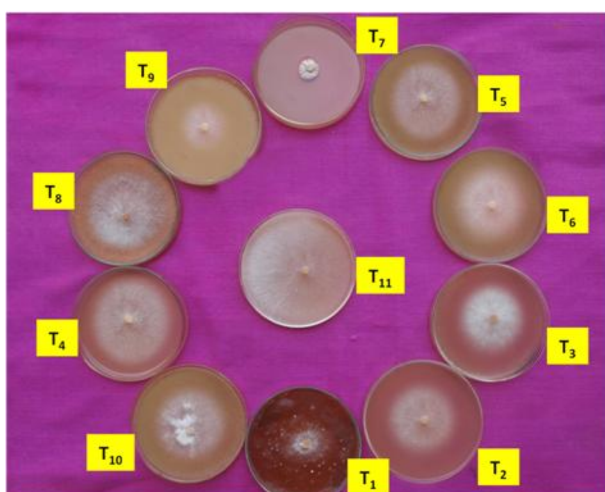


Plate 6. *In vitro* efficacy of plant extract/ botanicals at 10% concentration on radial growth of *P. ultimum.*

Plate 7. *In vitro* efficacy of plant extract/ botanicals at 15% concentration on radial growth of *P. ultimum.*

Radial mycelial growth

Results (Table 2) reveal that all the botanicals/plant extracts tested exhibited a wide range of radial mycelial growth of the test pathogen and it was varied with their concentrations used.

At 10%, (Plate 6) radial mycelial growth of the test pathogen ranged from 13.93 (Garlic) to 73.86 mm Turmeric). However, it was maximum with turmeric 73.86 mm). This was followed by Tulsi (67.90 mm), Shatawari (66.96 mm), Neem (66.06 mm), ginger (47.46 mm), Parthenium (47.50 mm) both of which were at par, (Datura (37.43 mm) and Mehandi (35.56 mm).

Comparatively, least growth was recorded with Adulsa (25.30 mm) and Garlic (13.93 mm).

At 15% (Plate 7), radial mycelial growth of the test pathogen ranged from 00.00 (Garlic) to 71.56 mm (Turmeric). The 10 and 15% plant extracts exhibited similar trend of radial mycelial growth. However, maximium radial mycelial growth was recorded with Turmeric (71.56 mm) followed by Tulsi (64.36 mm), Neem (63.73 mm) and Shatawari. (63.73 mm); all three were at par; Ginger (44.69 mm) and Parthenium (44.43 mm) were at par; for Mehandi (38.61 mm) and Datura (35.43 mm) significantly least mean mycelial growth was recorded with Adulsa (23.06 mm) and Garlic (00.00 mm). At 20 per cent, (Plate 8) radial mycelial growth was ranged

Plate 8. *In vitro* efficacy of plant extract/ botanicals at 20% concentration on radial growth of *P. ultimum.*

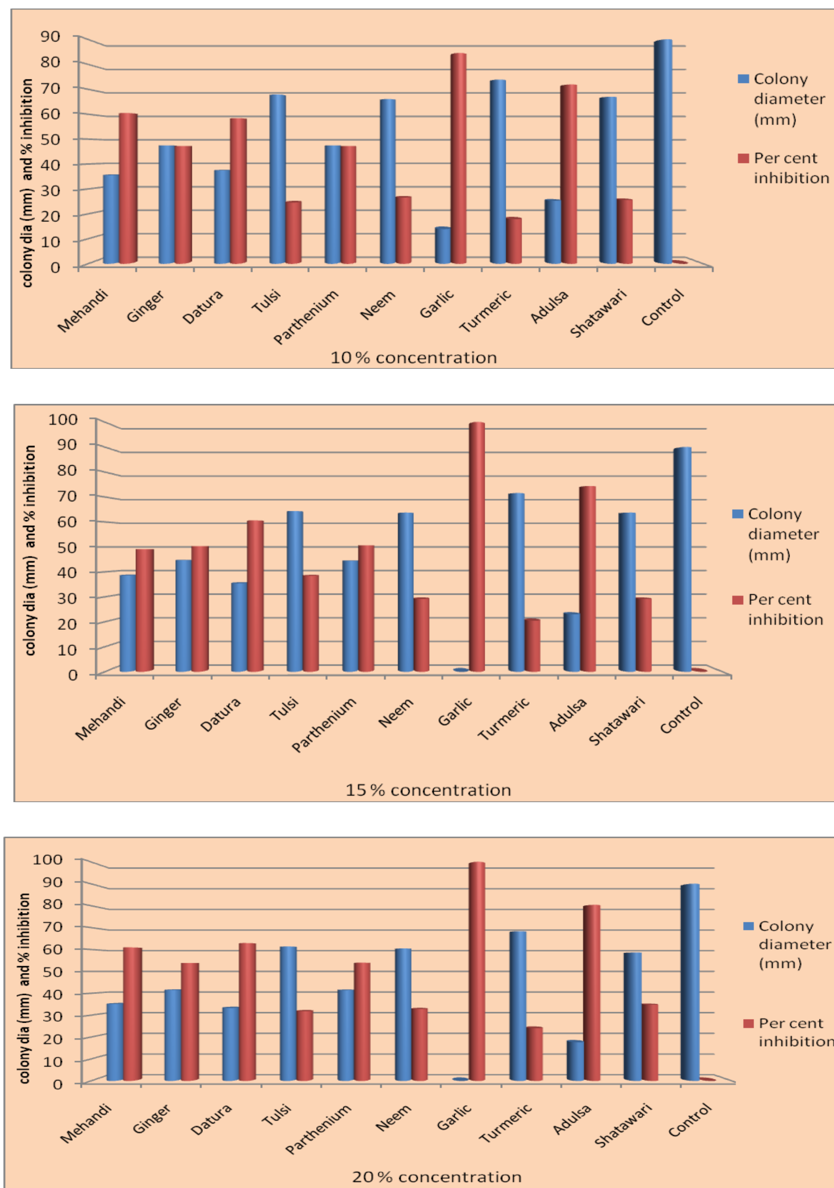


Figure 2. *In vitro* efficacy of plant extract/ botanicals at 10, 15, 20 % concentration on radial growth of *P. ultimum* Trow.

from 00.00 mm (Garlic) to 68.33mm (Turmeric). However, significantly highest mycelial growth was recorded with turmeric (68.33mm), This was followed by Tulsi (61.37mm),Neem (60.46mm)both of which were on par and Shatawari (58.63mm) significantly least growth was recorded with Garlic (00.00mm)and this was followed by Adulsa (17.66mm) Datura (33.34mm) Mehandi (35.13mm) Ginger and Parthenium (41.52 and 41.44) both of which were on par.

Average radial myceilial growth (Table 2) recorded with all the plant extract tested (at 10, 15, and 20%) ranged from 04.64 (Garlic) to 71.25 mm (Turmeric). However, highest mean radial mycelial growth was recorded with Turmeric (71.25 mm) and was followed by Tulsi (64.54 mm), Neem (63.41 mm), Shatawari (63.10 mm). Ginger (44.55 mm) and Parthenium (44.45 mm) were at par, and Mehandi (36.43 mm), and Datura (35.40 mm). Significantly least mean mycelial growth was recorded with Garlic (00.00 mm) and Adulsa (22.00 mm).

Mycelial inhibition

Result (Table 2) reveal that all the plant extracts tested (at 10, 15 and 20 per cent), significantly inhibited mycelial growth of the test pathogen over untreated control and per cent mycelial inhibition was increased with increase in concentrations of the botanicals tested.

At 10%, mycelial growth inhibition ranged from 17.92 (Turmeric) to 84.51% (Garlic). However, significantly highest mycelial inhibition was recorded with Garlic (84.51%) and was followed by Adulsa (71.88%), Mehandi (60.47%), Datura (58.40%) both of which were on par; Ginger and Parthenium (47.25 and 47.22%) each were on Par, Neem (26.58%), Shatawari (25.58%), Tulsi(24.55%) and all three were on par and Turmeric (17.92%).

At 15% similar trend of mycelial inhibition as that of 10% was recorded and it ranged from 20.47 (Turmeric) to 100% (Garlic). However, significantly highest mycelial inhibition was recorded with Garlic (100%), and was followed by Adulsa (74.36%), Datura (60.62%), Parthenium (50.62%) and Ginger (50.33%); both were on par, Mehandi (49.06%), Tulsi (38.47%) Neem and Shatawari (each 29.18%); which are on par with and Turmeric (20.47%).

At 20%, the percentage mycelial inhibition ranged from 24.07 (Turmeric) and 100% (Garlic). However, significantly highest mycelial inhibition was recorded with Garlic (100%) and was followed by Adulsa (80.36%), Datura (62.94%), Mehandi (60.96%), Ginger and Parthenium (each 53.95%), (both were on par), Shatarwari (34.84%), Neem (32.81%), Tulsi (31.80%) and Turmeric (24.07%).

Average percentage mycelial inhibition (Table 2) recorded with all the test botanicals was from 20.82% for Turmeric to 94.83% for Garlic. However, Garlic was found to be most fungistatic which recorded significantly highest mean mycelial inhibition (94.83%). This was followed by Adulsa (75.53%), Datura (60.65%), Mehandi (56.83%), Parthenium and Ginger (each 50.47%), which were on par, Tulsi (31.60%), Satawari (29.86%) and Neem (29.52%) both were on par and Turmeric (20.82%).

Thus, all the plant extracts tested were found as fungistatic/antifungal against *P. ultimum* and significantly inhibited mycelial growth of the test pathogen over untreated control However, Garlic recorded highest mean mycelial inhibition (94.83%) followed by Adulsa (75.53%) and Datura (60.65%). Result of the present study are in conformity with those reported earlier by several workers (Bhat and Shrivastava, 2003; Bhora et al., 2006; Muthukumar et al., 2010 Ambikapathy et al., 2011).

Efficacy of bioagents

Seven fungal antagonists *viz., T. viride, T. harzianum, T. hamatum, T. koningii, Gliocladium virens, Pseudomonas flueroscens,* and *Bacillus subtilis*were evaluated *in vitro* against *P. ultimum* Trow. applying dual culture technique (Stack et al.,1986) using PDA as basal medium and result obtained are presented in Table 3 (Plate 9) and Figure 3.

Result (Table 3) reveals that all the bioagents evaluated exhibited fungistatic /antifungal activity against *P. ythium ultimum* Trow. and significantly inhibited its mycelial growth over untreated control (Plate 9). Of the antagonist tested, *T. viride* was found most effective and recorded significantly least mycelial growth (27.49 mm) with highest mycelial inhibition (69.44%) of the test pathogen over untreated control (00.00%). The second and third best antagonists found were *T. koningii* and *T. hamatum* which recorded mycelial growth of 29.40 and 32.40 mm, respectively and inhibition respectively of 67.32 and 63.99%. This was followed by *Bacillus subtilis* and *P. flueroscens* with colony growth respectively of 36.25 and 39.35 mm and corresponding growth inhibition of 59.71 and 56.27%. *G. virens* recorded mycelial growth of 41. and corresponding growth inhibition of 54.14%. *T. harzianum* was found relatively less effective with 45.35 mm colony diameter and 49.60% inhibition of the test pathogen.

Thus all the fungal and bacterial antagonists/bioagents evaluated *in vitro* were found fungistatic /antifungal against *P. ultimum* and caused significant reduction in the linear mycelial growth of the test pathogen over untreated control.

The inhibition effects of *Trichoderma* spp., *P. fluorescens* and *B. subtilis* against *P. ultimum* may be attributed to the mechanisms *viz,.* antibiosis, lysis, mycoparasitism, competition and production of volatile substances, by the test bioagents/antagonists.

Results of the present study on inhibitory effects of the test antagonists: *Trichoderma* spp., *B. subtilis,* and

Table 3. Efficacy of the bioagents/antagonists against *P.ultimum* Trow.

Treatment number	Treatments	Mean colony diameter(mm)*	Per cent inhibition
T_1	*Trichoderma viride*	27.49	69.44 (56.43)
T_2	*T. harzianum*	45.35	49.60 (44.76)
T_3	*T. koingii*	29.40	67.32 (55.13)
T_4	*T. hamatum*	32.40	63.99 (53.12)
T_5	*Gliocladium virens*	41.27	54.14 (47.36)
T_6	*Pseudomonas fluorescens*	39.35	56.27 (48.59)
T_7	*Bacillus subtilis*	36.25	59.71 (50.59)
T_8	Control(untreated)	90.00	00.00 (00.00)
SE±	--	0.55	0.36
CD	--	1.66	1.09

*Mean of three replications. Figure in parenthesis are angular transformed values.

Plate 9. *In vitro efficacy of the bioagents against P. ultimum Trow.*

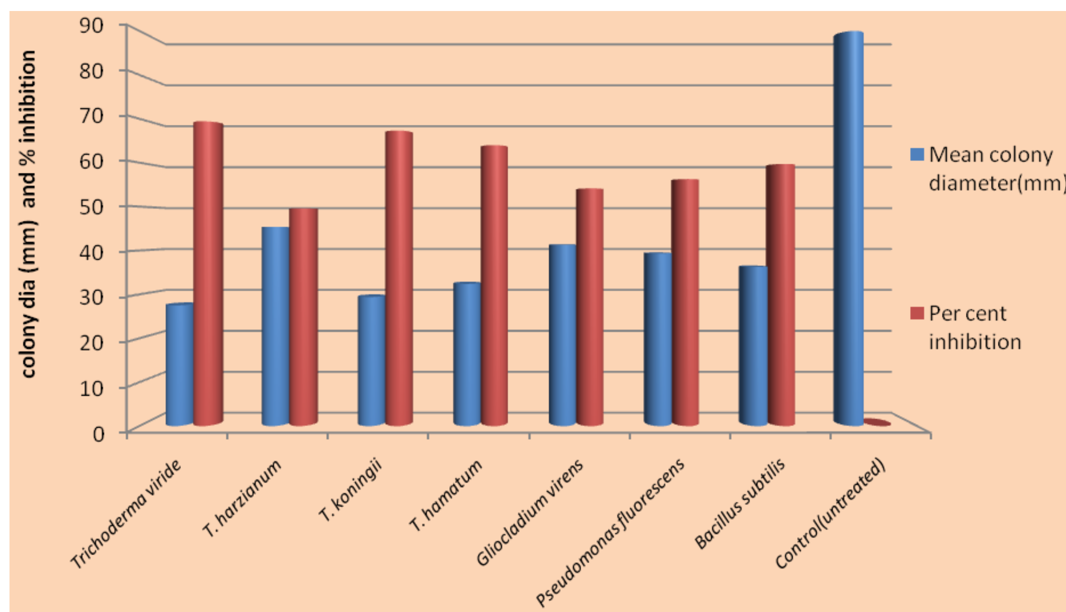


Figure 3. *In vitro* efficacy of bioagents against *P. ultimum* Trow.

Table 4. Integrated disease management with effective fungicides, botanicals and bioagents in pot culture.

Treatment number	Treatment	Rate of application	Pre emergence Damping Off (%)	Post emergence Damping Off (%)	Average mortality (%)
T1	Metalaxyl	6 g/kg seed	38.88(38.50)	36.11(36.91)	37.49
T2	Captan+ Metalaxyl	1.5g+3 g/kg seed	33.33(34.78)	26.11(30.60)	29.72
T3	Garlic	100 ml/kg soil	44.44(41.74)	41.66(40.18)	43.05
T4	*T.viride*	25 g/kg soil	55.55(48.23)	49.99(44.99)	52.77
T5	Metalaxyl + Garlic	6 g/kg seed + 100 ml/kg soil	33.33(34.78)	26.11(30.60)	29.72
T6	Metalaxyl + *T.viride*	6 g/kg seed + 25 g/kg soil	38.88(38.50)	36.11(36.91)	37.49
T7	Captan + Metalaxyl + Garlic	1.5 g + 3 g/kg seed + 100 ml/kg soil	22.21(27.81)	21.66(27.70)	21.93
T8	Captan + Metalaxyl + *T.viride*	1.5 g + 3 g/kg seed + 25 g/kg soil	33.33(34.78)	26.11(30.60)	29.72
T9	Metalaxyl + Garlic + *T.viride*	3 g/kg seed + 100 ml/kg soil + 25 g/ kg soil	33.33(34.78)	41.66(40.18)	37.49
T10	Captan + Metalaxyl + Garlic + *T.viride*	1.5 g + 3 g/kg seed + 100 ml/kg soil+25 g/kg soil	16.66(24.08)	20.00(26.56)	18.33
T11	Control	-	72.21(58.44)	66.66(54.77)	69.43
SE±	-	-	4.15	1.91	-
CD	-	-	12.16	5.62	-

Figure in parenthesis are angular transformed values.

P. fluorescens are in conformity with those reported earlier by several workers (Manoranjitham et al., 2000; Chakrabarti et al., 2005; Pandey and Pandey, 2005; Valerie et al., 2005, Abeysinghe, 2009).

Integrated disease management strategies

The results obtained on IDM *in vitro* studies (pot culture) of 11 treatments against *P. ultimum* of present study are presented in The Table 4 and depicted in Plate 10 and Figure 4.

Pre-emergence damping off

Results (Table 4) reveals that the percent pre-emergence damping off recorded with all the treatment ranged from 16.66 to 55.55% as against 72.21% in control. The least pre-emergence damping off percentage was recorded with the treatment (T_{10}) Captan + Metalaxyl + Garlic + *T. viride* (16.66%), followed by the treatment (T_7): Captan + Metalaxyl + Garlic (22.21%). The treatments T_2, T_5, T_8, T_9 were found on par with T_{10} and T_7.

The maximum pre-emergence damping off was recorded with the treatment (T_4, *T. viride)* (55.55%), followed by the treatment (T_3, Garlic) (44.44%) and depicted in Plate 10 and Figure 4.

Post emergence damping off

Results (Table 4) revealed that the percent post emergence damping off recorded with all the treatments ranged from 20% to T_4 as against 66.66% in control.

The similar pattern of result was recorded in post emergence damping off as observed in pre-emergence damping off. The least post emergence damping off was recorded with the treatment Captan + Metalaxyl + Garlic + *T. viride* (T_{10}) (20%) followed by Captan + Metalaxyl + Garlic (T_7) (21.66%). The treatments T_2, T_5 and T_8 were found on par with T_{10} and T_7. The maximum post emergence damping off, was recorded with the treatment T_4: *T. viride* (49.99%), followed by the treatment T_3: Garlic (41.66%) and treatment T_9: Metalaxyl + Garlic + *T. viride* (41.66%) and depicted in Plate 10 and Figure 4.

Average percent mortality

Results (Table 4) reveal that the average percent mortality of pre and post emergence damping off with all the treatments ranged from 18.33 to 52.77% as against 69.43% in control.

The least average per cent mortality was recorded with the treatment T_{10}: Captan + Metalaxyl + Garlic + *T. viride* (18.33%), followed by the treatment T_7: Captan + Metalaxyl (21.93%). The treatments T_2, T_5 and T_8 (29.72%) where found on par with T_{10} and $T_{7.}$

Comparatively maximum average mortality was recorded with the treatment T_4: *T.viride* (52.77%), followed by the treatment T_3: garlic (43.05%), T_1, T_6 and T_9 (37.49%).

Reduction in damping off

The results obtained on integrated disease management *in vitro* studies (pot culture) of 11 treatments against *P. ultimum* are presented in Table 4.

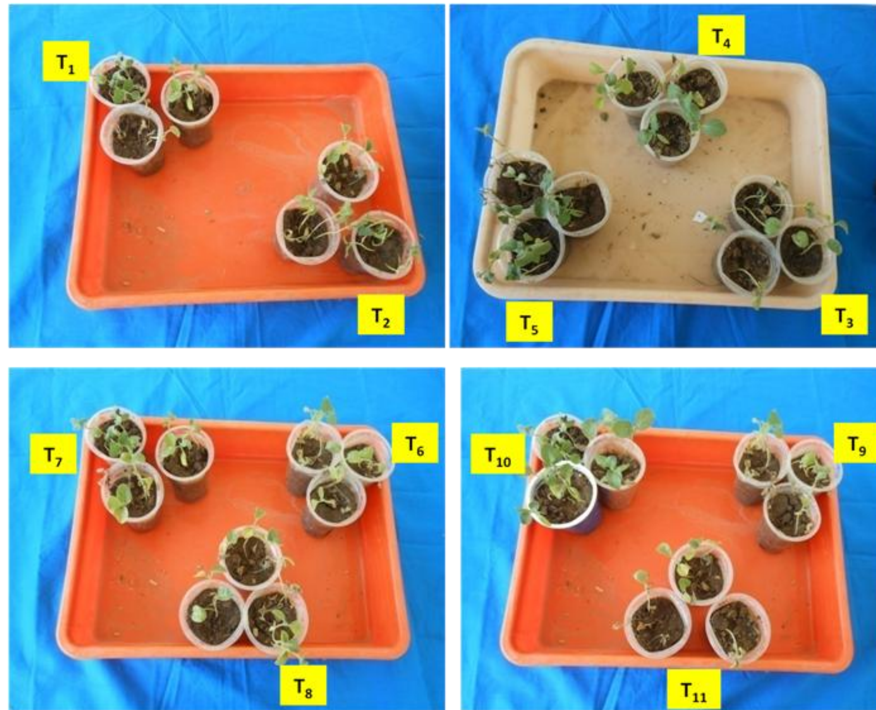


Plate 10. Experiment (pot culture) on integrated management of damping off in Brinjal Cv. Hadgaon local

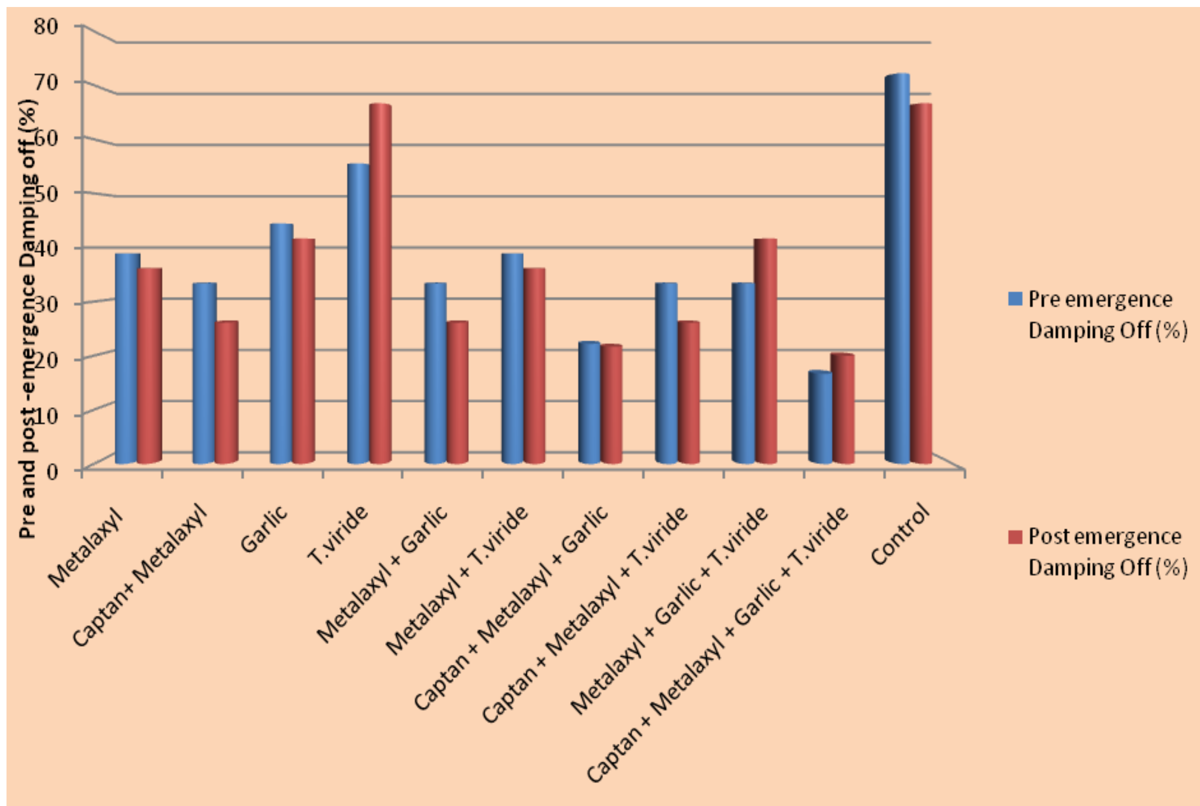


Figure 4. IDM (pot culture) of *P. ultimum* Trow. with effective Fungicides, botanicals and bioagents on brinjal cv. Hadgaon local.

Table 5. Efficacy of fungicides, bioagents and botanicals in reduction damping off in brinjal cv. Hadgoan local

T. No.	Treatments	Rate of application	Percent reduction in damping off		Average reduction (%)
			Pre emergence	Post emergence	
T_1	Metalaxyl	6 g/kg seed	46.15 (42.79)	45.82 (42.60)	45.98 (42.69)
T_2	Captan+ Metalaxyl	1.5 g + 3g/kg seed	53.84 (47.20)	60.83 (51.25)	57.33(49.21)
T_3	Garlic	100 ml/kg soil	38.45 (38.32)	37.50 (37.76)	33.97 (35.65)
T_4	*T.viride*	25 g/kg soil	23.07 (28.70)	25.50 (30.32)	24.28 (29.52)
T_5	Metalaxyl+ Garlic	6 g/kg seed + 100ml/kg soil	53.84 (47.20)	60.83 (51.25)	57.33 (49.21)
T_6	Metalaxyl+ *T.viride*	6 g/kg seed + 25g/kg soil	46.15 (42.79)	45.82 (42.60)	45.98 (42.69)
T_7	Captan+ Metalaxyl+Garlic	1.5 g + 3 g/kg seed + 100 ml/kg soil	69.24 (56.31)	65.50 (54.02)	67.37 (55.16)
T_8	Captan+ Metalaxyl+ *T.viride*	1.5 g + 3g/kg seed + 25 g/kg soil	53.84 (47.20)	60.83(51.25)	57.33 (49.21)
T_9	Metalaxyl + 0 Garlic + *T.viride*	3 g/kg seed + 100 ml/kg soil + 25 g/kg soil	53.84 (47.20)	37.50 (37.76)	45.67 (42.51)
T_{10}	Captan + Metalaxyl + Garlic + *T.viride*	1.5 g + 3 g/kg seed + 100ml /kg soil + 25 g/kg soil	76.92 (61.28)	69.99 (56.78)	73.45 (58.98)
T_{11}	Control (untreated)		00.00 (00.00)	00.00 (00.00)	00.00 (00.00)

Figure in parenthesis are angular transformed values.

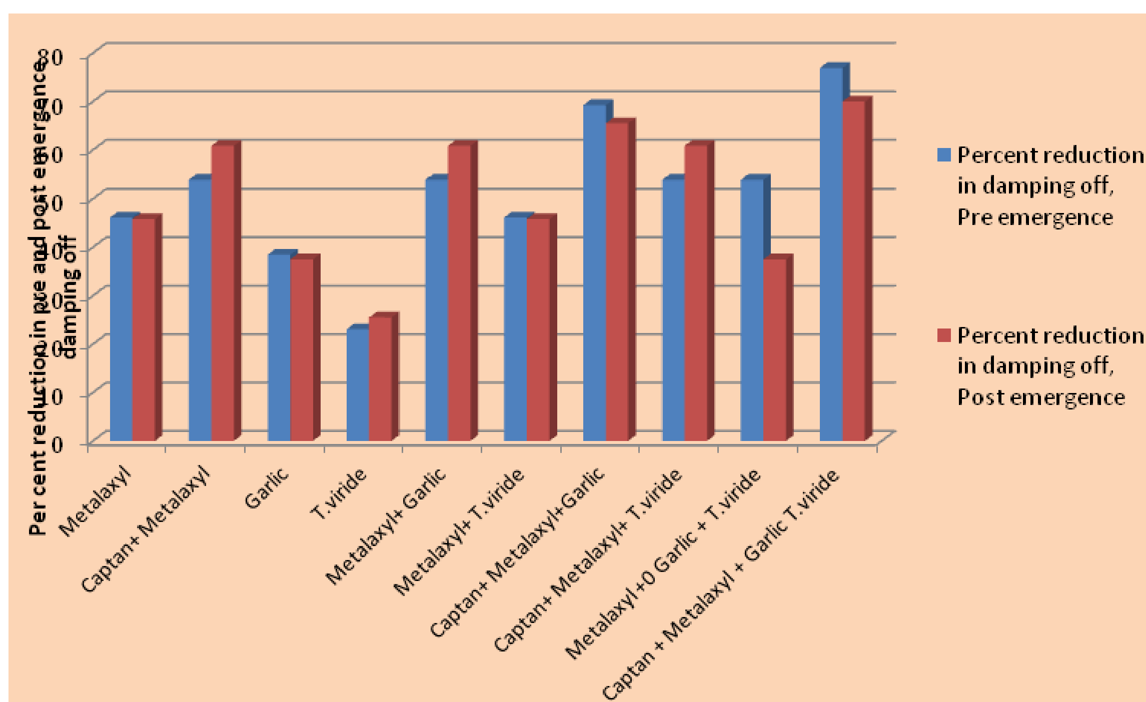


Figure 5. Efficacy of fungicides, bioagents and botanicals in reducing Pre and post emergence damping of in brinjal cv. Hadgaon local.

Percent reduction in pre-emergence damping off

Results (Table 5 and Figure 5) reveal that the percent reduction in pre-emergence damping off recorded with all the treatments ranged from 23.07 to 76.92% as against the control.

The maximum percent reduction in pre-emergence damping off was recorded with the treatment T_{10}:

Captan + Metalaxyl + Garlic + *T. viride*) (76.92%), followed by the treatment T_7: Captan + Metalaxyl + Garlic (69.24%). The treatment T_2, T_5, T_8 and T_9 were found on par with T_{10} and T_7.

The minimum percent reduction in pre-emergence damping off was recorded with the treatment T_4: *T. viride* (23.07%), followed by the treatment T_3: Garlic (38.45%), T_1: Metalaxyl (46.15%) and (T_6) Metalaxyl + *T. viride* (46.11%).

Percent reduction in post emergence damping off

Results (Table 5 and Figure 5) revealed that the percent reduction in post emergence damping off recorded with all the treatments ranged from 25.50 to 69.99%.

The maximum percent reduction in post emergence damping off was recorded with treatment T_{10}: Captan + Metalaxyl + Garlic + *T. viride* (69.99%) followed by the treatment T_7: Captan + Metalaxyl + Garlic (65.50%). The treatments T_2, T_5 and T_8 (60.83%) were found on par with T_{10} and T_7.

The minimum percent reduction in post emergence damping off was recorded with the treatment (T_4) *T. viride* (25.50%), followed by treatment T_3: Garlic (37.50%), T_9: Metalaxyl + Garlic (37.50%), $T_{1:}$ Metalaxyl (45.82%) and T_6 Metalaxyl + *T. viride* (45.82%).

Average percent reduction in damping off

Results (Table 5) reveals that the averaged percent reduction in pre and post emergence damping off with all the treatments ranged from 24.28 to 73.45%.

The maximum percent reduction in damping off was recorded with the treatment T_{10}: Captan + Metalaxyl + Garlic + *T. viride* (73.45%), followed by the treatment T_7: Captan + Metalaxyl + Garlic (67.37%). The treatment T_2, T_5 and T_8 (57.33%) were found at par with treatment T_{10} and T_7.

Comparatively minimum percent reduction in damping off was recorded with the treatment T_4: *T. viride* (24.28%) followed by the treatments T_3: Garlic (33.97%), T_9: Metalaxyl + Garlic + *T. viride* (45.67%), T_1: Metalaxyl (45.98%) and T_6: Metalaxyl + *T. viride* (45.98%). The result of the present study obtained in respect of IDM of *P. ultimum* Trow. with the fungicides, botanicals and plant extracts are in conformity with those reported earlier by several workers (Arya, 2004; Rakesh and Hooda, 2007; Muthukumar et al., 2010).

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Variability among the potato sclerotial isolates of *Rhizoctonia solani* of Mountainous Region, Gilgit-Baltistan, Pakistan

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An experiment was carried out to find the variability among twenty sclerotial isolates of *Rhizoctonia solani* collected from potato growing areas of Gilgit-Baltistan. These isolates were grown in different culture media, mycelial compatibility and fungus protein profile were investigated. Culture media were used to study radial colony growth and sclerotial production of isolates. Radial colony growth (RCG) and sclerotial production (SP) of isolates against culture media ranged from 12.31-21.55; 3.66-22.66 in potato dextrose agar, 12.67-18.56; 4.66-10.66 in czpedox agar, 12.02-20.42; 2.00-8.66 in corn meal agar and 10.54-14.16; 0.00-3.00 in water agar, respectively. These isolates were further classified into three categories on the basis of RCG and SP. Result revealed that out of total isolates, 60% showed medium RCG and 40% fast growth, while 10, 60 and 30% isolates showed low, medium and high SP. Furthermore, sclerotial characteristic such as size, shape and distribution pattern were also recorded. Mycelial compatibility and incompatibility among the *R. solani* isolates was also studied. The results indicated that out of 190 combinations, 72.10% were compatible, whereas 27.90% were incompatible. Fungus protein profile of twenty isolates of *R. solani* by sodium dodecyl suplphate gel electrophoresis (SDS-PAGE) recovered sixty one bands with different frequencies among the isolates. Cluster analysis of twenty isolates divided them into two major lineage groups, A and B. Lineage A contained 65% isolates whereas lineage B contained 35% isolates. These lineages were further divided into thirteen clusters (C_1-C_{13}); A was comprised of eight and B five clusters, respectively.

Key words: *Rhizoctonia solani*, sclerotial isolates, radial colony growth, sclerotial feature, mycelial compatibility, sodium dodecyl suplphate gel electrophoresis (SDS-PAGE), Gilgit-Baltistan.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the important vegetable crops in the world. In Pakistan, its production increases year by year. According to Agricultural statistics, potato is grown on an area of 145000 ha during

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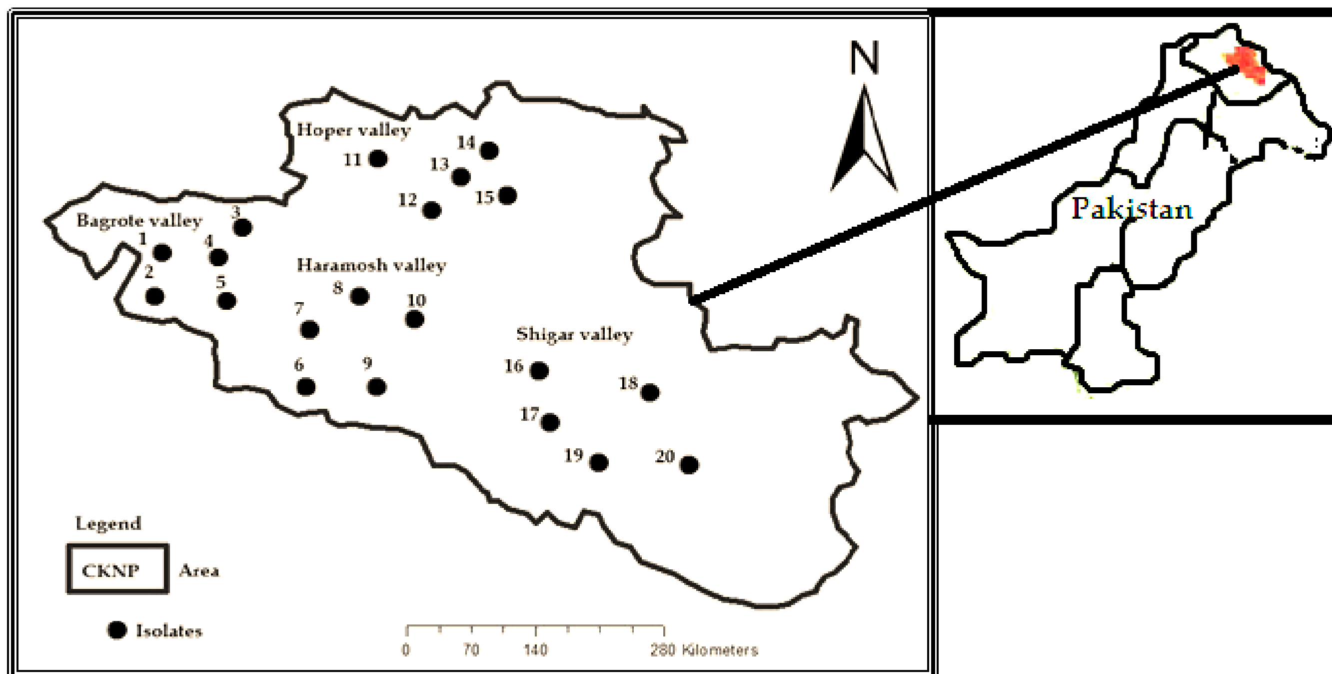


Figre 1. Map of the study area and dot showing collection site of isolates.

the year 2008-2009, with a production of 2941300 tons (Agricultural Statistics of Pakistan, 2008-2009) while in Gilgit-Baltistan, it is cultivated on an area of 8526 h with a production of 134031 Mt/ha (Agricultural Statistics Gilgit-Baltistan, 2009). In Gilgit-Baltistan, the production of potato is low as compared to its potential. Many biotic (fungal, bacterial and viral) and abiotic (temperature, pH, humidity and soil nutrients) constrains are involved. Many diseases especially black scurf caused by *Rhizoctonia solani* are the important biotic constraints (Bhutta et al., 2004). *Rhizoctonia solani* (teleomorph *Thanatephorus cucumeris*) was reported nearly 150 years ago as destructive pathogen of potato crop by Kuhn (1858). It is a widely distributed pathogen affecting different economically important crops, especially potato, where the tuber quality and production are highly decreased by the action of this fungus (Krechel et al., 2002).

Infection starts when mycelia or hyphae from a germinating sclerotium starts to grow towards a suitable host as a result of attracting chemical exudates, e.g., amino acids, sugars, organic acids and phenols, from the plants (Keijer, 1996). Inoculum sources of *R. solani* include seed tubers and soil, both of which can harbour mycelium and sclerotia (Tsror and Peretz-Alon, 2005). *R. solani* infects subterranean stems and stolons, and severe lesions can have a negative effect on plant growth and tuber development (Banville et al., 1996). Mycelia and sclerotia can grow and develop on plant debris as well as tubers, allowing inoculum to survive in the soil as well as on seed from season to season (Dijst, 1988; Gudmestad et al., 1979).

Several attempts have been made to group the isolates of this pathogen taxonomically. So far, anastomosis of mycelia is the criterion most widely accepted and used to group the isolates of this fungus (Anguiz, 1989). But grouping by anastomosis does not always correspond to grouping by colony morphology, pathogenicity or other physiological features (Carling, 1996).

R. solani is highly diverse, comprises of a number of genetically different groups, often varying in their cultural, morphological, pathological and physiological characters (Ou, 1985). The variation in the fungus *R. solani* has been reported from different parts of the world and can affect management of the disease (Basu et al., 2004). It occurs as an aggregate of strains varying in cultural appearance, anastomosis grouping and physiology (Parmeter and Whitney, 1970).

MATERIALS AND METHODS

Collection of potato tuber

Twenty isolates of *R. solani* used in the current study were isolated from potato tuber showing typical symptoms of black scurf. All samples were collected during the harvesting stage from four valleys and key potato growing villages of Central Karakoram Nation Park of Gilgit-Baltistan, Pakistan (Figure 1). The collected samples were packed in polythene bags and transferred to the laboratory for variability study among the isolates of *R. solani* designated as RS_1-RS_{20}. Samples were surface sterilized by 0.1% mercuric chloride for 2-3 min and extensively washed with sterile distilled water. Sclerotial spot on tuber surface were scratched and placed on Petri plates containing potato-dextrose-agar (PDA). Each isolates was further purified by hyphal tip method (Mundkur, 1959) and maintained as pure culture and stored at 4°C for further study.

Morphological and sclerotial variability of *R. solani* isolates

Morphological and sclerotial variability of *R. solani* isolates were studied on four solid media viz, PDA, CZPA, CMA and WA. Twenty millilitres of each medium was poured into sterilized Petri-dishes and 5 mm actively growing mycelial plug were inoculated. Experiment was repeated twice and maintained three replicates in each isolate. Radial colony growth was recorded every 24 h during 4 days at 25 ± 2°C. Mean radial colony growth rate per day was determined by dividing colony growth recorded at 96 h by 4 days. Radial colony growth rate (mmd^{-1}) was calculated by using following formula as described by Guleria et al. (2007).

$$\mathrm{RCG\ d^{-1}} = \frac{\mathrm{RCG\ 96h}}{4}$$

RCG = Radial colony growth.

Sclerotial variability was studied after three weeks and also number of sclerotia was counted under binocular microscope (Goswami et al., 2011). Based on radial colony growth rate (mmd^{-1}) and sclerotial production (cm^2), isolates were categorized into three groups as: RCG = low (<10), medium (10-20) and fast (>20) and SP = low (<5), medium (5-10) and fast (>10), respectively.

Size, shape and distribution pattern among the isolates were also recorded as described by Jayaprakashvel and Mathivanan (2012).

Mycelial compatibility

Mycelial discs (5 mm in diameter) taken from the edge of an actively growing colony (4th day old) of each isolate were placed 40 mm apart on opposite sides of Petri dishes (90 mm in diameter) and incubated at 25 ± 2°C. Two isolates were paired on one Petri dish and the test was repeated twice. The pairings were examined macroscopically for presence of an antagonistic (barrage or aversion) zone in the region of mycelial contact as described by Punja and Grogan (1983).

Extraction of proteins from *R. solani* isolates

Protein profile of *R. solani* isolates was carried out using SDS-PAGE as described by Laemmli (1970). Fungus protein was extracted from the mycelium mat of each isolate grown on potato dextrose agar plate for 6 days at 27± 2°C. Mycelium of each isolate was harvested then dried. A dry mycelium of each isolate was ground to make a fine powder with mortar and pestle.

Mycelium flour 0.01 g was added to 400 µl extraction buffer (0.5 M Tris-HCL (pH 6.8), 2.5 SDS, 10% glycerol and 5% 2-mercaptoethanol) mixed 400 µl in Eppendorf tube and vortexed (Automatic lab Mixer DH-10). Then, the samples were centrifuged at 15,000 rpm at least ten minutes at ambient temperature. The clear supernatant was transferred into 1.5 ml Eppendorf tubes and stored at 2°C until they were run on the polyacrylamide gel.

Electrophoretic procedure

Fungus protein was analyzed through slab type SDS-PAGE using 12.25% polyacrylamide gel PAGE (Model: AE-6530M, Japan), resolving gel (3.0M Tris-HCl) pH9, 0.4% SDS and 4.5% stacking gel (0.4M Tris-HCl pH 7.0, 0.4% SDS). Electrode buffer (0.025 M Tris, 129 M Glycine, 0.125 % SDS) was loaded top pool of apparatus. A 15 µl of the supernatant of isolates along with marker were loaded into the wells of the gel. Apparatus was connected with uninterrupted electric supply (100 V) until the bromophenol blue (BPB) reached the bottom of the gel plate.

Gel staining

Gel were placed in staining solution (0.2% Commassie Brilliant Blue dissolved in 10% glacial acetic acid, 40% methanol and water in the ratio of 10:40:50) for one hour then placed in destaining solution (5% acetic acid and 20% methanol). Destained gels were analyzed directly using photographic method or drying gel by gel-drying processor for about 2-4 h.

Statistical analysis

The design of *in vitro* experiment was a randomized complete block and analysis of variance (ANOVA) of the data was performed using the statistical package STATISTICA 8.1 and SPSS Version 16.0 for Windows 2007.

RESULTS

Effect of different culture media on colony growth rate and sclerotium production

In this study, different culture media and twenty isolates of *R. solani* were used to determine suitable medium and variability of radial colony growth, sclerotium production, size, shape and distribution of sclerotia. Four culture media viz., PDA, CZPA, CMA and WA were used. During the studies, it was observed that the colony growth rate and sclerotium production in the PDA ranged from 12.31-21.55; 3.66-22.66 in CZPA, 12.67-18.56; 4.66-10.66 in CMA 12.02-20.42; 2.00-8.66 and in WA (10.54-14.16; 0.00-3.00). Mean radial colony growth and sclerotial production were recorded in PDA (18.29, 9.94), CZPA (16.76, 6.83), CMA (15.80, 4.79) and WA (12.70, 1.33). The result observed that potato dextrose agar were suitable medium for culture of *R. solani* (Table 1 and Figure 5). Table 2 showed that the *R. solani* isolates grown in potato dextrose agar were further categorized on the basis of slow, medium and high. Result indicated that 60% of isolates had medium and 40% had fast growth on the basis of radial colony growth (Figure 2) while on the basis of sclerotium production, 10% isolate had low, 60% medium and 30% high (Figure 3). Besides these, significant variations among sclerotial size, shape and distribution pattern of *R. solani* isolates were observed (Table 2, Figure 4 and Figure 6).

Mycelial compatibility group

There were one hundred and ninety combinations of the twenty isolates of *R. solani.* Amongst only 53 showed incompatibility reaction and 137 showed compatibility (Table 3). For combinations which showed antagonistic reactions with each other, a thin band of living or dead mycelia was formed (Figure 7). Based on mycelial compatibility, 72.10% mycelial compatibility and 27.90% non compatible among the tested isolates were shown.

Table 1. Colony mycelial growth, rate/day and sclerotium production of twenty isolates of *R. solani* on different media culture at 25±2°C.

Isolates	Media							
	Colony growth rate (mmd^{-1})				Sclerotium production(cm^2)			
	PDA	CZPA	CMA	WA	PDA	CZPA	CMA	WA
RS_1	14.55^{K}	12.67^{L}	12.02^{O}	10.54^{I}	15.33^{D}	10.66^{A}	8.66^{A}	2.00^{ABC}
RS_2	21.12^{C}	14.66^{K}	13.86^{N}	11.04^{HI}	18.33^{C}	7.00^{BCD}	5.00^{BCDEF}	2.00^{ABC}
RS_3	21.36^{B}	17.08^{F}	14.56^{M}	11.32^{H}	21.00^{AB}	5.00^{EF}	5.00^{BCDEF}	2.66^{AB}
RS_4	21.55^{A}	18.56^{A}	15.64^{GH}	12.56^{FG}	22.66^{A}	8.00^{B}	7.33^{AB}	2.00^{ABC}
RS_5	21.09^{C}	18.20^{B}	16.12^{E}	12.80^{EF}	18.66^{BC}	6.00^{CDEF}	6.00^{ABCDE}	0.00^{D}
RS_6	14.58^{K}	15.52^{H}	16.64^{D}	12.58^{EFG}	9.00^{EFG}	4.66^{F}	5.33^{BCDEF}	2.66^{AB}
RS_7	13.12^{M}	17.58^{D}	17.12^{C}	12.92^{DEF}	7.00^{FGH}	7.00^{BCD}	4.00^{CDEFG}	1.66^{ABCD}
RS_8	14.02^{L}	15.06^{J}	17.26^{BC}	12.74^{EF}	6.00^{HIJ}	7.00^{BCD}	3.66^{DEFG}	0.00^{D}
RS_9	12.31^{N}	17.38^{E}	14.84^{KL}	13.04^{CDEF}	4.00^{IJ}	6.00^{CDEF}	6.33^{ABCD}	2.00^{ABC}
RS_{10}	21.09^{C}	16.83^{G}	20.42^{I}	13.56^{ABCD}	10.00^{E}	8.33^{B}	4.33^{CDEFG}	0.00^{D}
RS_{11}	18.14^{H}	18.08^{B}	17.54^{A}	14.16^{A}	7.00^{FGH}	6.00^{CDEF}	4.00^{CDEFG}	3.00^{A}
RS_{12}	20.52^{D}	18.56^{A}	17.44^{AB}	13.82^{AB}	6.33^{HI}	7.33^{BCD}	6.66^{ABC}	1.00^{BCD}
RS_{13}	16.10^{J}	16.66^{H}	15.82^{F}	13.60^{ABC}	3.66^{J}	7.66^{BC}	4.33^{CDEFG}	0.00^{D}
RS_{14}	19.58^{F}	15.28^{I}	15.08^{J}	12.66^{EFG}	6.66^{GH}	6.66^{BCDE}	3.33^{EFG}	1.00^{BCD}
RS_{15}	21.26^{B}	17.38^{E}	14.72^{L}	10.92^{HI}	9.33^{EF}	6.00^{CDEF}	3.00^{FG}	1.00^{BCD}
RS_{16}	21.05^{B}	14.69^{K}	15.08^{J}	13.21^{BCDEF}	11.00^{E}	5.00^{EF}	3.00^{FG}	1.00^{BCD}
RS_{17}	18.96^{G}	17.64^{D}	15.80^{FG}	13.68^{ABC}	7.00^{FGH}	7.33^{BCD}	2.00^{G}	2.00^{ABC}
RS_{18}	19.98^{E}	17.86^{C}	15.56^{H}	13.64^{ABC}	6.00^{HIJ}	7.33^{BCD}	5.00^{BCDEF}	0.33^{CD}
RS_{19}	17.53^{I}	18.04^{B}	14.98^{JK}	12.06^{G}	5.00^{HIJ}	5.66^{DEF}	4.66^{BCDEFG}	0.66^{CD}
RS_{20}	18.05^{H}	17.52^{DE}	15.52^{H}	13.22^{BCDE}	5.00^{HIJ}	8.00^{B}	4.33^{CDEFG}	1.66^{ABCD}
Mean	18.29	16.76	15.80	12.70	9.94	6.83	4.79	1.33
Minimum	12.31	12.67	12.02	10.54	3.66	4.66	2.00	0.00
Maximum	21.55	18.56	20.42	14.16	22.66	10.66	8.66	3.00
SD	3.11	1.59	1.70	1.03	5.92	1.39	1.60	0.96
CV	17.00	9.49	10.79	8.16	59.52	20.35	33.36	72.58

All the values are means of three replications and values with same letters are not statistically different at LSD 0.05%. PDA: Potato dextrose agar; MYE: malt yeast agar; CMA: corn meal agar; WA: water agar. RS = isolate of *Rhizoctonia solani.*

SDS-PAG gel electrophoresis

Twenty isolates of *R. solani* were subjected to SDS-PAGE gel electrophoresis producing sixty one fungal protein bands. On the basis of presence or absence of protein bands in individual isolates, RS_{17} exhibited highest number of protein bands followed by RS_6, RS_8, RS_{10}, RS_{13}, RS_{19} and RS_{20} whereas on the basis of molecular weight, highest number of bands were produced at 18 kDa and lowest were observed in 45 kDa (Figures 8 and 9). Cluster analysis divided twenty isolates into two major lineage groups, groups A and B. Lineage A contains 65% of isolates whereas lineage B contains 35% isolates (Figure 10). These lineages were further divided into thirteen clusters (C_1-C_{13}). Lineage A consists of eight cluster while lineage B comprises five clusters. Among the lineage A, C_1 contains three isolates, C_3, C_4, and C_6 has two isolates, while C_2, C_5, C_7 and C_8 comprises single isolate each. Similarly, in the lineage B, C_9 consists of three isolates while C_{10}, C_{11}, C_{12} and C_{13} contain single isolate each (Table 4).

DISCUSSION

Different culture media, mycelial compatibility and protein profile through SDS-PAGE were used to study variability of *R. solani* isolates. All isolates of *R. solani* showed variation in radial colony growth and sclerotial production against culture media. Maximum radial colony growth and sclerotial production was found in PDA which was followed by CZPA and CMA, while least radial colony growth and sclerotial production was recorded in WA. The results were in agreement with that of Lalan et al. (2013) who studied colony diameter, growth, colour and sclerotia formation of six isolates of *R. solani* of soybean and concluded that PDA was best for growth and development among the tested culture media. It has been further observed that PDA is a frequently used medium, due to its simple formulation and supportive nature of different plant pathogenic fungi (Maheshwari et al., 1999; Saha et al., 2008). Numerous researchers have confirmed that PDA is most excellent for colony growth of different fungi (Xu et al., 1984; Meera et al., 2012;

Table 2. Classification of *R. solani* isolates on the basis of radial colony growth, sclerotial production, size, shape and distribution pattern.

S/N	Category	Number	Isolates
Radial colony growth			
1	Low < 10	0	None
2	Medium 10-20	12	RS_{1}, RS_{6}, RS_{7}, RS_{8}, RS_{9}, RS_{11}, RS_{13}, RS_{14}, RS_{17}, RS_{18}, RS_{19} and RS_{20}
3	High >20	8	RS_{2}, RS_{3}, RS_{4}, RS_{5}, RS_{10}, RS_{12}, RS_{15} and RS_{16}
Sclerotial production		**Number**	**Isolates**
1	Low <5	2	RS_{1} and RS_{9}
2	Medium 5-10	12	RS_{6}, RS_{7}, RS_{8}, RS_{10}, RS_{11}, RS_{12}, RS_{13}, RS_{14}, RS_{17}, RS_{18}, RS_{19} and RS_{20}
3	High >10	6	RS_{2}, RS_{3}, RS_{4} RS_{5} , RS_{15} and RS_{16}
Sclerotial characteristics		**Number**	**Isolates**
	Macro	10	RS_{1}, RS_{6}, RS_{7}, RS_{8}, RS_{9}, RS_{10}, RS_{11}, RS_{12}, RS_{13}, RS_{14},
Size	Micro	6	RS_{2}, RS_{3}, RS_{17}, RS_{18}, RS_{19} and RS_{20}
	Mixed	4	RS_{3}, RS_{4}, RS_{5}, RS_{15} and RS_{16}
	Spherical	3	RS_{1}, RS_{6} and RS_{8}
Shape	irregular	12	RS_{2}, RS_{3}, RS_{5}, RS_{7}, RS_{12}, RS_{13}, RS_{14}, RS_{17}, RS_{18}, RS_{19}, RS_{20}, and RS_{15}
	Mixed	5	RS_{4}, RS_{6}, RS_{9}, RS_{10} and RS_{11}
	Scatter	7	RS_{1}, RS_{5}, RS_{6}, RS_{11}, RS_{12}, RS_{14} and RS_{15}
Distribution	Concentrated	9	RS_{4}, RS_{7}, RS_{8}, RS_{13}, RS_{17}, RS_{18} RS_{16}, RS_{19} and RS_{20}
	Mixed	4	RS_{2}, RS_{3}, RS_{9} and RS_{10}

RS = isolate of *Rhizoctonia solani.*

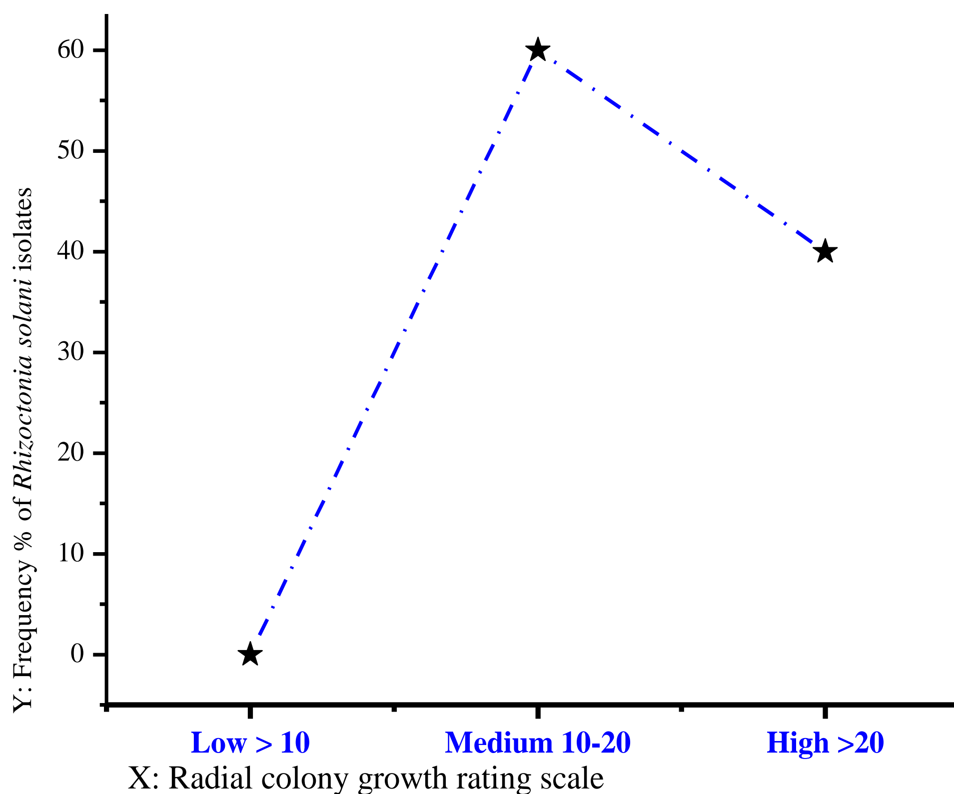


Figure 2. *Rhizoctonia solani* isolates classified into three groups on the basis of colony growth rate mmd^{-1} on PDA medium.

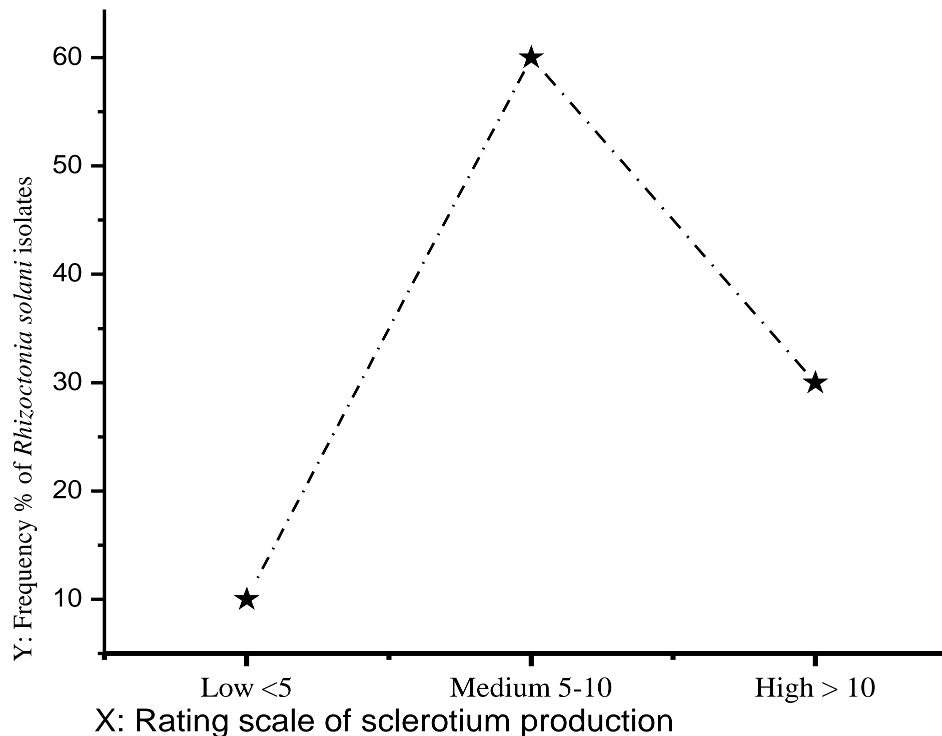


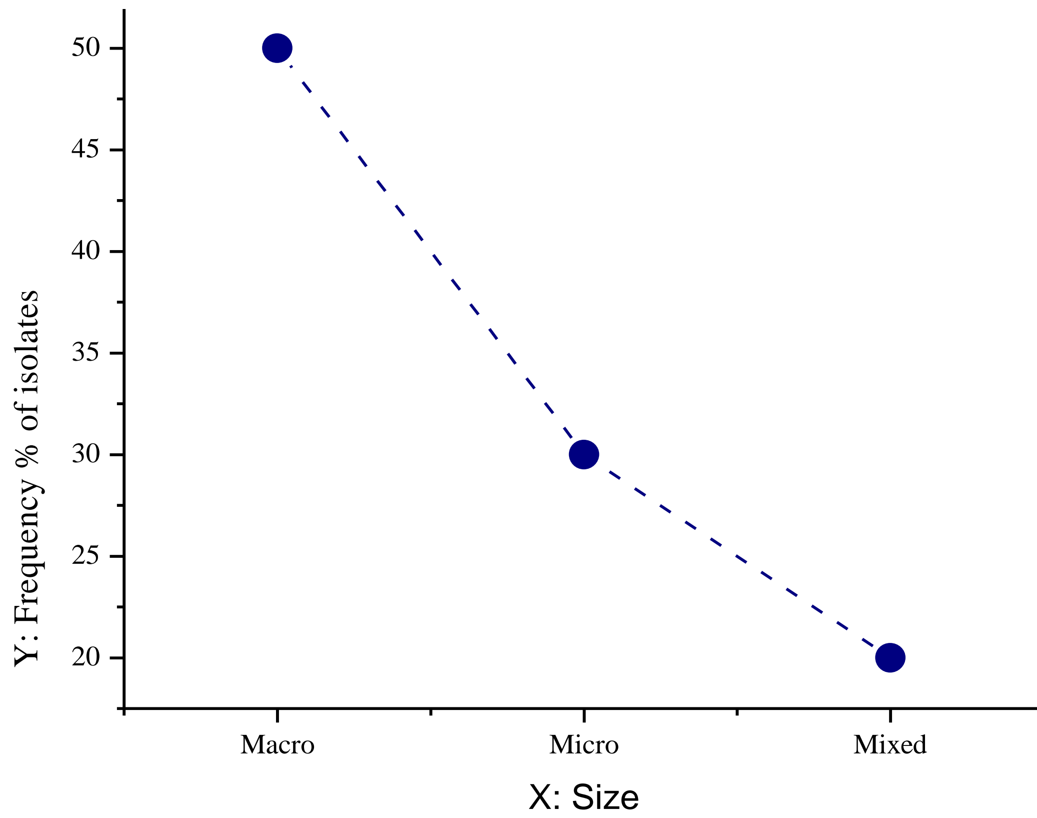
Figure 3. *Rhizoctonia solani* isolates classified into three groups on the basis of sclerotium production cm^2 on PDA medium.

Saha et al., 2008). *R. solani* is a complex pathogen with wide host range. Due to their ill-define taxonomy and poor understanding of natural history, its identification and study is always a challenging tasks (Cubeta and Vilgalys, 1997). Variability within the isolates of *R. solani* has been reported by many researchers (Sherwood, 1969; Neeraja et al., 2002; Linde et al., 2005; Guleria et al., 2007; Thind and Aggarwal, 2008). In our current study, different isolates of *R. solani* showed considerable variation in terms of redial colony growth, number, size, shape, and distribution pattern of sclerotia which is in agreement with report of Thind and Aggarwal (2008) who studied morphological and sclerotial characteristics of potato *R. solani* isolate. Yadav and Anamika (2005) studied morphological and culture variation of different isolates of *R. solani* that caused damping off fenugreek vegetable and concluded that all isolates differ in colony growth, colour, width and sclerotial production.

The finding of the current study revealed that majority of the isolates showed compatibility reaction by pairing. Mycelial compatibility and incompatibility reactions are a useful way of categorizing intraspecific heterogenecity. It is a self and non-self-recognition system controlled by multiple loci, but knowledge of the underlying genetic mechanisms is limited in most filamentous fungi (Glass and Kaneko, 2003). In contrast, isolates that are different at one or some or more of these loci will not anastomose. Rosa et al. (2012) studied compatibility/incompatibility of 433 *R. solani* isolates and found that about 91% isolates were incompatible, while 9% of the pairing were compatible. Majority of isolates showed compatibility in the same county except few isolates.

In the current study, different fungus protein band were recovered from mountaneous isolates of *R. solani*. The results also agree with previous finding of Monica et al. (1983) who studied protein pattern of different anastomosis group of *R. solani* and showed that there was sufficient variability among the isolates particularly AG3 having distinct protein band regardless of isolates sources. Zuber and Manibushanrao (1982) also reported that polyacrylamide gel electrophoresis showed marked variation among five virulent isolates of *R. solani*. Similarly, El-Akkad (1997) also recorded fungus protein band heterogeneity among the *R. solani* isolates of AG-4. Hussein et al. (2000) reported that cluster analysis of protein band recoverd by SDS-PAGE form seventeen isolates of multinucleate and binucleate *R. solani* showed clear-cut differentiating features between *Rhizoctonia* spp. The results of the current study showed variation in fungus protein banding. However, some isolates gave similar binding pattern, they could be differentiated by some components. Iqbal et al. (2005) reported that similarity might be due to comigration of different peptides on the SDS-PAGE and closely related ancestry.

The grouping on the basis of morphological characters was quite different from that which was on the basis of



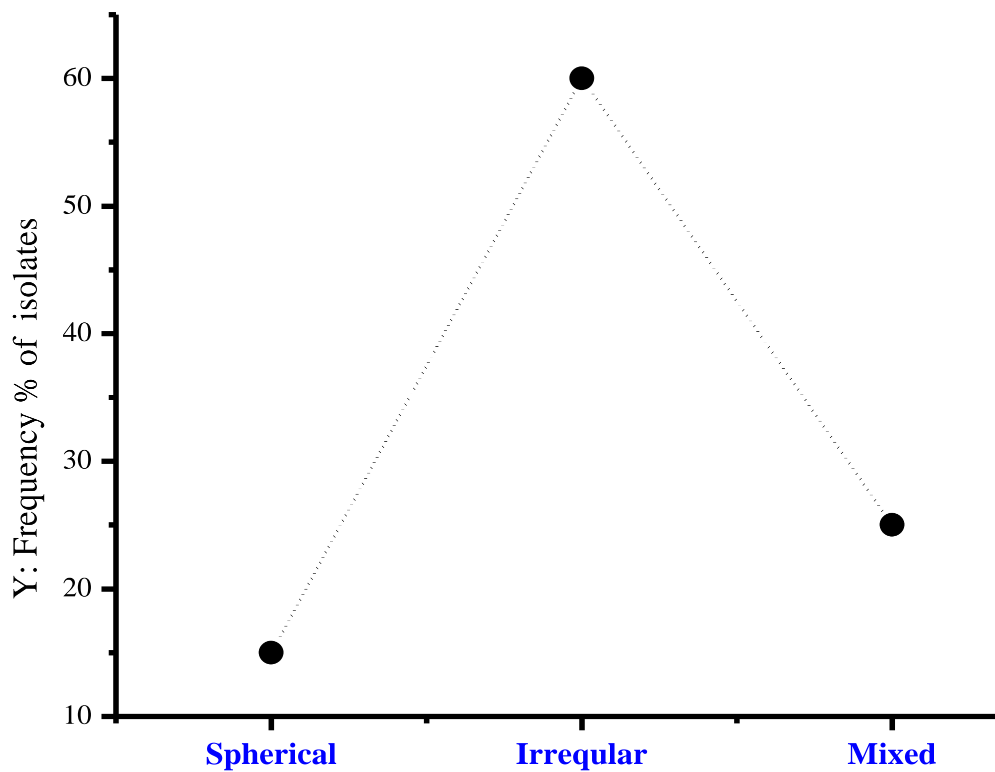
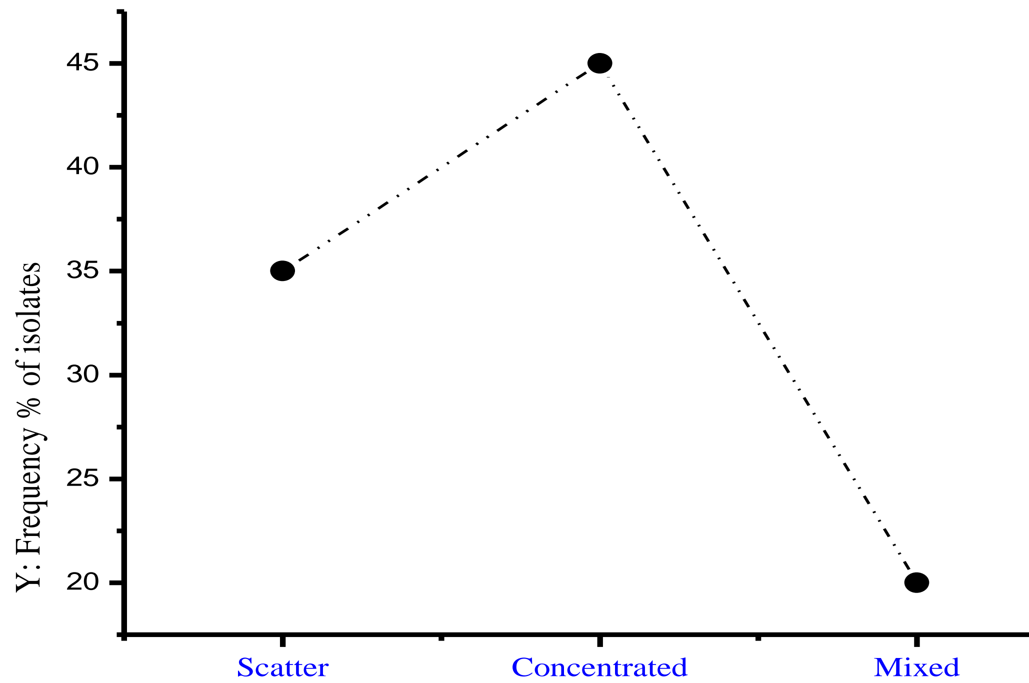


Figure 4. Sclerotial variability (size, shape and distribution pattern) of *Rhizoctonia solani* isolates.



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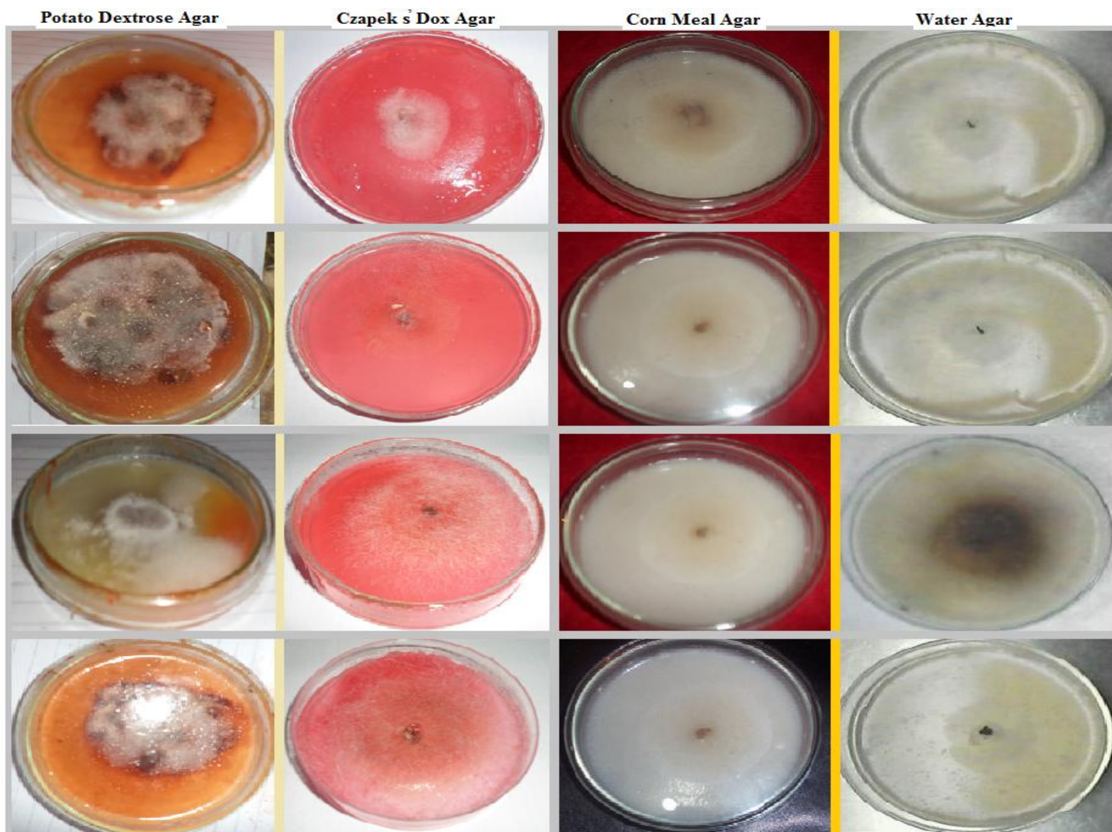

Figure 5. Radial colony growth of different isolates of R. *solani* on different culture media.

Table 3. Mycelial compatibility (MCGs) of different isolates of *Rhizoctonia solani* collected in CKNP region, Gilgit-Baltistan.

Isolates	RS_2	RS_3	RS_4	RS_5	RS_6	RS_7	RS_8	RS_9	RS_{10}	RS_{11}	RS12	RS_{13}	RS_{14}	RS_{15}	RS_{16}	RS_{17}	RS_{18}	RS_{19}	RS_{20}
RS_1	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve
RS_2		-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
RS_3			+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
RS_4				+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
RS_5					-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
RS_6						-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
RS_7							+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
RS_8								+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
RS_9									+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
RS_{10}										-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
RS_{11}											+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
RS_{12}												+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
RS_{13}													-ve	+ve	+ve	+ve	+ve	+ve	+ve
RS_{14}														+ve	+ve	-ve	+ve	+ve	+ve
RS_{15}															+ve	+ve	+ve	+ve	+ve
RS_{16}																-ve	+ve	-ve	+ve
RS_{17}																	+ve	+ve	-ve
RS_{18}																		+ve	+ve
RS_{19}																			+ve

+Ve = Mycelial compatible; -Ve = mycelial incompatible.

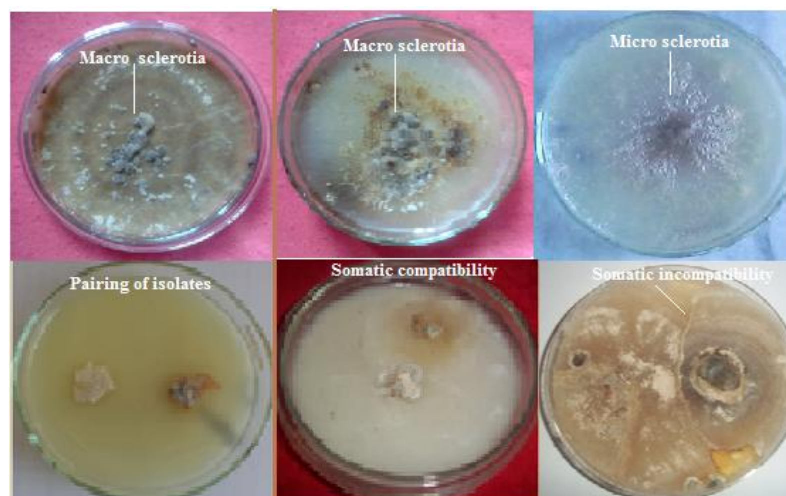

Figure 6. Sclerotial growth of different *R. solani* isolates.

Figure 7. Macro, micro sclerotia, pairing, mycelial compatibility/incompatibility.

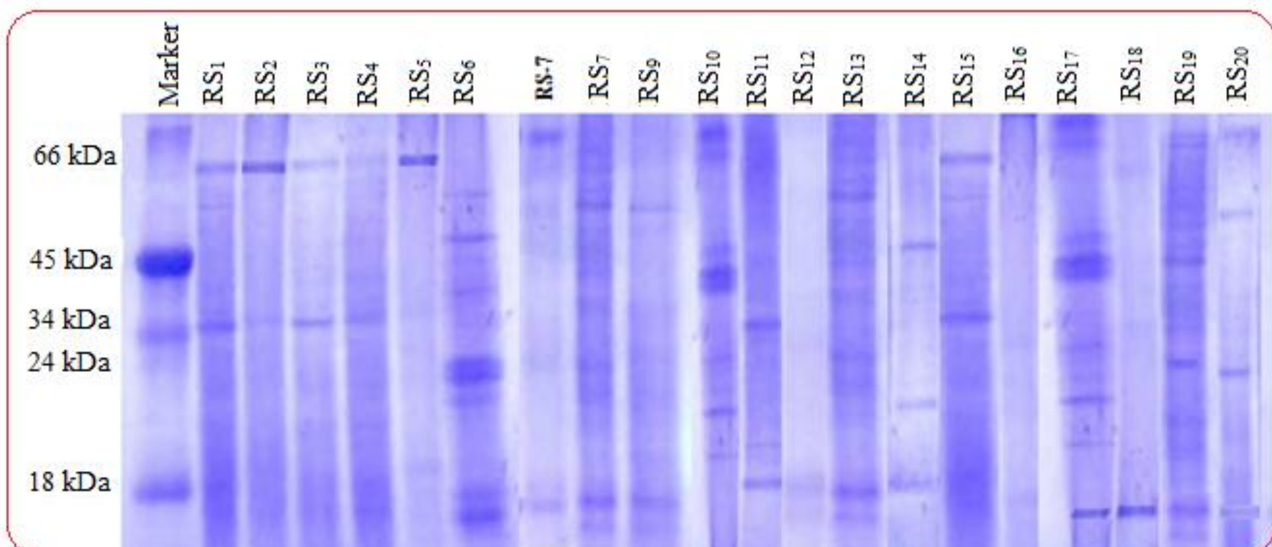

Figure 8. Electrophoregram of 20 isolates of *Rhizoctonia solani* showing polymorphic protein bands.

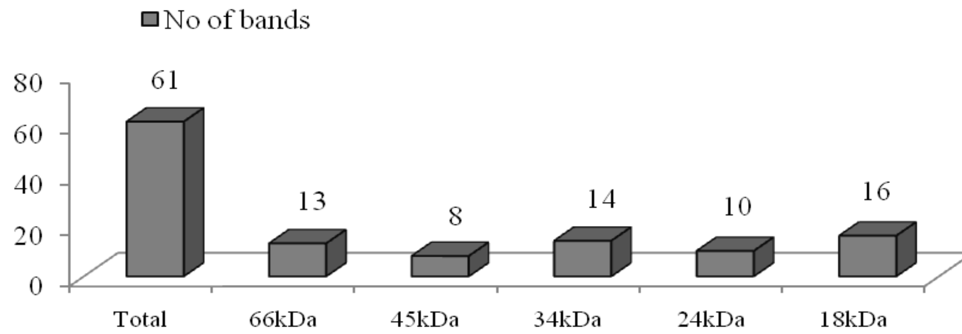

Figure 9. Number of protein bands present on the basis of molecular weight.

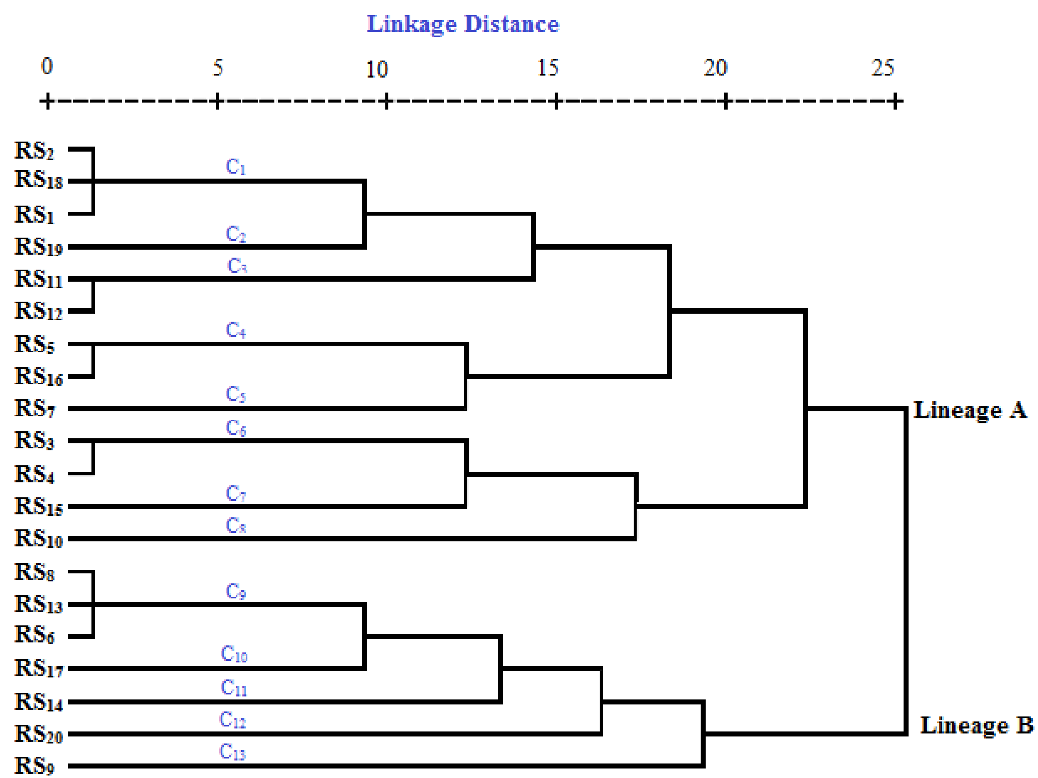

Figure 10. Dendrogram of twenty isolates of *Rhizoctonia solani* on the basis of SDS-PAGE.

Table 4. Cluster analysis of *R. solani* isolates based on SDS-PAGE electrophoresis.

	Cluster	Isolates		Cluster	Isolates
	C_1	RS_2, RS_{18}, RS_1		C_9	RS_8, RS_{13}, RS_6
	C_2	RS_{19}		C_{10}	RS_{17}
	C_3	RS_{11}, RS_{12}		C_{11}	RS_{14}
Lineage A	C_4	RS_5, RS_{16}	**Lineage B**	C_{12}	RS_{20}
	C_5	RS_7		C_{13}	RS_9
	C_6	RS_3, RS_4			
	C_7	RS_{15}			
	C_8	RS_{10}			

SDS-PAGE. This is because of the fact that all the genes are not expressed in a particular environment. However, variability among the isolates was observed. The result of the current study indicates that *R. solani* of mountainous region Gilgit-Baltistan is composed of pathotype and morphological variability and genetic diversity exists. More studies are needed by using different molecular techniques for understanding of diversity among the isolates.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Comparative analysis of 16S ribosomal RNA of '*Candidatus* Liberibacter asiaticus' associated with Huanglongbing disease of Persian lime and Mexican lime reveals a major haplotype with worldwide distribution

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Huanglongbing (HLB) is considered as one of the most destructive and devastating disease of citrus trees worldwide. In the present study, we analyzed the genetic diversity of the 16S rDNA gene sequence of '*Candidatus* Liberibacter asiaticus' ('*Ca.*L. asiaticus') strains from symptomatic trees of *Citrus latifolia* and *Citrus aurantifolia* with HLB and performed comparative analysis with sequences in the GenBank database. Two hundred and fourteen (214) samples of trees collected in the Yucatán Peninsula were analyzed using PCR amplification and real-timeq PCR. We detected twenty-five positive trees by PCR, although the positive trees were increased to seventy by real-time q PCR. Blast searches of 75 sequences of 1167 bp in size obtained by PCR-positive trees revealed 99 to 100% similarity with sequences of '*Ca.*L. asiaticus'. Multiple alignment showed 2 clusters of sequences, wherein the first group were 100% identical, and the second group was characterized by the presence of polymorphic sites distributed across the gene sequence. The consensus sequence of the first group was named H36PENINSULAR. Analysis of the 16S rDNA sequences from GenBank revealed the same behavior, wherein one group exhibited 100% similarity (within the group). Alignment of the first groups from each of the two analyses above revealed a complete match between them (100% similarity). Accordingly, we suggest that sequence H36PENINSULAR be considered the principal haplotype of the 16S rRNA gene of '*Ca.*L. asiaticus' because it exhibits worldwide distribution and dominance.

Key words: Huanglongbing, 16s rRNA gene, '*Candidatus* Liberibacter asiaticus', Mexican lime, Persian lime.

INTRODUCTION

Huanglongbing (HLB) or citrus greening, is considered the most destructive and devastating disease of citrus

trees worldwide (Gottwald et al., 2007). The disease affects almost all major citrus fruit trees, with sweet oranges, mandarins and mandarin hybrids being most affected (Bové, 2006). HLB has spread throughout the majority of citrus-producing countries with millions of dollars lost for growers. The disease is feared worldwide because citrus trees, once infected, will irrevocably deteriorate. In the course of many years, no effective treatments for the disease existed and successful control involves preventing trees from becoming infected (Teixeira et al., 2008). HLB disease management involves three principal components, control of the insect vector *Diaphorinacitri* by chemical and biological methods, planting pathogen-free nursery stock and removing the inoculum by destroying infected trees (Grafton-Cardwell et al., 2013). Currently, the use of controlled heat treatments to cure HLB caused by '*Candidatus* Liberibacter asiaticus' ('*Ca*.L. asiaticus') using continuous thermal exposure to 40 to 42°C for a minimum of 48 h was sufficient to significantly reduce titer or eliminate '*Ca.* L. asiaticus' entirely in HLB-affected citrus seedlings (Hoffman et al., 2013).

The HLB disease caused by a phloem-limited bacterium was originally described by Garnier et al. (1984) using electron microscopy as an intracellular pathogen, and was included in the α-*Proteobacteria* subdivision. Three identified species are the causative agents, '*Candidatus* Liberibacter africanus' ('*Ca.* L.africanus'), '*Candidatus* Liberibacter americanus' ('*Ca*. L. americanus'), and '*Ca.L*. asiaticus' (McClean and Oberholzer, 1965; Capoor et al., 1967; Bové, 2006). Nonetheless, given that axenic cultures of these bacteria have been difficult to obtain because it is an obligate pathogen, molecular techniques are essential tools for identifying and analyzing the phylogeny and taxonomy, by means of amplification of 16S rDNA. Diagnosis of HLB is made by means of PCR in leaves of diseased trees with various symptoms such as blotchy mottling, yellowing veins and green islands, wherein the bacterial titer is generally high (Teixeira et al., 2008). Real-time quantitative PCR (qPCR) is another method that has been used for detection and quantification of the pathogen. In plants and insect vectors, positive amplification was achieved with as few as 10 cells per PCR reaction and the presence is detected even at low levels of the pathogen (Li et al., 2006, 2007, 2008; Wang et al., 2006; Teixeira et al., 2008).

Before the complete genome sequence of '*Ca.* L. asiaticus' (ASM2376V1) was reported, information on the genetic diversity of HLB pathogens was scarce (Duan et al., 2009). Diversity studies were restricted to the analysis of sequences from 16S/23S genes, the *omp* gene region, or the rplKAJL-rpoB operon (Villechanoux et al., 1992; Planet et al., 1995; Jagoueix et al., 1997; Subandiyah et al., 2000; Bastaniel et al., 2005). Particularly, analysis of the 16SrDNA region has been used to estimate the genetic diversity among worldwide strains with many Asian strain having identical 16SrRNA sequences, e.g., sequences from Japan, Taiwan, Indonesia, Philippines, Vietnam, and Thailand (Jagoueix et al., 1994; Subandiyah et al., 2000; Tomimura et al., 2009). Furthermore, numerous single nucleotide polymorphisms (SNPs) identified using restriction fragment length polymorphism (RFLP) have been reported in one Chinese and two Indian strains collected in Karnataka in the southwest of India (Adkar-Purushothama et al., 2009).

In México, the first HLB-infected tree was detected in 2009 in the municipality of Tizimin, Yucatán and subsequently in the states of Quintana Roo, Nayarit, and Jalisco. Afterward, the occurrence of HLB was confirmed in different localities of Campeche, Colima, Sinaloa and Michoacán (Senasica-Sagarpa, 2010). We initiated a study with the aim to detect the pathogen '*Ca.* L. asiaticus' in citrus trees on the Yucatán Peninsula with the classical symptoms of the HLB disease. Here we reported the genetic diversity of the16S rRNA gene of '*Ca.* L. asiaticus' strains from symptomatic citrus plants of the species *Citrus latifolia* and *Citrus aurantifolia*. Analyzing the sequences from Mexico and other countries, we identified a universal haplotype (H36PENINSULAR) with worldwide distribution and detected in both the citrus species and the insect vector.

MATERIALS AND METHODS

Plant samples

The plant samples were collected during the years 2010 and 2011 in plantation fields and backyard trees of *C. latifolia* Tanaka (Persian lime) and *C. aurantifolia* Christmann (Mexican lime) located in Yucatán, Quintana Roo, and Campeche States, México. Leaves were sampled from the citrus plants with the characteristic HLB symptoms such as blotchy mottling, yellowing veins and green islands. Vegetal material was stored at 4°C and transported to the laboratory for DNA extraction. The leaves of five healthy citrus plants were used as a control.

Total genomic DNA extraction

Leaves were rinsed twice with sterile distilled water and twice with 95%v(v/v) ethanol and midribs were cut with a sterile scalpel. One-tenth of a gram of midribs was macerated with a pestle in a mortar containing liquid nitrogen. DNA was extracted using the CTAB method (Murray and Thompson, 1980). To eliminate impurities from the DNA preparation, samples were processed twice with one volume of phenol-chloroform (1:1) and once with chloroform. Precipitation of the DNA was performed with 3 M sodium acetate at pH 5.0 ($^{1}/_{10}$ of volume) plus 1 volume of 2- propanol.

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The resulting DNA solution was treated with RNAse H and stored at -70°C.

Cloning and sequencing of the 16S rRNA gene

PCR amplification of the 16S rRNA gene was carried out using REDTaq polymerase (Sigma-Aldrich) and oligonucleotides OI1/OI2C (Jagoueix et al., 1994). The reaction was performed in a total volume of 25 µl containing 1X PCR buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 5 ng of template DNA, and 1 unit of DNA polymerase. The PCR conditions were as follows: an initial denaturation step of 94°C for 2 min; 40 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; finally, 72°C for 10 min. Amplified fragments of 1167 bp were purified using the Qiaex II Gel Extraction Kit (Qiagen) and then cloned into the pGEM T-Easy Vector (Promega). Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing of the plasmids was performed on both DNA strands.

Escherichia coli cells were grown in the Luria-Bertani medium and standard procedures for the growth and transformation of the cells were used (Sambrook and Rusell, 2000). Ampicillin was added at a final concentration of 100 µg/mL.

Analysis of 16S rRNA sequences of 'Ca.L. asiaticus' and phylogenetic tree construction

The sequences were assembled and trimmed using the Sequencher software, version 5.0 (Gene Codes Corporation, Ann Arbor, MI USA [http://genecodes.com]). Edited sequences were analyzed for similarity using the BlastN program, and 16S rDNA gene sequences were retrieved from the non redundant NCBI database (http://blast.ncbi.nlm.nih.gov/).Selected sequences were aligned using the ClustalW software configured for highest accuracy (Larkin et al., 2007). The phylogenetic relationships were determined using the Neighbor-Joining algorithm in the Mega 4 software (Tamura et al., 2004), and the Kimura 2-parameter statistical model was applied (Kimura, 1980). The confidence of the grouping was verified using bootstrap analysis (1000 replications). *Sinorhizobium meliloti* RFP1 (EU271786), *Rhizobium etli* CFN42 (NR029184), and *Escherichia coli* (J01859) were used as outgroup.

Ninety-three sequences of the '*Ca.*L. asiaticus' 16SrRNA gene, at least 1000 bp in size were retrieved from the GenBank sequence database (Benson et al., 2005). Sequences were edited and phylogenetic analysis was performed as described above.

Identification of single nucleotide polymorphic sites and haplotype designation

All the DNA sequences of 16S rRNA gene were aligned using Sequencher software version 5.0. The identical sequences were separated and the group was realigned to confirm the percentage of identity. A representative sequence was used for realignment with the sequences with an identity value below 99.5%. Single nucleotide polymorphisms (SNPs) were identified visually (den Dunnen and Antonarakis, 2001). A sequence was considered a haplotype when 2 or more samples had a mutation in the same position (Arias et al., 2010). The SNPs and haplotypes was confirmed by means of the DnaSP software version 5.0 (Librado and Rosas, 2009). Definition of SNPs for the 16S ribosomal gene sequences of '*Ca.* L. asiaticus' obtained from the GenBank database was performed with a multiple alignment by using the Mexican sequence as reference to identify the positions of the SNP's.

Real-time qPCR

TaqMan amplification reactions were performed on a Real-Time PCR StepOnethermocycler (Applied Biosystems, Foster City, California, USA). PCR amplifications were performed with EXPRESS qPCRSupermix Universal (Invitrogen) in a 20 µl reaction containing 10 µl 2X qPCRSuperMix, 25 µM ROX reference dye, 250 nM of each target primer (HLBas and HLBr), and 150 nM of target probe (HLBp). For positive internal control, 300 nM (each) internal control primers (COXf and COXr), 150 nM internal control probe (COXp) were used (Li et al., 2006).The cycling amplification conditions were 95°C for 2 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. To exclude false-positive results some control reactions with genomic DNA sample from a PCR-positive HLB-diseased plant, DNA from five healthy citrus plants and distillated water were done. A second real-time PCR assay was carried out with positive reactions.

RESULTS AND DISCUSSION

Detection of '*Ca.*L. asiaticus'

PCR amplification of the 16S rRNA gene of '*Ca.*L. asiaticus' was carried out for 214 genomic DNA samples purified from leaf samples collected from HLB-symptomatic and asymptomatic citrus trees from Campeche, Yucatán, and Quintana Roo. '*Ca.* L. asiaticus' were detected in 25 trees; 6 of the leaf samples were collected from citrus plants in Campeche, 7 in Quintana Roo, and 12 in Yucatán. The presence of '*Ca.*L.asiaticus' in HLB-symptomatic citric plants was detected mostly in *C. latifolia* trees. The HLB-positive samples were confirmed by means of real-time PCR and C_T values are shown in Table 1. This analysis, however, showed an unexpectedly low number of PCR-positive samples from HLB-symptomatic citrus plants. To increase the reliability of the results, real-time qPCR was carried out for all the 214 samples. The results showed an increase in the positive samples from 25 to 70 (35 from Campeche, 15 from Quintana Roo and 20 from Yucatán). The real-time qPCR test improved the detec-tion of diseased plants by identifying false negative samples. The above results are suggestive of a low titer of the bacteria in the phloem tissue of the leaves and uneven distribution in the host (Hung et al., 1999; Li et al., 2008).

In México, the presence of '*Ca.*L. asiaticus' on citrus plantations was first reported in Yucatán, particularly in the municipality of Tizimin (Senasica-Sagarpa, 2009). Initially, PCR was the method of choice to detect the pathogen, but the low percentage of detection, even in HLB-symptomatic trees made it necessary to use real-time qPCR method. Nowadays, although the HLB vector (*Diaphorina citri)* has been collected in the 23 citricultural states of México, the disease has not yet been reported at all. In this regard, it is worth mentioning that Mexican authorities established Official Mexican Guidelines (NOM-EM-047-FITO-2009, http://www.senasica.gob.mx/?Idioma=2&doc=9366), which specifies actions, such as total destruction of trees,

Table 1. Detection of '*Candidatus* Liberibacter asiaticus' in leaf samples from Huanglongbing-symptomatic citrus trees.

Sample ID	Year of collection	Host	PCR[a]	C_T value[b]
QUINTANA ROO¥				
H002Q	2010	*Citrus latifolia*	+	19.63
H64AQ	2010	*Citrus latifolia*	+	21.3
H64BQ	2010	*Citrus latifolia*	+	20.51
H229Q	2010	*Citrus latifolia*	+	20.43
H232Q	2010	*Citrus latifolia*	+	20.32
H238Q	2010	*Citrus latifolia*	+	21.46
H243Q	2010	*Citrus latifolia*	+	20.68
YUCATÁN§				
H88Y	2010	*Citrus latifolia*	+	19.86
H89Y	2010	*Citrus latifolia*	+	21.95
H92Y	2010	*Citrus latifolia*	+	32.87
H93Y	2010	*Citrus latifolia*	+	20.63
H117Y	2010	*Citrus aurantifolia*	+	20.84
H143Y	2010	*Citrus latifolia*	+	20.2
H147Y	2010	*Citrus latifolia*	+	20.31
H163Y	2010	*Citrus latifolia*	+	19.09
H166Y	2010	*Citrus aurantifolia*	+	19.3
H259Y	2010	*Citrus aurantifolia*	+	20.62
H261Y	2010	*Citrus latifolia*	+	20.93
H267Y	2010	*Citrus latifolia*	+	21.27
CAMPECHE†				
H025C	2011	*Citrus latifolia*	+	26.6
H026C	2011	*Citrus aurantifolia*	+	21.0
H027C	2011	*Citrus aurantifolia*	+	25.2
H028C	2011	*Citrus aurantifolia*	+	22.6
H076C	2011	*Citrus latifolia*	+	19.09
H077C	2011	*Citrus latifolia*	+	21.36

¥: Total samples, 49; positive samples by PCR, 7; positive samples by real time-PCR, 9. §: total samples, 130; positive samples by PCR, 12; positive samples by real time-PCR, 20. †: total samples, 35; positive samples by PCR, 6; positive samples by real time-PCR, 35. [a] Samples used for cloning. [b] C_T values of positive PCR samples.

fruits and derivatives, that must be implemented immediately once the bacteria are detected.

Sequence and phylogenetic analysis

DNA fragments of 1167 bp from the 16S rDNA gene amplified from the 25 conventional PCR-positive samples were cloned, and three independent transformed cell clones harboring the constructs were selected from each positive sample. Sequencing of 75 independent plasmids was performed, and individual sequences were trimmed, edited, and analyzed as mentioned earlier. The multiple alignment showed sequences with similarity of 99 to 100% to sequences of '*Ca.* L. asiaticus', 96% with '*Ca.* L. africanus' and '*Ca.* L. solanacearum', and 94% with '*Ca.* L. americanus'. Phylogenetic tree construction with representative sequences from the Yucatán Peninsula and sequences deposited in the GenBank database is showing Figure 1. As expected, the sequences of our strains clustered with sequences of '*Ca*.L.asiaticus' from different countries. It is clearly a close relation with sequences of '*Ca*.L. africanus' rather than to sequences of '*Ca*.L. americanus'.

The above alignment also showed the clustering of sequences into two groups. The first group contained 26 sequences with an identity of 100%, and the second one contained 43 sequences with 1 to 5-sites of sequence polymorphism. The most important feature of the first subset of sequences was their geographical distribution

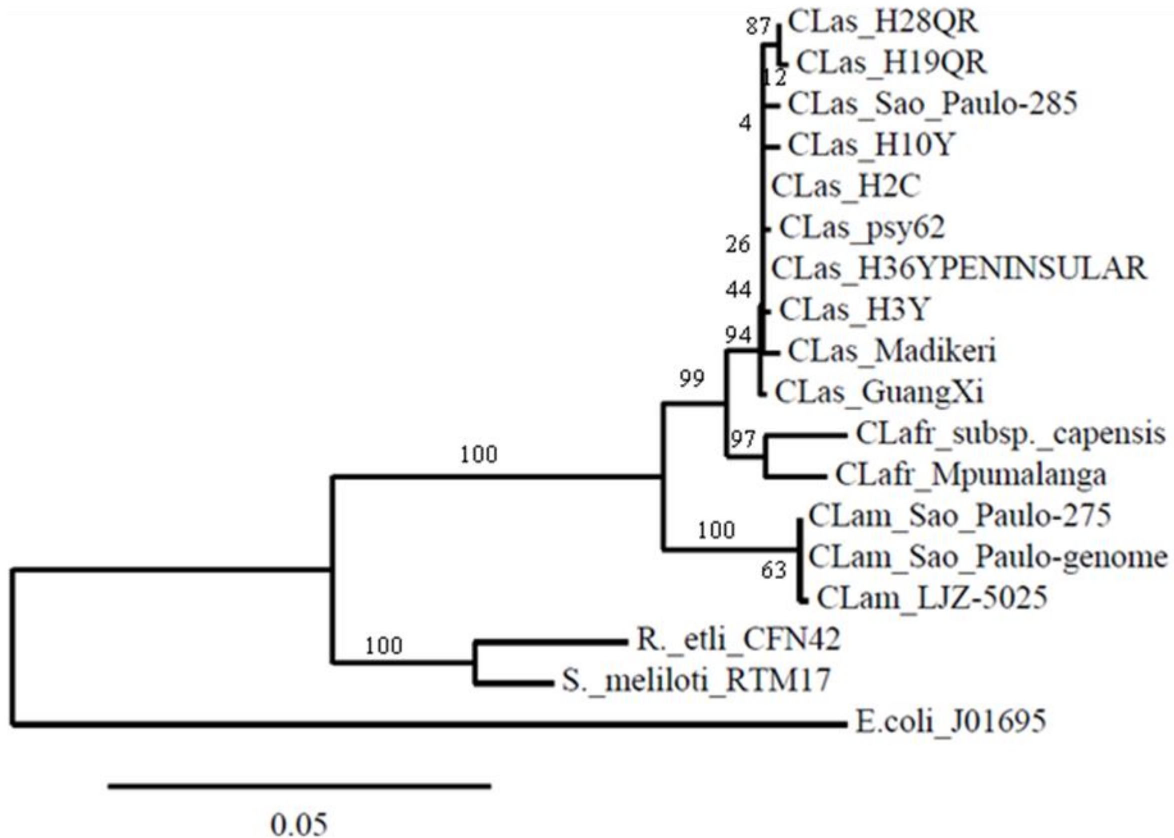

Figure 1. Phylogenetic relationships based on the 16s rRNA gene of '*Ca.* L. asiaticus' strains from the Yucatán Peninsula with closely related HLB species. The tree was constructed with the Neighbor-Joining method. The numbers at the nodes are percentages that indicate the levels of bootstrap support (1000 iterations). The sequences from '*Ca.* L. asiaticus' strains used in the analysis were Sao Paulo 285 (EU921613), Madikeri (FJ827777), Guang Xi-GL-10-CHN (EU921615), and Psy62 (CP00167). The sequences used from '*Ca.* L. africanus' were Capensis (AF137368), and Mpumalanga-UPCRI-06-0071 (EU921621). The sequences used from '*Ca.* L. americanus' were Sao Paulo 275 (EU921623), Sao Paulo 2 (AY742824) and LJZ-5025 (FJ263695). *Sinorhizobium meliloti* RTM17 (EU271786), *Rhizobium etli* CFN42 (NR_029184), and *Escherichia coli* (J01695) were used as the outgroup. Sequences of '*Ca.* L. asiaticus' of the present study were CLas H2C (JQ867437), H3Y (JQ867412), H10Y (JQ867417), H19QR (JQ867449) and H28QR (JQ867452).

into the three Mexican States. For this reason, we considered the sequence as the major haplotype identified on the peninsula, which we named H36PENINSULAR (GenBank accession No. JQ867409).

Worldwide distribution of the haplotype H36PENINSULAR

A similar analysis as previously mentioned was carried out with 93 16S rRNA sequences of'*Ca.*L. asiaticus' retrieved from GenBank sequence database with at least 1000 bp in length (Table 2). Results of the multiple alignment showed the formation of two groups of sequences, wherein the principal group contained 41 sequences with 100% similarity. For simplicity sake, this consensus sequence was named provisionally HLB-CLas, The second group contained sequences with1 to 15-sites (nucleotide) variations in different parts of the gene. Metadata of the members of the first group showed that all sequences originated from samples of different species of citrus trees and from the *D. citri* insect vector collected in countries such as Dominican Republic, Florida (USA), Brazil, Indonesia, Vietnam, Thailand, Taiwan and Japan. Identical sequences of 16S rDNA were obtained in Asiatic strains from Japan, Taiwan, Indonesia, the Philippines, Vietnam and Thailand (Subandiyah et al., 2000; Tomimura et al., 2009). Further studies based on the analysis of the 16S rRNA gene and the *omp* gene region of '*Ca.L.* asiaticus' found the closest relationship of sequences from northeastern India with sequences from Japan, Southeast Asia, USA (Florida) and Brazil, rather than with sequences from other Indian regions. Additionally, the study showed that common Asian strains are distributed in India together with other atypical strains (Miyata et al., 2011). Finally, a sequence comparison of

Table 2. Worldwide 16S rDNA sequences of '*Candidatus* Liberibacter asiaticus' included in the haplotype HLB-CLas and sequences with polymorphic sites.

ID Isolate	Source	Host	Accession	Reference
Haplotype HLB-CLas				
OKA901	Japan	*Citrus jambhiri*	AB480072	Okuda et al., 2005
KIN1	Japan	*Citrus jambhiri*	AB480073	Okuda et al., 2005
Y02-57	Japan	*Citrus tankan*	AB480074	Okuda et al., 2005
ISHI1	Japan	*Citrus*	AB480075	Tomimura et al., 2009
TW1	Taiwan	*Citrus sinensis*	AB480076	Tomimura et al.
TW2	Taiwan	*Citrus reticulata*	AB480077	Tomimura et al.
TW3	Taiwan	*Citrus grandis*	AB480078	Tsai et al., 2008
TW5	Taiwan	*Citrus grandis*	AB480079	Tsai et al.
TW6	Taiwan	*Citrus reticulata*	AB480080	Tsai et al.
V1	Vietnam	*Citrus nobilis*	AB480081	Tsai et al.
V2	Vietnam	*Citrus nobilis*	AB480082	Tsai et al.
VN50	Vietnam	*Citrus nobilis*	AB480083	Tominura et al., 2009
V61	Vietnam	*Citrus nobilis*	AB480084	Tominura et al.
V62	Vietnam	*Citrus nobilis*	AB480085	Tominura et al.
THA1	Thailand	*Citrus reticulata*	AB480086	Tominura et al.
IDN03-2	Indonesia	*Citrus reticulata*	AB480087	Okuda et al., 2005
IDN03-5	Indonesia	*Citrus reticulata*	AB480088	Okuda et al.
IDN03-7	Indonesia	*Citrus reticulata*	AB480090	Okuda et al.
B3T1	Indonesia	*Citrus reticulata*	AB480092	Okuda et al.
B3T2	Indonesia	*Citrus reticulata*	AB480093	Okuda et al.
B2T3	Indonesia	*Citrus reticulata*	AB480094	Okuda et al.
B3T4	Indonesia	*Citrus reticulata*	AB480095	Okuda et al.
B3T5	Indonesia	*Citrus reticulata*	AB480096	Okuda et al.
FC	Indonesia	*Citrus reticulata*	AB480097	Tominura et al., 2009
FD	Indonesia	*Citrus reticulata*	AB480098	Tominura et al.
EJ5	Indonesia	*Citrus reticulata*	AB480099	Tominura et al.
K1T2	Indonesia	*Citrus reticulata*	AB480101	Tominura et al.
PM13	Indonesia	*Citrus grandis*	AB480102	Tominura et al.
PM18	Indonesia	*Citrus grandis*	AB480102	Tominura et al.
IDN03-17	Indonesia	*Citrus reticulata*	AB480091	Tominura et al.
IDN03-6	Indonesia	*Citrus reticulata*	AB480089	Tominura et al.
MDL1398-DR002-12	Domininican Republic	NH[a]	FJ821716	Tominura et al.
MDL1399-DR002-13	Domininican Republic	NH	FJ821717	Tominura et al.
MDL1391-DR002-5	Domininican Republic	NH	FJ821710	N
MDL1396-DR002-10	Domininican Republic	NH	FJ821715	N
MDL1400-DR002-14	Domininican Republic	NH	FJ821718	N
F11Dade	Florida, USA	*Diaphorina citri*	EU130552	N
F16Nassau	Florida, USA	*Diaphorina citri*	EU130553	Manjunath et al., 2008
F17PALMBEACH F	Florida, USA	*Diaphorina citri*	EU130554	Manjunath et al.
18POLK	Brazil	*Diaphorina citri*	EU130555	Manjunath et al.
LSg1	Brazil	*Citrus*	AY919311	N
CLUSTER OF SEQUENCES WITH SNP´s				
A* Guangdong	Guangdong, China	NH	DQ157273	N
Guangxi	Guangxi, China	NH	DQ157274	N
B Guizhou	Guizhou, China	NH	DQ157275	N
C Sao Paulo 285	Sao Paulo, Brazil	NH	EU921613	Lin et al., 2009
D Psy62	Florida, USA	*Diaphorina citri*	CP00167	Duan et al., 2009
Flord1	Florida, USA	Citrus	DQ471900	N

Table 2. Contd.

	NC (Brasil 1)	Sao Paulo, Brazil	Citrus	DQ471901	Texeira et al., 2005
E	Kumquat 1	Taiwan	NH	DQ302750	N
F	Lastm06	Louisiana, USA	Satsuma plant	FJ750459	N
G	Guangxi Gl1	China	NH	DQ778016	Lin et al., 2009
	Guangxi Guilin China	China	NH	FJ914620	N
H	Guangxi Gl7 Chn	Guanxi, China	NH	EU921614	Lin et al., 2008
I	Flirc08		Orange sweet	FJ750456	N
	Flclm09	Florida, USA	*Calamondin citrus*	FJ750457	N
J	Spd53	Sao Paulo, Brazil	NH	EU921622	Lin et al., 2009
K	M20	China	*Diaphorina citri*	GU553033	N
L	Guangxi Gl10 Chn	Guangxi, China	NH	EU921615	Lin et al., 2009
M	Guangxi Gl19 Chn	Guangxi, China	NH	EU921616	Lin et al., 2009
N	Florida 1808	Florida, USA	NH	EU921618	Lin et al., 2009
O	Florida 8	Florida, USA	NH	EU921617	Lin et al., 2009
P	Mpw643	East Timor	*Citrus aurantifolia*	AB555706	Miyata et al., 2011
	Mpw1319a	Papua New Guinea	*Citrus aurantium*	AB555707	Miyata et al., 2011
Q	Satkara Tripura	India	*Citrus macroptera*	GQ369792	Das et al., 2010
	LJZ 4621	Florida, USA	*Citrus spp.*	FJ263698	N
R	LJZ 4622	Florida, USA	*Citrus spp.*	FJ263699	N
	LJZ 575	Florida, USA	*Citrus spp.*	FJ263702	N
S	374 15	California, USA	*Atalantia ceylanica*	GU991651	N
T	373 4	California, USA	*Severina buxifolia* (Poiret)	GU991650	N
U	GFB-T	Malasya	Citrus	EU371106	N
V	LJZ 451	Florida, USA	Citrus	FJ263696	N
W	SIHUI	Florida, USA	NH	EU644449	Deng et al., 2009
X	LAAPC06	Louisiana, USA	*Diaphorina citri*	FJ750458	N
Y	373.1	California, USA	*Severina buxifolia*	GU991649	N
Z	Hunan-NG	Hunan, China	NH	DQ432002	Ding et al., 2009
AA	LJZ 5719	Florida, USA	NH	FJ263703	N
BB	LJZ 4620	Florida, USA	NH	FJ263697	N
CC	CGBNM1	India Nagpur	*Citrus* spp.	EU939452	N
DD	Clona 139	Florida, USA	*Citrus* spp.	EU130556	Manjunath et al., 2008
EE	Chongqing ZG	China Chongqing	NH	DQ432004	Ding et al., 2009
	Karnataka	India Karnataka	*Citrus reticulata*	FJ765088	Adkar-
FF	Madikeri	India Karnataka	Tangerine	FJ827777	Purushothama
	Polibetta 1	India Karnataka	Tangerine	FJ827779	et al., 2012
GG	371	Belize	*Diaphorina citri*	GQ502291	Manjunath et al., 2010
HH	432.26	Belize	*Citrus* spp.	GU061003	Manjunath et al., 2010
II	POONA	Poona India	*Citrus* spp.	L22532	Jagoueix et al., 1994
JJ	LJZ 745	Florida, USA	*Citrus* spp.	FJ236554	N
KK	LJZ4730	Florida, USA	NH	FJ263700	N
	LJZ 5670	Florida, USA	NH	FJ263701	N
LL	GUANGDONG	Guangdong, China	*Citrus* spp.	DQ303210	N
MM	LJZ 5818	Florida, USA	*Citrus* spp.	FJ263704	N
NN	12166	Florida, USA	*Citrus reticulata*	EU265646	N
OO	GFB Pahangkelantan (So)	Pahan Kelatan, Malasya	*Citrus reticulata*	EU224394	N

[a] NH, no host mentioned; [b] N, No reference.

16S rRNA, 23S rRNA and 16S/23S intergenic region of 'Ca L. asiaticus' associated with yellowing disease of

Kinnow mandarin carried out in India showed lowest identities of 94.7, 95.4 and 95.9 percentage with '*Ca L.* asiaticus' from India (Poona strain), China and, USA and Brazil, respectively (Gupta et al., 2012).

Comparison between the sequences H36PENINSULAR and HLB-Clas was performed using the CLC Workbench software, version 6.1 (data not shown). Alignment of the two sequences showed a perfect match (100% identical), which suggests that the H36PENISULAR sequence can be considered the principal haplotype with worldwide distribution, which includes the Mexican states of Yucatán, Campeche and Quintana Roo. Therefore, we believe that this universal sequence could be used as reference for the analysis of 16S rRNA sequences of '*Ca.* L. asiaticus'.

Genetic diversity of 16s rRNA sequences of 'Ca. L. asiaticus'

To analyze the genetic diversity of the 16S rRNA gene in '*Ca.* L. asiaticus' a nucleotide comparison between the H36PENINSULAR sequence with the two subset of polymorphic sequences described above was carried out. For the Mexican sequences, the SNPs were identified as mentioned in Materials and Methods and the variants names were assigned. Forty-two variants were identified and their sequences were deposited in the GenBank database (Table 3). The table shows the position and type of mutation for each sequence, wherein it is clear, a predominance of sequences with 1or 2 polymorphic sites. The modifications were transitions, transversions, insertions and deletions with dominance of transitions. In the case of the subset of sequences from the GenBank, the variations detected ranged from 1 to 15 SNPs across the gene, thus showing a diversity of single and multiple polymorphic sites (Table 4). Besides, the GenBank sequences showed a higher number of polymorphic sites than the Mexican variants: the predominant nucleotide mutation was transitions (there were also some transversions, insertions, deletions and substitutions (Figure 2).

In relation to the SNPs, our findings show that Mexican strains have a small number of polymorphic sites in the 1167 bp DNA fragment distributed in a random fashion. In contrast, most GenBank sequences show high variability, which is suggestive of misreads during sequencing rather than genuine SNPs. To our knowledge, the '*Ca.*L. asiaticus' variants of Mexican isolates described in this study are the largest set of reported sequences with SNP sites in the 16s rDNA gene; as a result, 43 sequences were identified and added to the GenBank sequence database. Looking for possible phylogenetic relations between the polymorphic sequences, we constructed a tree using the Neighbor-Joining method (Figure 3). It was not possible to distinguish clusters of related polymorphisms, because only 3 well-supported clades were evident: a monophyletic clade that includes all the sequences corresponding to the 16S rRNA gene of '*Ca.*L. asiaticus' irrespective of the geographical region, and 2 independent clades corresponding to '*Ca.*L. africanus' and '*Ca.*L. americanus', as expected. Other studies have analyzed the genetic diversity of the 16S rRNA sequences. Based on the RFLP technique, Adkar-Purushothama et al. (2009) identified 14 genetic lineages by analyzing the SNPs present in the 16S rRNA, which revealed a new lineage on the Indian subcontinent. Recently, a study was conducted as a reanalysis of all '*Ca.*L. asiaticus' 16S rDNA sequences deposited in GenBank database to determine whether the discrepancy in reports of 16S variation can be resolved and whether this variation has a geographic origin (Nelson, 2012). The authors used geographic designations available in the metadata of the deposited sequences. A 302 bp segment common to 175 sequences was used to asses SNPs and the distribution, and the researchers found 118 identical sequences with different accession numbers and another 47 records exhibited 73 SNPs, most of them corresponding to a single accession number. A few SNPs occurred in more than one database record. The authors concluded that the reanalysis does not show sufficient confidence to confirm haplotypes of '*Ca.*L. asiaticus' based on the 16S rDNA sequences because the low percentage of SNPs in the segment studied suggested misreads during sequencing rather than genuine haplotypes. Differing from the above idea is that heterogeneity exists in the 16S rRNA genes detectable in the variable regions of the gene (V1, V2, V3, etc). The location of the differences in the most variable part of the 16S rRNA corroborates that the differences are true differences and not mere sequencing errors (Coenye and Vandamme, 2013).

Our results suggest that the sequence of the haplotype H36PENINSULAR may be a suitable reference sequence for the analysis of the 16s rRNA gene of novel '*Ca.* L. asiaticus' strains instead of the 16s rDNA sequences normally used for the detection of '*Ca.* L. asiaticus', such as the Poona, Karnataka and Madikeri strains. The Poona strain has commonly been used as a reference sequence for phylogenetic analysis, even though its 16S rRNA sequence still contains undetermined nucleotides (Miyata et al., 2011). The amplification and analysis of the 16S rDNA sequences are useful for describing new nucleotide variations across the gene sequence.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Table 3. Haplotype designation and SNP´s on the 16S rDNA sequences from Mexico.

ID*	1-200	201-400	401-600	601-800	801-1000	1001-1200	Accession
H1C						1162G>A;1164G>C	JQ867436
H2C	34A>G					1149delG	JQ867437
H3C				704C>T		1076delG	JQ867430
H22C				790A>G			JQ867438
H24C			560A>G				JQ867439
H29C	56-57insT	329T>C					JQ867431
H30C		322delC					JQ867432
H33C	74G>A	256A>G					JQ867433
H35C	34A>G						JQ867434
H1Y				601T>C		1142A>G	JQ867410
H2Y						1110G>A	JQ867411
H3Y				622-623insC		1110G>A	JQ867412
H4Y				622-623insC		1100G>A	JQ867405
H5Y						1065G>A	JQ867413
H6Y					837T>C	1053T>C	JQ867414
H8Y	71A>G	263delG		681G>A	896G>A	1011A>G	JQ867415
H9Y					925G>A	1009T>C	JQ867416
H10Y	45delA			753A>G	1000T>C		JQ867417
H16Y	43G>A				945A>G		JQ867418
H17Y					870G>A;921C>A		JQ867406
H22Y				788T>C			JQ867419
H23Y				700A>G;780A>G			JQ867420
H24Y				622-623insC			JQ867421
H25Y			491-492insC				JQ867407
H26Y		317A>G	464C>T				JQ867422
H27Y			447C>G;448G>T				JQ867423
H29Y		357A>G					JQ867408
H30Y	45delA	286-287insA					JQ867424
H31Y		203A>G; 263delG					JQ867425
H32Y		263G>A					JQ867426
H34Y	45delA						JQ867427
H35Y	34A>G						JQ867428
H7QR							JQ867442
H11QR					998T>C	1021T>C	JQ867443
H12QR			596A>G		995G>A		JQ867444
H13QR					967delG		JQ867445
H14QR				735G>A	967delG		JQ867446
H15QR					876T>C;967delG		JQ867447
H18QR					843G>A		JQ867448
H19QR		230A>G	432T>C		815A>G		JQ867449
H20QR					812A>G		JQ867450
H21QR		230A>G	432T>C				JQ867451
H28QR		230A>G	432T>C				JQ867452

* C, Campeche; Y, Yucatán; QR, Quintana Roo

Table 4. Haplotype designation and SNP’s on the 16S rDNA sequences from worldwide sequences.

ID*	1-200	201-400	401-600	601-800	801-1000	1001-1200
A	9G>C					
B	9G>C				840T>C	
C	10C>T; 10insG	315A>T; 372A>C		792A>G	806G>A	
D	10insG					
E	10insG; 49T>C					
F	10insG; 76A>G	254A>G; 281G>A	535T>C;		804delG; 816A>T; 847A>C; 866insC	1126C>T
G	10insG; 88A>G		549T>C; 562T>G; 598G>A	682A>G; 762C>T; 764T>G		
H	10insG; 97G>C			769A>G		1040T>G
I	10insG	205A>T; 398C>T		675C>T		1103insC
J	10insG	274A>C				
K	10insG	283A>G; 358A>G	403G>A			
L	10insG	365T>C	448delA			
M	10insG		572A>G	637A>G		1036T>A
N	10insG				892A>G; 975T>A	1101T>A
O	10insG			787A>T	820G>A; 991insC	
P	17A>T					
Q	35A>G		448A>T; 458T>A; 459A>T		834T>A; 988G>A	
R	73T>C; 175A>G	217A>G		668T>C; 762C>T; 764T>G		1075A>G
S	77A>T; 114A>G; 137T>C	372A>G		752A>G		
T	101T>C; 107G>A; 112A>G	202A>G; 347A>G				
U	168G>A		460G>A; 467C>G; 468G>A; 469T>A: 473G>C; 544G>T; 545A>G; 549G>A; 553delT; 577T>G; 579G>A; 580A>G	602G>A		
V	187T>A			672A>G		
W	187insA; 195G>T; 199insA	213delC; 225insG; 227A>G; 229insC; 234delC; 247delG; 274delA; 283delA; 285C>G				
X	196T>C	315delA		749T>C	803insG; 847A>C; 866insC	

Table 4. Contd.

Y		250A>G; 306T>C		746G>A	827C>T; 885C>G	
Z		327T>C; 350A>T			973A>T	1164G>C
AA		338T>C				
BB		348G>A	454C>T; 562T>C	641G>A	936T>C; 979G>A	
CC		353T>A	429G>T; 442G>T; 448A>T; 459A>T; 461G>C	617C>T; 650G>T; 774G>A		1110T>C
DD		369A>G			817A>G	
EE		374A>G				1024T>C; 1114G>C
FF			404C>T	633G>C		1041T>C
GG			413G>A			
HH			430T>C; 527A>G			
II			463delG; 491delG; 532insC	740G>C; 741C>G;	820delG	1102delG; 1103insC; 1104C>G
JJ			520T>C			
KK				798 A>G		
LL	9G>C				847delA	1149G>A
MM						
NN					944G>C	
OO					988G>A	

*Accession number of the GenBank database are shown in Table 2.

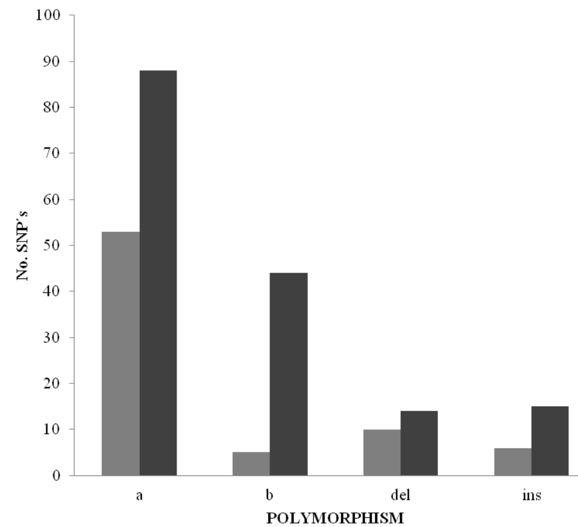

Figure 2. Comparison of polymorphic changes in 16s rRNA sequences of '*Ca.* L. asiaticus' from the Yucatán Peninsula (black bar) and sequences from GenBank database (gray bar). Nucleotide changes: a, transitions; b, transversions; del, deletions; ins, insertions.

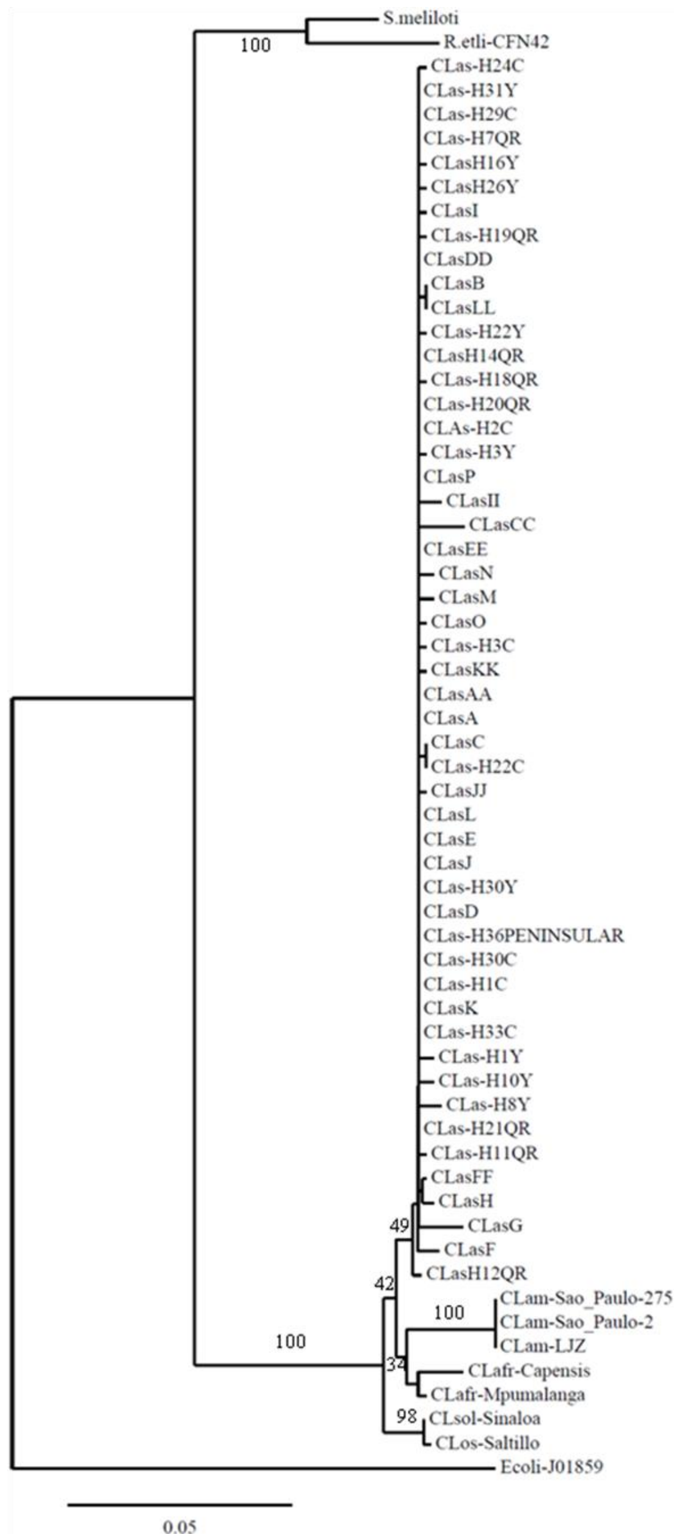

Figure 3. Phylogenetic tree including 16s rRNA sequences from Yucatán Peninsula haplotypes and closely related sequences using the Neighbor-Joining method. The numbers at the nodes are percentages that indicate the levels of bootstrap support (1000 iterations). The sequences used from GenBank were '*Ca.* L. africanus' Mpumalaga UPCRI-05-0232 (FJ914622), Mpumalaga UPCRI-06-0071 (EU921621), Nelspruit (L22533), and subsp. Capensis (AF137368); '*Ca.* L. solanacearum' Sinaloa (FJ957897), NZLS0002 (HM246509), NZ082226 (EU834130), and Saltillo 9 08 (FJ490086); and '*Ca.* L. americanus' LJZ5110 (FJ263689), Sao Paulo 275 (EU921623), Sao Paulo 2 (FJ914621), and Sao Paulo State (AY742824). The rhizobial group consisted of *Agrobacterium tumefaciens* RFP1 (GU902302), *Rhizobium etli* CFN42 (NR029184), and *Shinorizobium meliloti* RTM17 (EU271786). *Escherichia coli* (J01859) and *Pseudomonas* sp. NJ (AY339889) were used as the outgroup.

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Full Length Research Paper

Effect of growth conditions on glutathione accumulation, *gshR* gene expression and resistance to the lyophilization process in *Lactococcus lactis* LVA. 2

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The functionality of glutathione (GSH) and the effect of growth conditions on GSH accumulation, *gshR* gene expression and viability following lyophilization were examined in *Lactococcus lactis* LVA. 2. The accumulation of GSH under anaerobic and continuously stirred aerobic conditions enabled a higher GSH accumulation than a static aerobic culture, which is the growth condition normally used. The *gshR* gene expression under the growth conditions in the anaerobic, static aerobic and continuously stirred aerobic media was similar. Although, GSH failed to provide an apparent protection to *L. lactis* LVA. 2 following the lyophilization process, the fact that this strain accumulates GSH and expresses the *gshR* gene makes it a promising strain to cope with other stress situations.

Key words: *Lactococcus lactis*, reduced glutathione, lyophilization, survival.

INTRODUCTION

Lyophilization is the method most commonly used in the preservation of lactic acid bacteria intended for cheese, yogurt and fermented-milk production (Champagne et al., 2009; Turchi et al., 2013). Although the lyophilization process is used to maintain the viability of bacteria for a long storage period, much of the cell viability is compromised. Lyophilization exposes cells to stress conditions due to freezing and to osmotic stress caused by water loss (Santivarangkna et al., 2008). Various treatments are applied to the cell suspension to protect it from the damages caused by lyophilization, the most common being the addition of cryoprotectant substances and the application of sublethal stress treatments (Savini et al., 2010).

Glutathione (γ-glutamyl-cysteine-glycine [GSH]) is a tripeptide found in eukaryotes and Gram-negative bacteria

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and to a lesser extent in Gram-positive bacteria (Copley and Dhillon, 2002). GSH is found in the reduced (GSH) and oxidized (GSSG) forms in cells. An increased proportion of GSSG is found when cells are exposed to oxidative stress conditions. One of the main functions of GSH in cells is the maintenance of intracellular homeostasis. In addition to GSH accumulation, the presence of glutathione reductase (GshR), whose role is to convert GSSG into GSH to maintain intracellular homeostasis, is also important (Pophaly et al., 2012).

The ability to transport GSH into the intracellular environment and the presence of glutathione reductase have already been noted in some strains of *Lactococcus lactis* (Li et al., 2003). The functionality of GSH in *L. lactis* was identified as contributing to protection from conditions of oxidative stress (Li et al., 2003), acid stress (Zhang et al., 2007) and osmotic stress (Zhang et al., 2010). However, the functionality of GSH has not been evaluated in *L. lactis* regarding protection from the lyophilization process.

Thus, the purpose of this study was to evaluate whether GSH has a protective effect following the lyophilization process in *L. lactis* LVA. 2. Furthermore, the effect of several growth conditions on GSH accumulation, expression of the *gshR* gene (which encodes the glutathione reductase enzyme), and resistance to lyophilization was evaluated.

MATERIALS AND METHODS

Detection of synthesis and/or transport of GSH

L. lactis LVA. 2 is a strain isolated from cow's milk, belonging to the collection of cultures from the Laboratory of Industrial Microbiology, Federal University of Viçosa (Universidade Federal de Viçosa - UFV), Brazil. The culture was transferred from the stock at -80°C into M17 broth containing 5 g/L of glucose and incubated at 30°C for 14 h to prepare the preculture. The preculture was inoculated (1%, v/v) in a chemically defined medium (CDM) supplemented and unsupplemented with reduced GSH (Sigma-Aldrich Co., St Louis). The final concentrations of GSH in the CDM were 2, 4 and 6 mM in the supplemented treatments. The inoculated media were statically incubated at 30°C for 7 h. The detailed composition of CDM is described by Li et al. (2003).

Growing conditions for physiological characterization

M17 supplemented with GSH (final GSH concentration of 4 mM) and without GSH supplementation was incubated statically and in a rotary shaker at 200 rpm at 30°C for 7 h. Anaerobic M17 medium (supplemented and unsupplemented with GSH) obtained by heating and injecting nitrogen gas for 30 min was prepared in addition to those conditions. The inoculated media were incubated at 30°C for 7 h.

Cell-free extract preparation

The cells derived from the different growth conditions were recovered by centrifugation (8,000 *xg*, 10 min, 4°C). The cell pellet was washed twice with 0.1 M potassium phosphate buffer supplemented with EDTA (5 mM), pH 7.5 (KPE) and re-suspended in 4 mL of KPE buffer. Glass beads (Sigma-Aldrich Co., St Louis) were added to the cell suspension for cell lysis in Precellys 24 (Bertin Technologies®, Bioamerica Inc.) at 4,000 rpm for 15 s. The cell extracts were pelleted by centrifugation for 10 min (10,000 *xg*, 4°C), and the supernatant was used to assess the GSH. The protein concentration in the supernatant was assessed using the Lowry method, and bovine serum albumin was used as standard.

GSH assessment

Total GSH, which includes both the reduced form (GSH) and the oxidized form (glutathione disulfide - GSSG), and GSSG were quantified using the enzymatic recycling method (Tietze, 1969), and the absorbance at 412 nm was assessed in a 96-well microdilution plate in an enzyme-linked immunosorbent assay (ELISA) reader (Biotek) (Rahman et al., 2006). The amount of GSH was calculated as the difference between total glutathione and GSSG (total glutathione-GSSG = GSH). The glutathione content was expressed as nmol/mg protein in the sample.

gshR gene detection

The primer pair GSHRf/GSHRr, based on the nucleotide sequence of *L. lactis* subsp. *lactis* Il1403 (NC_002662.2) available in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database, was designed to amplify the *gshR* gene encoding the protein glutathione reductase. The Primer-Blast tool, available at NCBI, was used for primer selection, and the primer quality was assessed using the OligoAnalyzer 3.1 software (http://www.idtdna.com). The PCR product obtained was sequenced. The primer sequence is shown in Table 1.

Analysis of *gshR* gene expression

The *L. lactis* LVA. 2 cells derived from growth in M17 medium (static growth, in a rotary shaker, in anaerobic medium) were recovered by centrifugation at 8,000 *xg* and 4 C for 5 min. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA purity was assessed by measuring the absorbance values at 260 and 280 [nm] (approximately 1.8 to 2.0) in a Nanodrop spectrophotometer (Thermo Scientific, San Jose, CA, USA), and the RNA integrity was evaluated using electrophoresis. The contaminant genomic DNA present in the total RNA was removed upon treatment with RNase-free DNase (Promega, Madison, WI) at 37°C for 60 min, and the resulting RNA was used to synthesize cDNA using the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer's instructions.

The partial sequence of the *gshR* gene, previously obtained by sequencing, was used to design the GS912f/GS912r primer pair to evaluate the *gshR* gene expression. The GYRB4f/GYRB4r primer pair was used to evaluate the expression of the *gyrB* gene, which encodes the protein DNA gyrase, used as an internal control. GenScript Real-time PCR tools were used for primer selection, and the primer quality was assessed using the OligoAnalyzer 3.1 software (http://www.idtdna.com). The primer sequences are shown in Table 1.

The quantitative analyses were performed on a CFX96TM Real-Time PCR detection system and a C100TM Thermal Cycler (Bio-Rad) thermocycler to evaluate the *gshR* gene expression at a transcriptional level, using Platinum® SYBR® Green qPCR supermix-UDG (Invitrogen) to monitor the synthesis of double-strand DNA. The melting curve was analyzed to verify the absence of non-specific amplification of the PCR product. All the samples

Table 1. Sequences of primers used.

Gene	*Primer* (5'-3')	Amplicon size	Use
gshR	GSHRf - TGATGGCAGTTGTCGAAGCC GSHRr - ATGAATCCCCTCACCTGCCA	917	PCR
gshR	GS912f - CGCTTGAATTTCCCGCTCA GS912r - TCGCTTCCAACCGCTTTAGCA	91	qRT-PCR
gyrB	GYRB4f - GGGATGATGTCCGCGAAGGA GYRB4r - TGTTGTCAAAGCTTCCGCAAAGA	143	qRT-PCR

were analyzed in triplicate, and the mean values were used in the subsequent calculations. The reaction efficiencies were measured (Bustin et al., 2005; Pfall, 2001) and the amplification specificity was assessed through analysis of dissociation curves. The gshR gene expression was normalized using the expression of the internal control (gyrB) gene. The relative expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Lyophilization

Aliquots (1 mL) from the *L. lactis* LVA. 2 culture grown in CDM and M17 media, under different growth conditions, were centrifuged at 10,000 *xg* and 4°C for 5 min. The pellet formed was washed twice with 0.1% peptone water, re-suspended in 1 mL of peptone water and stored at -80°C for 3 h. Subsequently, the samples were dehydrated in a lyophilizer (Enterprise II, Terroni) for 7 h. After the lyophilization process, the dehydrated cells were reconstituted in 1 mL of 0.1% peptone water for 15 min at room temperature. The viability was assessed using the flow cytometry method immediately after reconstitution.

Analysis of viable cells

The cell integrity following the lyophilization process was analyzed using two dyes, which stain nucleic acids, in combination with the flow cytometry analyses. The fluorescent dyes syto9 and propidium iodide (PI) from the bacterial viability kit LIVE/DEAD® *Bac*Light™ (Molecular probes, Invitrogen) were used for that purpose. The syto9 dye stains the DNA of living and damaged cells. The PI dye only stains the DNA of cells with compromised plasma membranes. The cell-staining protocol provided by the manufacturer was modified in a few steps. Aliquots of stock solutions of the Syto (3.34 mM) and PI (20 mM) dyes were diluted in 0.85% saline to prepare a 0.83 and 5 mM concentration of Syto9 and PI, respectively. The dehydrated cells reconstituted in 0.1% peptone water were diluted in 0.85% saline to reach 10^4 to 10^5 colony-forming units (CFU) mL^{-1}. Aliquots (100 µL each) of the diluted cell suspension were supplemented with 900 µL of 0.85% saline and 1.5 µL of each dye. The samples were incubated for 15 min at room temperature and in the dark and were then centrifuged (13,000 *xg*, 3 min, 4 °C), and the pellet was resuspended in 300 µL of 0.85% saline prior to the flow cytometry analysis. The analyses were performed in a Guava Easycyte Plus® flow cytometer. The red fluorescence of the PI-stained cells and the green fluorescence of the syto9-stained cells were detected using 583/26 and 525/30 nm filters, respectively. The gates of the forward scatter/side scatter (FSC/SSC) graphs were used to distinguish the bacteria. The analyses were performed using low flow (0.24 µl $sec.^{-1}$), and the event data (10,000 events per sample counted in the bacterial gate in the FSCxSSC plots) were collected and analyzed using Cytosoft 5.3 Guava®. The results found are derived from three replicates.

Nucleotide sequence accession number

The nucleotide sequence for the *gshR* gene described in this study was deposited in GenBank under accession number KC821740.

RESULTS

Presence of GSH in *L. lactis* LVA. 2

The intracellular GSH content of *L. lactis* LVA. 2 grown in CDM with and without GSH supplementation was evaluated (Figure 1). The absence of intracellular GSH in *L. lactis* LVA. 2 in the CDM without GSH supplementation indicated that this strain is not able to synthesize GSH and is only able to transport it when the CDM is supplemented with GSH (Figure 1a). It was also observed that the cells accumulated almost the same amount of GSH that was found in the CDM at the 4-mM GSH concentration when the final concentration of GSH in the CDM was 6 mM. Furthermore, the tested strain does not use GSH from the culture medium as a source of that nutrient for its growth (Figure 1b).

Increased concentration of intracellular GSH

Different growth conditions were evaluated to assess their effect on the intracellular GSH concentration in *L. lactis* LVA. 2 (Figure 2). A low concentration of intracellular GSH was obtained when the cells were grown in M17 medium without GSH supplementation, regardless of the growth condition used, and no noticeable difference was found. The supplementation of the M17 medium with GSH increased the intracellular GSH concentration in the three growth conditions, although such increase was more marked in the anaerobic medium and in the aerobic growth in a rotary shaker.

gshR gene expression in different growth conditions

The relative expression analysis using real-time quantitative polymerase chain reaction (RT-qPCR) of the *gshR* gene was evaluated to assess whether the gene's expression is altered in the different growth conditions in

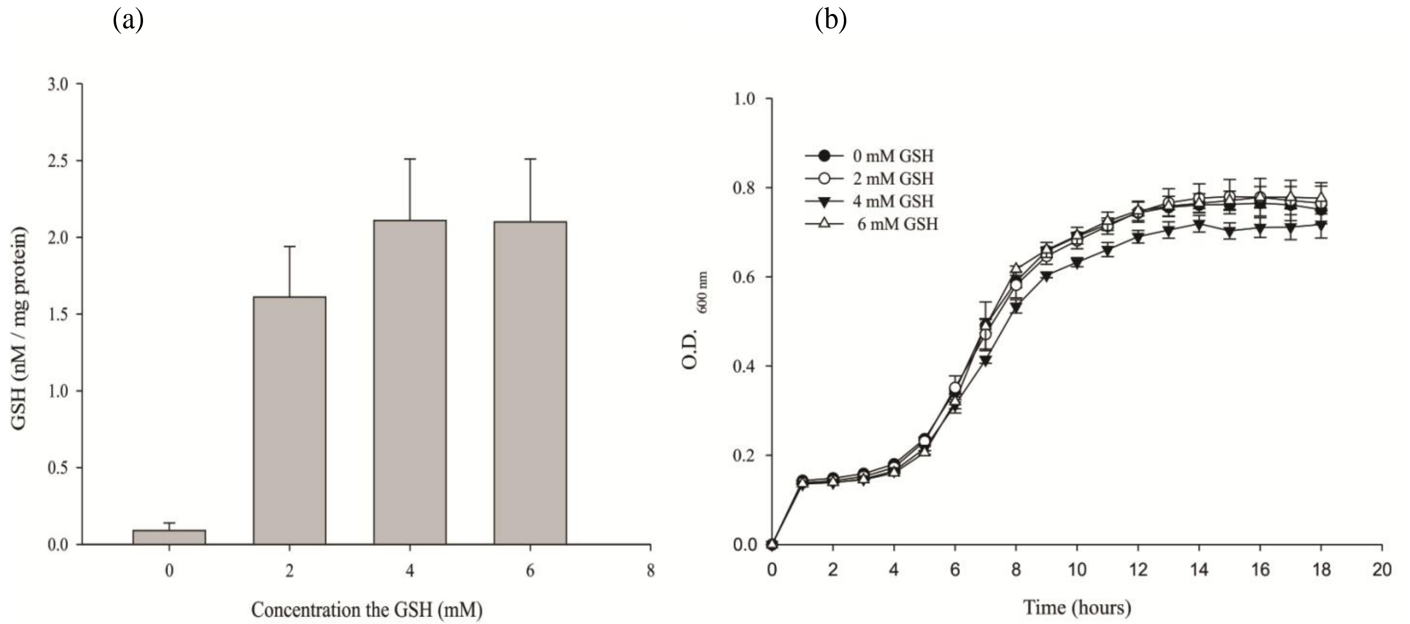

Figure 1. Effect of adding different GSH concentrations to CDM on the intracellular GSH concentration (a) and growth of *L. lactis* LVA. 2 (b) cultured at 30°C for 7 h. The intracellular GSH concentration and cellular growth were assessed using a spectrophotometer measuring the absorbance at 412 and 600 nm, respectively. The mean values are based on three sample replicates. The error bars indicate standard deviations.

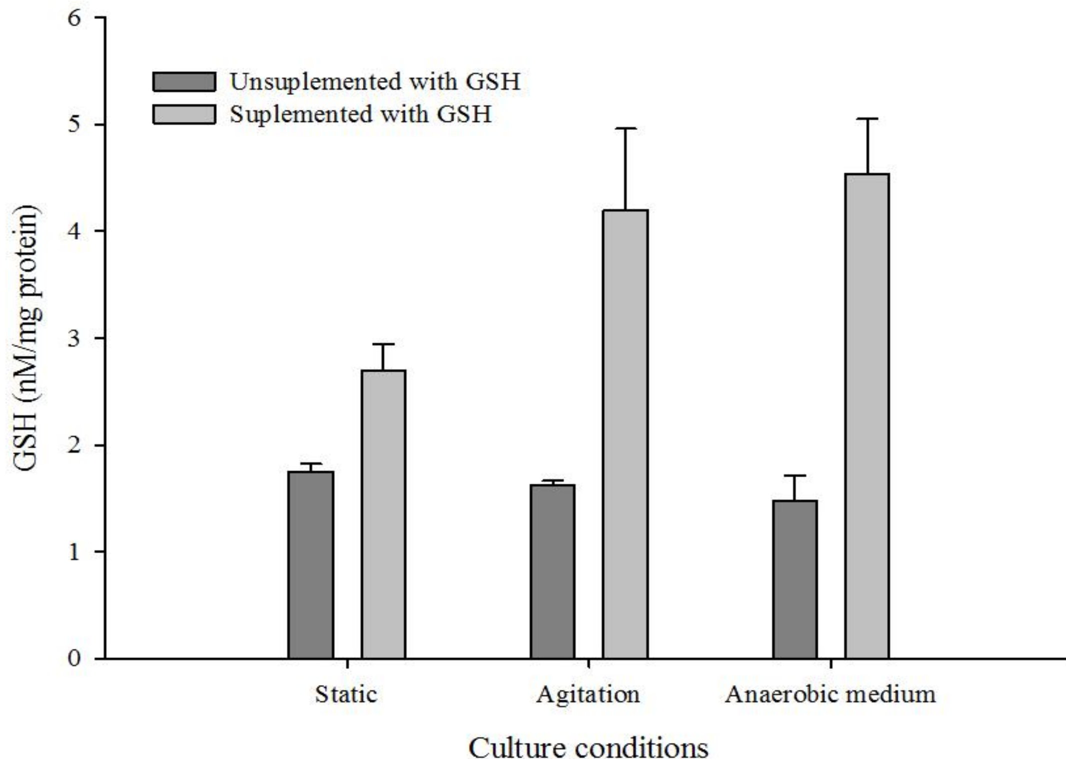

Figure 2. Intracellular GSH concentration of *L. lactis* LVA. 2 in M17 medium supplemented and unsupplemented with GSH. The cells were incubated statically or in a rotary shaker (200 rpm) at 30°C for 7 h. The cells were also seeded in anaerobic M17 medium statically incubated at 30°C for 7 h. The intracellular GSH concentration was assessed using a spectrophotometer measuring the absorbance at 412 nm. The mean values are based on three sample replicates. The error bars indicate standard deviations.

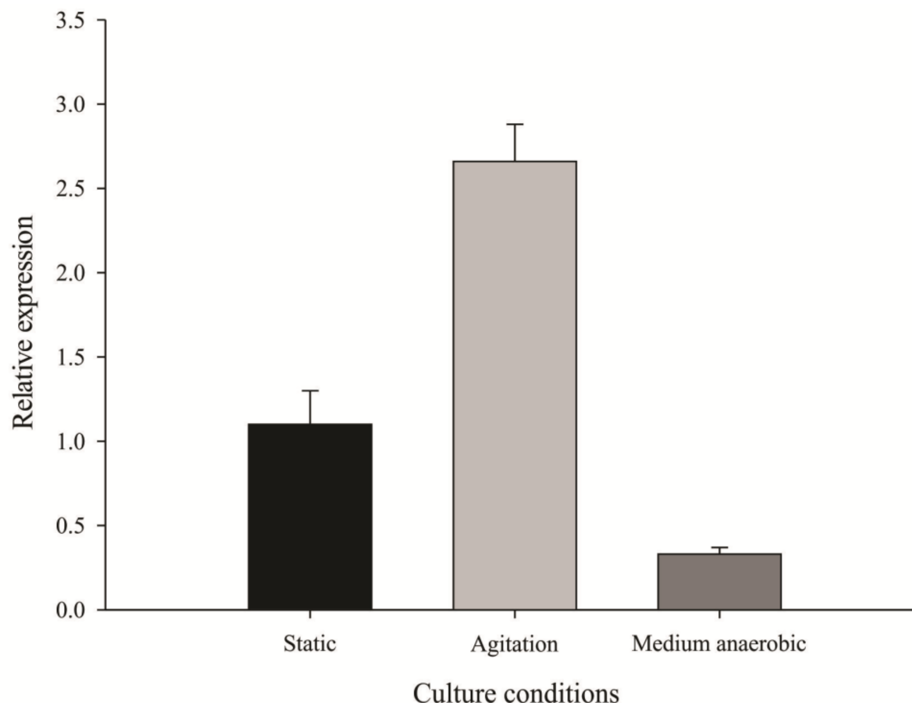

Figure 3. Normalized relative expression of the *gshR* gene of *L. lactis* LVA. 2 under different growth conditions in M17 medium supplemented with GSH. The inoculated medium was incubated at 30°C for 7 h. The error bars represent the standard deviations.

M17 medium supplemented with GSH. The growth of *L. lactis* LVA. 2 under the different growth conditions caused different variations in the relative expression of the *gshR* gene (Figure 3). The gene expression under continuously stirred aerobic growth conditions increased 2-fold as compared to the growth in static aerobic conditions (control), and its expression increased 8-fold as compared to the growth in the anaerobic medium.

Viability assessment

The PI and syto9 dyes were used simultaneously to assess the viability of *L. lactis* LVA. 2 prior to and following lyophilization (Figure 4). Differences in the distribution of the cell population were observed among the cell populations prior to (Figure 4a) and following lyophilization (Figure 4b). Most of the cell population following the lyophilization process was damaged.

The growth in CDM with and without GSH was used to examine whether the GSH accumulation in *L. lactis* LVA. 2 had a protective effect on the cells subjected to the lyophilization process. The GSH accumulation in *L. lactis* LVA. 2 provided no apparent protection when the cells were exposed to the lyophilization process (Figure 5a). The cells grown in the anaerobic medium were apparently more sensitive to the lyophilization process than the cells grown in the static aerobic and continuously stirred aerobic conditions when *L. lactis* was grown in M17 medium supplemented with GSH under different growth conditions (Figure 5b).

DISCUSSION

The selection of *L. lactis* strains with the ability to transport GSH from the growth medium into the intracellular environment has been regarded as an alternative to prepare starter cultures with more stable viability under stress conditions (Pophaly et al., 2012). The existence of *L. lactis* strains with the ability to biosynthesize GSH has not been reported in the literature, and there are only reports of strains with the ability to transport GSH into the intracellular medium (Li et al., 2003). Given the functional role that GSH has in cells, it is interesting that GSH must be accumulated in large amounts in cells to play its protective role. Thus, the use of strains using GSH as a nutrient source is not recommended. GSH is noticeably consumed as a cysteine source during growth in *Streptococcus mutans* (Sherrill and Fahey, 1998) and *Leuconostoc mesenteroides* (Kim et al., 2012).

The microorganism growth conditions must be optimized to prepare a freeze-drying or spray-drying culture or a frozen culture with a higher proportion of viable cells, to provide physiological changes favorable to

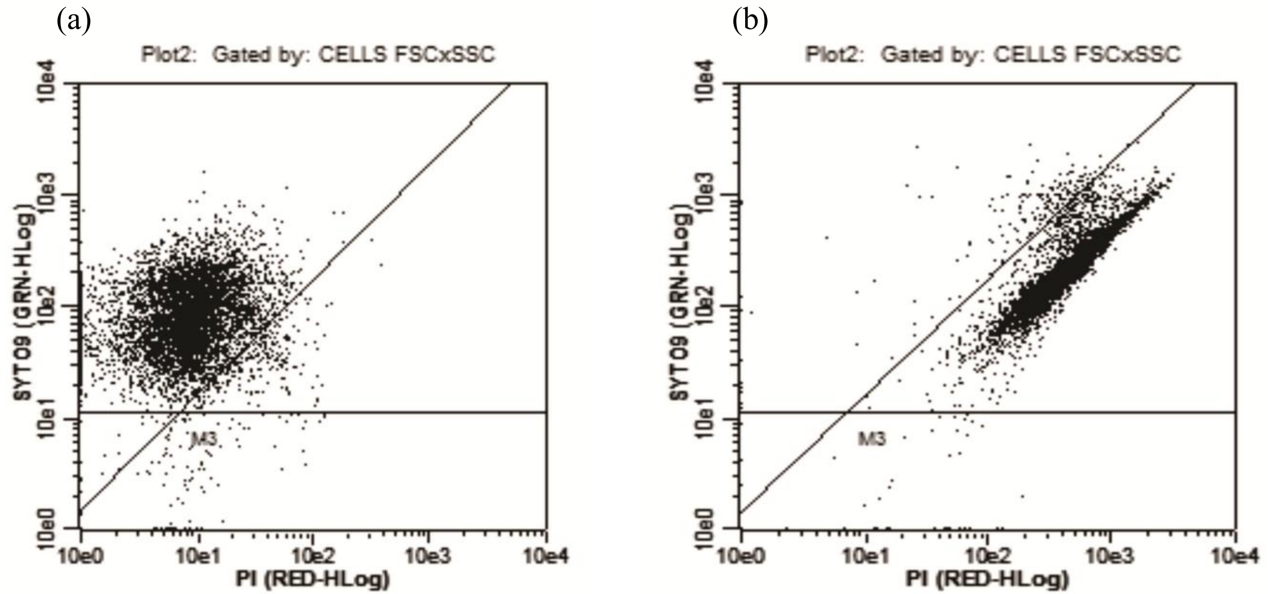

Figure 4. Multiparametric dot plot obtained after the double staining with syto9/PI in the labeling of *L. lactis* LVA. 2 (a) cells collected prior to and (b) following lyophilization.

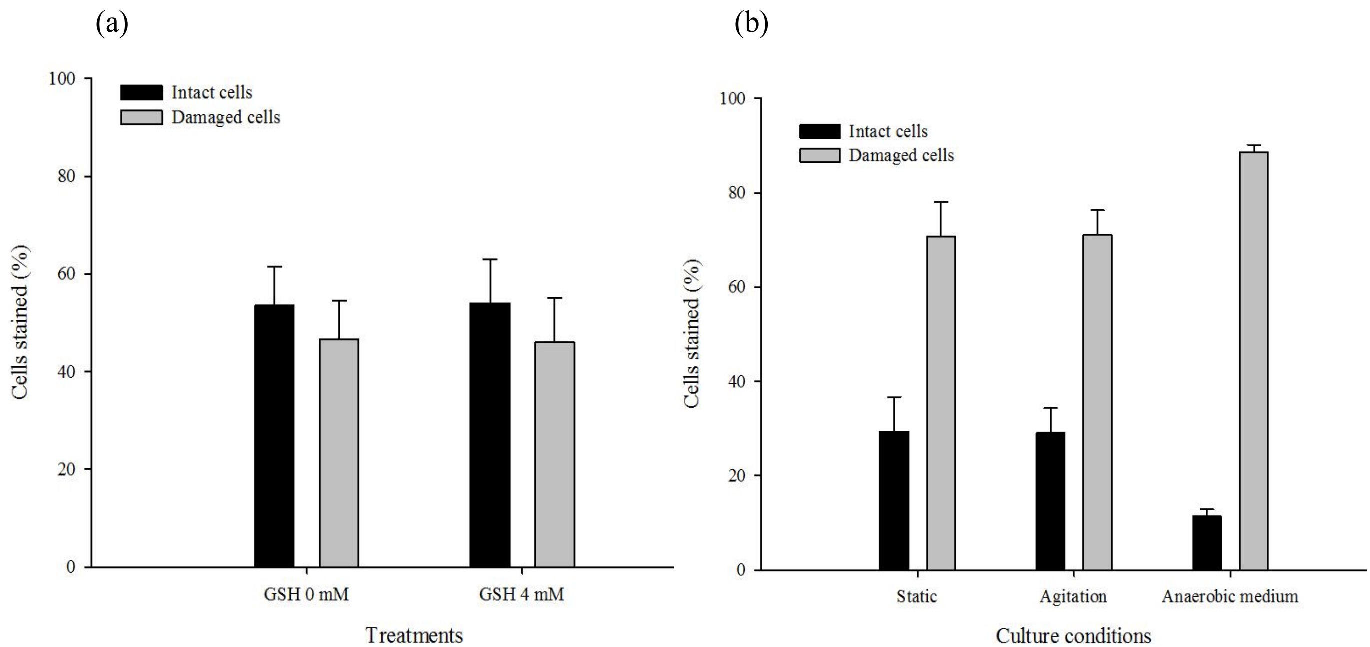

Figure 5. Percentage of undamaged and damaged cells of *L. lactis* LVA. 2 following the lyophilization process, analyzed using flow cytometry. The cells were grown in CDM (supplemented and unsupplemented with GSH) (a) and in M17 medium supplemented with GSH (static, continuously stirred or in anaerobic medium) (b). The data are mean values based on three replicates.

the cells, making them more resistant to those conditions. The increased accumulation of GSH in *L. lactis* LVA. 2 under the continuously stirred aerobic and NaCl-supplemented aerobic growth conditions appears to act as a cellular regulatory defense mechanism when the cells are exposed to stress conditions. It has already been found that the accumulation of GSH in *Escherichia coli* is high when the cells are exposed to NaCl-induced hypertonic conditions (Mclaggan et al., 1990). A similar result was not found when *L. lactis* LVA. 2 was grown in

static aerobic conditions because the regulatory mechanism most likely did not need to be very active given the lower oxygen incorporation. However, we believe that the large accumulation of GSH noted in the anaerobic-medium condition resulted from the low cellular consumption of GSH to maintain intracellular homeostasis.

The physiological status of *L. lactis* LVA. 2 cells can be monitored by the assessment of *gshR* gene expression. It has already been shown that the presence or absence of GSH has no effect on the activity of the GshR enzyme (Li et al., 2003). Thus, the factor that most likely led to an alteration in the *gshR* gene expression in the present study was the oxygen concentration of the culture medium. Therefore, the expression of the gene is expected to increase in continuously stirred aerobic growth conditions, and its expression would be reduced in anaerobic medium. Conversely, the *gshR* gene may have been repressed in the *L. lactis* LVA. 2 cells grown under osmotic stress conditions upon medium-level supplementation with NaCl.

GSH appears not to trigger any protective mechanism in the *L. lactis* LVA. 2 plasma membrane during lyophilization. There is no reference of any study in the literature that evaluated the protective effect of GSH in *L. lactis* following lyophilization, and only the protective effect of GSH on the *Lactobacillus sanfranciscensis* strain has been evaluated and verified (Zhang et al., 2010a). The detection of the protective effect provided by GSH might have been favored by the fact that the *L. sanfranciscensis* strain is very sensitive to the lyophilization process. However, we believe that GSH may have provided a type of protection by physiological regulation, as found in *L. sanfranciscensis* (Zhang et al., 2010b), and that protection cannot be found in *L. lactis* LVA. 2 immediately following lyophilization. The use of flow cytometry, a very sensitive technique that evaluates the integrity of the cell membrane in combination with dyes, showed that GSH had no protective effect on the membrane regarding the damage caused by ice-crystal formation and osmotic stress. The same result was also found using the plate-count method (data not shown).

Although the anaerobic medium provided better growth conditions to *L. lactis* LVA. 2, the absence of oxygen during growth may have inactivated some mechanisms involved in protection against the lyophilization process.

Conclusion

Although GSH failed to provide an apparent protection to *L. lactis* LVA. 2 following the lyophilization process, the fact that this strain accumulates GSH and expresses the *gshR* gene makes it a promising strain to cope with other stress situations, and GSH functionality has already been confirmed in other *L. lactis* strains.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Resistance of *Xanthomonas campestris* pv. *vesicatoria* isolates from Tanzania to copper and implications for bacterial spot management

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Thirty isolates of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) from Tanzania were evaluated for sensitivity to copper sulphate ($CuSO_4$:5$H_2$0) using dilution plate counts. Ninety-three percent (28/30) of the isolates produced countable colonies on nutrient glucose agar amended with 0.8 mM $CuSO_4$. All the *Xcv* isolates (7/7) from Arusha (northern Tanzania) grew on the copper medium. Isolates of the pathogen from Iringa (southern Tanzania) produced variable results (15/21). However, neither of the two *Xcv* isolates from Morogoro (Eastern Tanzania) grew on the copper medium. These results indicate long-term exposure of *Xcv* isolates from Tanzania to selection pressure for copper tolerance.

Key words: *Xanthomonas campestris* pv. *vesicatoria*, copper resistance, Tanzania.

INTRODUCTION

Bacterial leaf spot (BLS) of tomato (*Solanum lycopersicum* L.), caused by *Xanthomonas campestris* pv. *vesicatoria* (Syn. *Xanthomonas axonopodis* pv. *vesicatoria* (Vauterin et al., 2000) or *Xanthomonas euvesicatoria* (Jones et al., 2004)) is one of the most destructive and widely distributed diseases of tomatoes (Hovarth et al., 2012; Yu et al., 1995). The disease affects every above-ground part of the tomato plant. Attack on leaves causes defoliation, resulting in exposure of fruits to sun scald (Dougherty, 1978; Pohronezny and Volin, 1983). However, the main economic effect of the disease is the reduction in fruit weight and quality. Bacterial spots on the fruits have been reported to account for up to 52% weight loss in infected fruits (Jones et al., 1986). Disease control is exceedingly difficult to achieve when environmental and weather conditions are conducive for pathogen proliferation (Jones et al., 1986).

It is not clear when tomato bacterial spot was first

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identified and recorded in Tanzania, but Black et al. (2001) reported that the disease was widespread in tomato and pepper fields in all the vegetable-growing regions in Northern and Southern mainland Tanzania. Other surveys by Kaaya et al. (2003) confirmed wide-spread occurrence of bacterial spot in the country. Although most tomato growers in Tanzania rely on fixed copper chemicals to manage tomato bacterial spot, the disease has continued to spread in spite of the chemical application (Shenge et al., 2007). In spite of these reports, the long-term impact of these chemicals on the disease, the environment and agricultural production in Tanzania has not been assessed. The present study, therefore, aimed at assessing isolates of *Xcv* from Tanzania for resistance to copper, and determining if copper-based chemicals were an effective way of managing the disease in Tanzania.

MATERIALS AND METHODS

Seven samples were collected from Arusha, 2 from Morogoro, and 21 from Iringa region (Figure 1). Presumptive *Xanthomonas campestris* pv. *vesicatoria* isolates from tomato fruit lesions, and one reference *Xcv* strain from The Göttingen Collection of Phytopathogenic Bacteria (GSPB), GSPB 2043 were tested for *Xcv* determinative characteristics, including Gram reaction, nitrate reduction, Kovac's oxidase reaction, starch hydrolysis, pectin degradation, Biolog and hypersensitive reaction on tobacco leaves, following methods and classifications outlined in Schaad et al. (1988), Holt et al., (2000), O'Garro et al. (2003) and Woodland, (2004). Pathogenicity of the isolates was confirmed by misting an inoculum suspension of the bacteria adjusted to 10^8 cfu ml^{-1} onto 35-day-old tomato (cv. Tanya) plants and scoring disease symptoms 14 days after inoculation. Suspensions of 48 h old bacterial isolates were prepared in phosphate buffered saline (PBS) and adjusted to an optical density (OD) of 0.06 at 620 nm, corresponding to ca 10^8 cfu ml^{-1}. Twenty microliters of each suspension were spread evenly onto the 0.8 mM copper-containing medium (200 mg $CuSO_4.5H_2O$/1000 ml of NGA). NGA served as the untreated control. Inoculated plates were incubated at 28°C and enumerated 36 to 48 h later. Counts on the copper-amended medium and NGA were compared statistically using t-tests at $P \leq 0.05$.

RESULTS AND DISCUSSION

Yellow-pigmented *Xcv* isolates from lesions on tomato fruit samples were rod-shaped, unipolar flagellated, Gram-negative, oxidase-negative and catalase-positive. All the isolates hydrolyzed starch and degraded pectin, but were unable to reduce nitrates. Tobacco leaves inoculated with suspensions of the isolates showed a hypersensitive reaction within 24 h, and typical bacterial spot symptoms that were similar to natural symptoms developed on inoculated tomato (cv. Tanya) plants following inoculation. The isolates differed widely in their carbon source utilization profiles in Biolog; the dendrogram showed three distinct clusters that confirmed a strong relationship with geographic origin (Shenge et al., 2007).

The response of Xcv isolates from Tanzania to 0.8 mM CuSO4 is presented in Figure 2. Seventy-three percent of the isolates (22/30) produced countable colonies on the copper-amended medium, with all the isolates from Arusha Region (7/7) showing resistance to copper. Out of 21 isolates from Iringa Region, 15 grew on the copper-amended medium; neither of the isolates from Morogoro produced colonies on the copper medium.

The findings of this study demonstrated that resistance to copper-based chemicals was widespread within *Xcv* populations from the northern and southern highlands of Tanzania. These regions are also the leading tomato production areas in Tanzania, indicating that long-term use of copper-based chemicals in tomato production inadvertently led to *Xcv* selection for resistance to copper in the regions. In addition to tomato production, Arusha Region also has a long history of coffee production, which also receives heavy applications of copper compounds for coffee disease management.

Copper is required in trace quantities by many bacteria for their structural composition, as a co-factor in enzymatic functions (Bai et al., 2007), and electron transport and redox reactions (Cervantes and Guitierrex-Corona, 1994). However, at high concentrations, copper molecules become toxic to bacteria, interfering with the energy transport system and disrupting enzyme active sites, as well as the integrity of cell membranes (Cervantes and Gutierrez-Corona, 1994; Garcia-Horsman et al., 1994). High cellular copper concentrations have also been shown to damage lipids, proteins and DNA (Bai et al., 2007). Owing to these toxic properties, copper formulations have been used against a wide range of bacterial and fungal plant pathogens for more than 100 years (Cooksey and Azad, 1992). However, copper ions are not degraded in soil and can accumulate to high levels at locations with a history of intensive copper application (Koller, 1998). Long-term microbial exposure to sub-lethal concentrations of the chemical leads to adaptation through the development of plasmid/chromo-somally-borne cellular copper sequestration (Cooksey, 1990) and detoxification systems that protect the bacteria from toxic concentration levels of copper, while at the same time ensuring that their nutritional copper require-ments are met (Voloudakis et al., 2005).

In general, the ability of *Xcv* strains to tolerate copper in artificial growth media is used to measure copper resistance in the field. Several instances of this approach have been documented in literature (Cooksey et al., 1990; Gore and O'Garro, 1999; Tesoriero et al., 1997; Zevenhuizen et al., 1979; Martin and Hamilton, 2004) with similar levels of effectiveness. In studies by Zevenhuizen et al. (1979), strains of bacteria were considered to be resistant to $CuSO_4.5H_20$ if they were able to survive in a medium containing 1.0 mM of the compound. In other studies, Gore and O'Garro (1999) reported that ability of *Xcv* strains to express confluent growth on NA amended with copper sulphate at a concentration of 200 μg ml^{-1} (0.80 mM) was an expression of resistance to the

Figure 1. Geopolitical map of Tanzania. Red stars indicate locations where *Xanthomonas campestris* pv. *vesicatoria* isolates were collected. The locations were selected based on a history of tomato production, and also to cover a wide range of ecological conditions.

chemical. In the current study, 73% of *Xcv* isolates from Tanzania produced countable colonies in NGA amended with 0.8 mM $CuSO_4$. Based on the conclusions of previous studies, the *Xcv* strains from Tanzania can, therefore, be classified as resistant to copper.

The findings of this study are in agreement with other reports, which showed that increasing occurrence of copper resistance within populations of *Xcv* was becoming a serious problem in many tomato production areas (Gore and O' Garro, 1999; Martin and Hamilton, 2004; Mirik et al., 2007). These findings highlight the negative environmental impact of long-term use of copper-based chemicals as a plant disease management option. With particular reference to Tanzania, these results demonstrated that copper pesticides are no longer an effective means for tomato bacterial spot management. Identification of alternative natural and synthetic antimicrobial agents against *Xcv* is therefore, exigent. Recent reports by Mbega et al. (2012) indicated that some plant extracts were effective in reducing seed-borne xanthomonads associated with bacterial leaf spot. It seems that tomato bacterial spot management approaches that

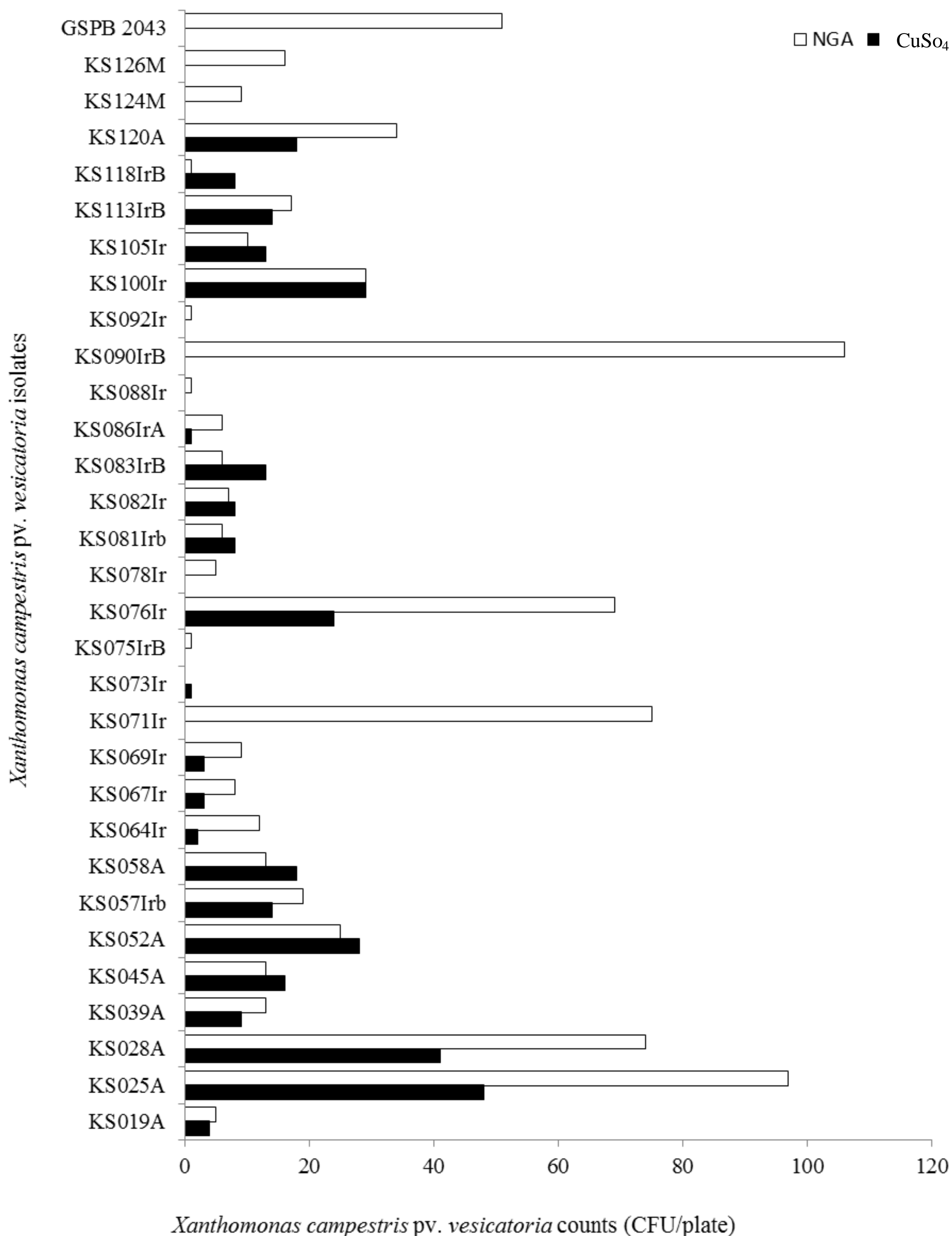

Figure 2. Resistance of *Xanthomonas campestris* pv. *vesicatoria* to $CuSO_4$. Solid, black bars indicate the number of countable *Xcv* colony forming units (CFU) on nutrient glucose agar (NGA: Nutrient agar + 5% glucose) medium amended with 0.8 mM $CuSO_4{:}5H_2O$ while unshaded bars indicate the number of *Xcv* CFU on NGA medium without $CuSO_4$. Labels on the vertical axis represent isolates used in the study. Isolate names ending with A indicates that the isolates were collected from Arusha Region; Ir, IrA and IrB were collected from Iringa Region, while those ending with M were collected from Morogoro region. IrA and IrB indicate that the diseased tomato fruit samples from which the pathogens were isolated came from the same field.

integrate the use of such plant products, with synthetic chemicals, disease-free seeds, field sanitation and resistant tomato varieties would be an effective way of managing the disease in a sustainable manner. In general, successful management of bacterial leaf spot in Tanzania would require the design of spray programs that accommodate the pesticide sensitivity status of pathogen populations. One component of such a program should consist of regular field surveys to determine the likelihood that farmers will encounter resistant strains of the pathogens, and a system that combines one or two antimicrobial compounds to eliminate the likelihood of the pathogens developing resistance to any one of them.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Effect of the inoculation of *Axonopus affinis* plantlets with immobilized rhizobacteria exposed to cadmium

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The interactions between plants with rhizosphere and root associated microorganisms have been considered because they are potentially useful in phytoremediation and are used as microbial inoculates (biofertilizers) that give bioprotection to plants against biotic and abiotic stresses. One of the methods safe and effective for introducing bioinoculants is the encapsulation of cells in biodegradable gel matrices like alginate. The aim of this study is to evaluate the effect of three rhizobacteria (*Achromobacter* sp. strain C1, *Pseudomonas* sp. strain sp7E and *Serratia* sp. strain 6), immobilized in alginate beads (bioinoculants) on growth of carpet grass (*Axonopus affinis*) plantlets against a heavy metal. The results showed the maintenance of the *A. affinis* plantlets growth against cadmium exposed to immobilized rhizobacteria in alginate beads with a higher cell density under protected micro environment. The results of this study show that the immobilized *Achromobacter* sp. strain C1 and *Serratia* sp. strain 6 are suitable bioinoculants because they maintain a higher cell density under protected micro environment as well as the growth of *Axonopus affinis* plantlets against cadmium.

Key words: Immobilization, plant growth-promoting rhizobacteria, cadmium, *Axonopus affinis.*

INTRODUCTION

The interactions between plants with rhizosphere and root associated microorganisms have been considered in phytoremediation technologies (Abhilash et al., 2012; Singh, 2004; Weyens et al., 2009). Plant growth-promoting rhizobacteria (PGPR) establish direct and indirect interactions with plant roots, favoring the increase of nutrient availability, health and plant growth (Glick, 1995; Park et al., 2005; Vassilev et al., 2001).

Rekha et al. (2007) and Saxena (2011) noted the importance in the use of inoculant formulation: it provides

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more suitable microenvironment for the prolonged survival of bacteria that guarantee the release of high density bacterial inoculums that reduces cell loss. This technique could be a successfully bioaugmentation process that can be improved by microbial cell immobilization in a carrier matrix (Jézequel and Lebeau, 2008; McLoughlin, 1994).

Albareda et al. (2008, 2009), Dary et al. (2010), Staley and Brauer (2006), Vassilev et al. (2001) and Vilchez and Manzanera (2011) mention that one of the methods safe and effective for introducing bioinoculants in soil is the encapsulation of cells in biodegradable gel matrices. Bashan et al.,(2002), Compant et al. (2010) and alginate beads Guimaraes et al. (2010) recommend the use of non toxic natural polymers like alginate beads and alginate based preparation systems that preserve the beneficial and potential properties of bacteria, maintained under storage. These formulations used to encapsulate living cells can protect and maintain the microorganisms against many environmental stresses. When the polymers are degraded, the bacteria are released in large quantities and it will be very important for seed germination or emergence of plants seedling.

The aim of this study is to evaluate the effect of inoculation with three rhizobacteria (*Achromobacter* sp. strain C1, *Pseudomonas* sp. strain sp7E and *Serratia* sp. strain 6) immobilized in alginate beads on growth of carpet grass (*Axonopus affinis*) plantlets against a heavy metal.

MATERIALS AND METHODS

Inoculum preparation of the employed rhizobacteria

Three siderophore producing rhizobacteria strains were used as inoculants: *Achromobacter* sp. strain C1 isolated from the rhizosphere of *Sporobolus indicus*, *Pseudomonas* sp. strain sp7E isolated from the rhizosphere of *Viguiera dentata* (Cav.) Spreng. and *Serratia* sp. strain 6 isolated from the rhizosphere of *Sphaeralcea angustifolia*; the three plant species are grown in a metal contaminated soil located in Villa de la Paz in the State of San Luis Potosí, México (Melo et al., 2011).

Bacterial inoculum was obtained from each rhizobacteria strain cultured on plates with Luria-Bertani (LB) agar medium for 48 h at 28°C. Calibrated loops (1/100 cells) were re-suspended ina nefelometric flask with 25 mL of LB liquid medium and adjusted by optical density to $9x10^8$ $CFUmL^{-1}$; the rhizobacterial cultures were incubated in a shaker incubator at 28°C for 24 h. Later, the inoculums were finally adjusted to an optical density of $5x10^9$ CFU mL^{-1} for immobilization.

Immobilization of rhizobacteria in sodium alginate

Twenty five milliliters sterile solution of sodium alginate (2.75 g sodium alginate/75 mL of distilled water, autoclaved at 120°C, during 15 min) was mixed thoroughly with the cell suspension and vigorously stirred for 30 min to allow a homogenous dissolution of alginate. Ca-alginate beads were obtained by dropping the alginate cell mixture with 5mL sterile pipette (10 cm) into a 200 mL stirred-sterile solution of $CaCl_2$. Beads remained in this solution for 15 min. The $CaCl_2$ solution was drained and then the alginate beads were rinsed three times with sterile distilled water. The resultant alginate beads had a mean diameter of 3 mm used for entrapping the bacterial cells. The bacterial-alginate beads were collected and allowed to dry at room temperature for 30 min until they were further used for bioassays.

Assessment of the growth of *A. affinis* plantlets inoculated with the selected immobilized rhizobacteria exposed to cadmium

Commercially obtained certified seeds of *A. affinis* (Chase) were surface-sterilized with 10% sodium hypochlorite, rinsed with sterile distilled water and deposited on sterile plastic pots of 50 mL capacity (5 seeds/pot) filled with 28 g of sterile vermiculite. Pots were wet with 15 mL of mineral solution (0.20 M $NH_4H_2PO_4$, 0.50 M NH_4NO_3, 1.15 M $Ca(NO_3)_2$, 0.26 M $CaCl_2$, 0.2 MMg $Cl_2{\bullet}6H_2O$, 0.20 M $Mg(NO_3)_2{\bullet}6H_2O$, 0.40 M $MgSO_4{\bullet}7H_2O$, 0.20 M KH_2PO_4, 1.2 M KNO_3, 0.5 M K_2SO_4, 0.04 M $FeCl_3{\bullet}6H_2O$, 1.2×10^{-2} M H_3BO_3, 1.2×10^{-4} M $CuCl_2{\bullet}H_2O$, 2.3×10^{-3}M $ZnCl_2$, 4.4×10^{-4}M $MnCl_2{\bullet}4H_2O$, 6×10^{-6} M $Na_2MoO_4{\bullet}H_2O$, EDTA and $FeSO_4{\bullet}7H_2O$, pH = ±6.0). The established experiments were done with and without the immobilized rhizobacteria; they were supplemented with cadmium ($3CdSO_4{\bullet}8H_2O$) concentrations of 0.5, 1.0 and 1.5 mM. Rhizobacterial strains encapsulated in alginate beads were sown for each inoculated pot in 15 alginate beads per 5 seeds (4 x 10^8CFU $mL^{-1}$of inoculum). All the experiments were performed by quintuplicate and plantlets of *A. affinis* were cultured and maintained at 28°C in a growth chamber of a 12:12 photoperiod for 10 days.

Determination of the tolerance index of *Axonopus affinis* plantlets to cadmium

Epicotyl and radicle parts of the plantlets were separated at the end of the cultures, weighted as fresh biomass and then dried at 70°C for 24 h to obtain the dry biomass. The Tolerance Index (TI) was calculated by using the formula of Wilkins (1978) and Burd et al. (1998): TI = RBm / RBc; where, RBm is the mean fresh biomass of plantlets grown in the presence of cadmium and RBc is the mean fresh biomass of control plantlets grown without the metal. The experiments with the rhizobacteria alginate beads were done using this formula: IT = RBim + m/RBim, where RBim + m is the mean fresh biomass of plantlets with rhizobacteria immobilized in alginate beads grown in the presence of cadmium and RBim is the mean fresh biomass of plantlets with rhizobacteria immobilized in alginate beads without the metal.

Evaluation of the immobilized rhizobacteria at the end of the experiments: bacterial survivor and growth on alginate beads

For the evaluation of the immobilized rhizobacteria tested at the end of the experiments, one rhizobacteria alginate bead from each pot (quintuplicate) was recovered and the calcium alginate matrix was dissolved according to the method of Yoon et al. (2002). The alginate beads were deposited in assay tubes with 5 mL EDTA-Na_2 50 mM, for 24 h at room temperature. After this, the tubes were incubated for 1 h in a boiled bath at 40°C with occasional hand shake. The rhizobacteria cell concentrations were determined by measuring the optical density (OD) of the samples with a Klett-Summersonfotocolorimeter with Blue Filter No.42 (Absorbance = Klett Units (KU/500).

All the results were analyzed by ANOVA one way test and Tukey-Kramer method using Graph PadInstat Ver. 3.10 program. A linear regression equation between plants' dry biomass and bacterial growth was done using the Microcal Origin Ver. 3.5.

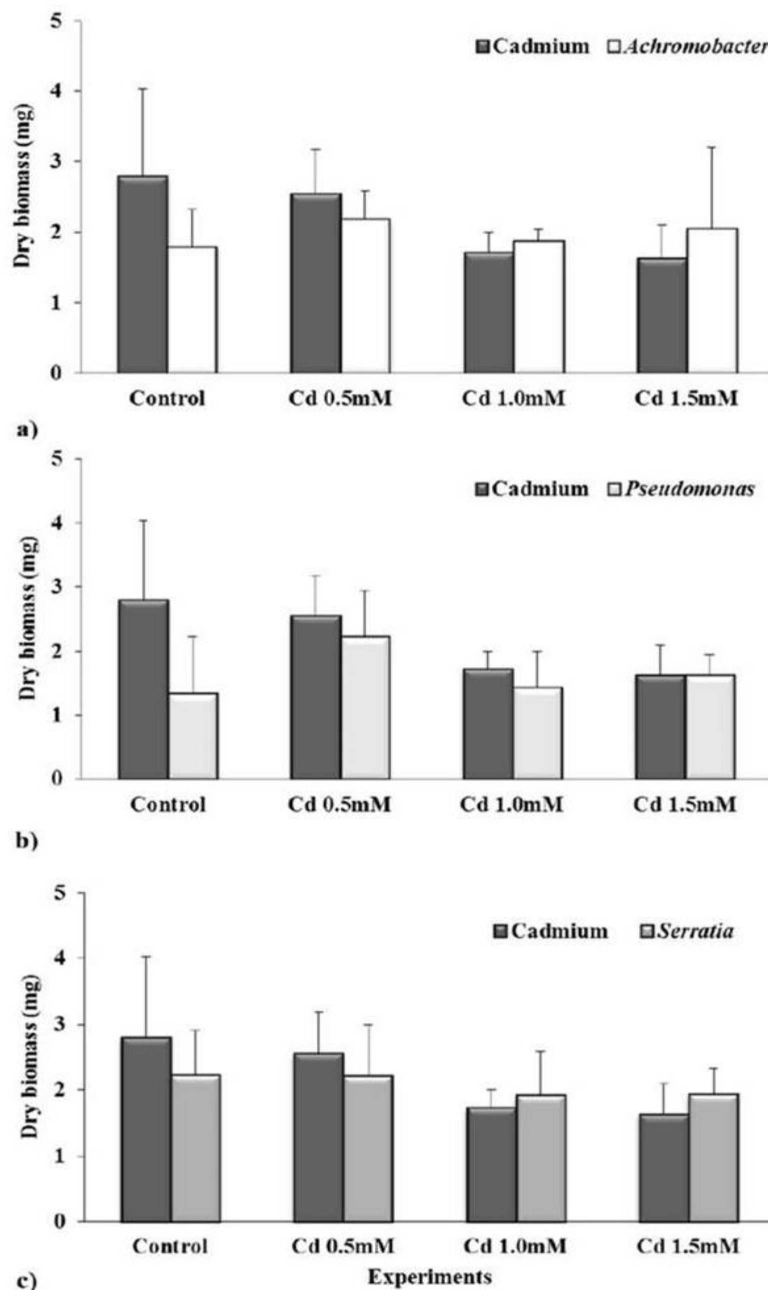

Figure 1. Dry biomass of *Axonopus affinis* plantlets inoculated with the immobilized rhizobactera, exposed to cadmium; control experiments means without cadmium and with inoculum. a) *Achromobacter* sp. strain C1, b) *Pseudomonas* sp. strain Sp7E and c) *Serratia* sp. strain 6. Mean values + S.D., n=25. No significant differences between experiments (p>0.05).

RESULTS AND DISCUSSION

The presence of cadmium in this species had an inhibitory effect on plantlets' growth; there was a decrease in dry biomass as the cadmium concentration increased. Figure 1 shows the increase in the plantlets' dry biomass obtained in the presence of the two immobilized rhizobacteria (*Achromobacter*sp. strain C1 (Figure 1a) and *Serratia* sp. strain 6) (Figure 1c), in the experiments exposed to 1.0mM and 1.5mM concentrations of cadmium. But, rhizobacteria *Pseudomonas* sp. strain Sp7E (Figure 1b) had low effect on plant biomass. The protection given to plantlets by the three immobilized rhizobacteria are analyzed by the

Table 1. Tolerance index (TI) of non inoculated and inoculated *Axonopus affinis* plantlets to cadmium*.

0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
No inoculated plantlets		
1.19±1.2	1.01±0.92	1.01±0.95
Inoculated plantlets with *Achromobacter* sp. strain C1		
1.22±0.78	1.15±0.53	1.17±0.63
Inoculated plantlets with *Pseudomonas* sp. strain Sp7E		
1.36±0.63	1.18±0.71	1.06±0.6
Inoculated plantlets with *Serratia* sp. strain 6		
1.29±0.74	1.23±0.69	1.23±0.63

*No significant differences between experiments (p>0.05).

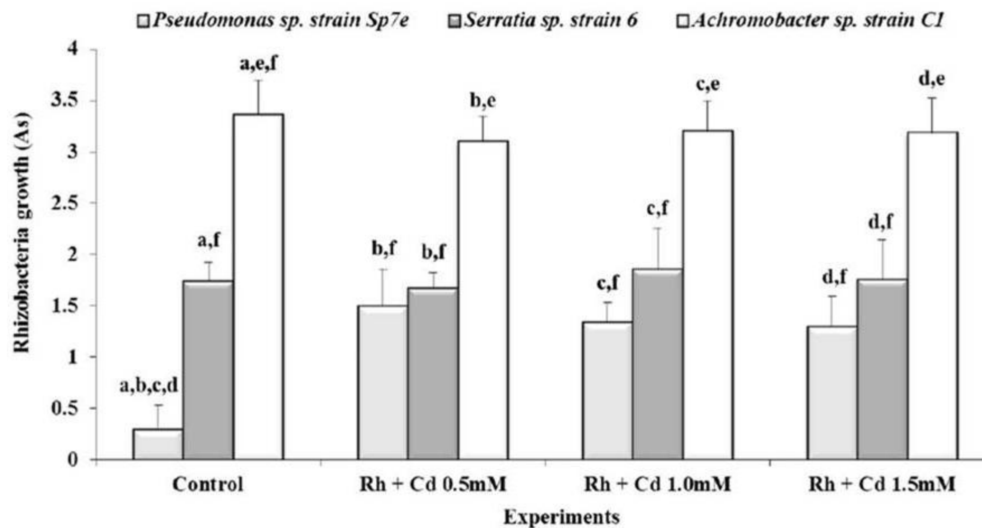

Figure 2. Rhizobacteria final growth (turbidity measurement) analyzed from the alginate beads after the exposition to cadmium. Mean values + S.D., n=5. The different lower-case letters shows the significant differences between experiments (p<0.001).

Tolerance Index (TI) to cadmium (Table 1) based on fresh weight of plantlets, compared to the control experiments. They are as follows: *Serratia* sp. strain 6 with TI of 1.29, 1.23 and 1.23; *Achromobacter* sp. strain 6, TI: 1.22, 1.15 and 1.17 and *Pseudomonas* sp. strain Sp7E, TI: 1.36, 1.18 and 1.06 to 0.5, 1.0 and 1.5 mM cadmium concentrations, respectively. Rhizobacteria *Serratia* sp. strain 6 that maintains the plantlets' growth at three cadmium concentrations led to an increase of 29 to 23% of fresh biomass.

Saxena (2011) mentions that the entrapment of microorganisms is an effective strategy for the application of bioinoculants, because it provides a protective niche together with nutrient source. Some works like those of El-Katatny et al. (2003) and El-Komy (2005) showed that the alginate encapsulation of *Azospirillum* and *Bacillus megaterium* prolonged the durability of their inoculum and also the entrapment of microbial cells improved their metabolic activities. The encapsulation of microorganisms into a polymer matrix is still experimental in the field of bacterial-inoculation technology and alginate is the most commonly material used. In our study, the immobilized rhizobacteria resisted the micro-environmental conditions against the presence of cadmium in the experiments. They not only maintained the survival of the PGPRs, they also allowed the bacterial growth and released them close to the plantlets' roots. The growth measured after the treatment to release them from the dissolved alginate beads at the end of the experiments (10 days) (Figure 2) showed the conservation and increase of two effective rhizobacteria tested: *Achromobacter* sp. strain C1 with 3.1×10^9, 3.2×10^9 and 3.1×10^9CFU mL^{-1} and *Serratia* sp. strain 6 with 1.7×10^9, 1.8×10^9 and 1.8×10^9CFU mL^{-1}, with cadmium concentrations of 0.5, 1.0 and 1.5 mM,

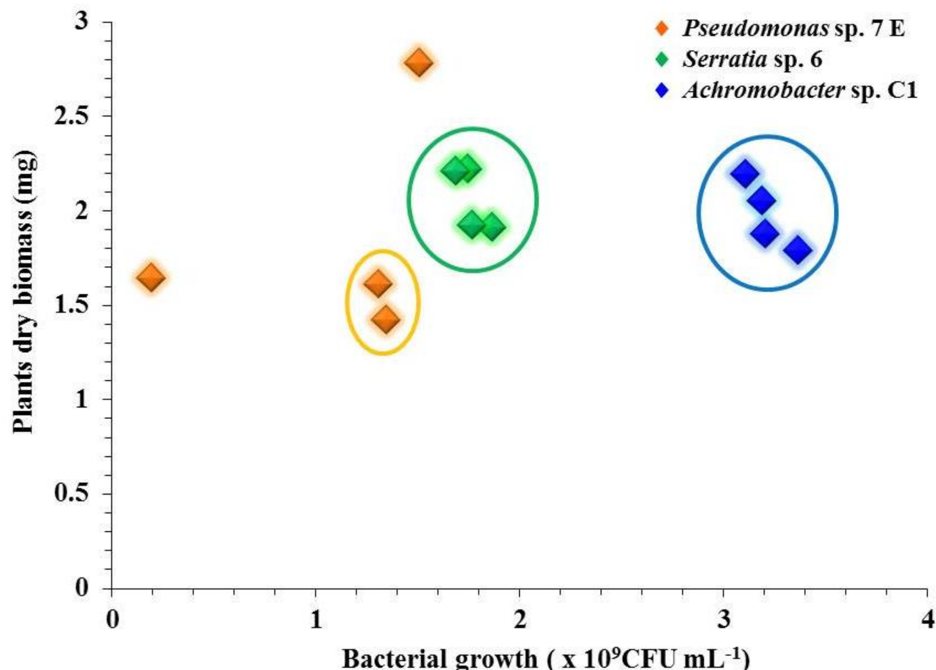

Figure 3. Linear regression curve showing the relationship between plants dry biomass (mg) and bacterial growth (10^9CFU mL^{-1}), experiments are shown in the plot (r= 0.16).

respectively. The micro environments of the alginate beads were not only successful, but it is also important to mention that the size of the alginate beads produced (3 mm diameter) was adequate for the rhizobacteria. These alginate beads entrapped a significant number of rhizobacteria (approximately 10^8 to 10^9CFU), similar to the level obtained in alginate macro beds by Bashan et al. (2002).

Bashan et al. (2002), Trejo et al. (2012) and Yabur et al. (2007) mention that alginate does not inhibit plant growth and that is why it is widely used in synthetic inoculants for PGPRs. Saxena (2011) inoculated wheat plants with immobilized bacterial strains; these significantly increased their shoot weight and length. The authors suggest that this increase may be due to the production of growth promoting substances by the inoculated bacteria. Their results were also corroborated by Chabot et al. (1993) who inoculated lettuce and corn plants with *Pseudomonas* sp. and observed an increase in plant height due to the siderophores and auxins produced by these bacteria.

The presence of immobilized rhizobacteria led to the maintenance of the *Axonopus affinis* plantlets growth against cadmium. Even though this growth was small compared to the control plantlets, it suggests that the immobilized rhizobacteria protect. Figure 3 shows the correlation between the gains of the total dry biomass of *A. affinis* inoculated with the three immobilized rhizobacteria and the bacterial growth measured at the end of the experiments. The correlation showed the particular effect of the control and cadmium experiments inoculated with *Serratia* sp. strain 6 and *Achromobacter* sp. strain C1; there was an increase in the biomass and growth of both rhizobacteria compared to *Pseudomonas* sp. strain Sp7E. This rhizobacteria showed the lowest plant biomass and bacterial growth with two extreme responses; plants inoculated without the heavy metal are at the left side and middle of the figure; it shows their response in the experiment inoculated with the bacteria and Cd (1 and 1.5 mM). Plants at the top of the figure had higher total plant biomass in the experiments inoculated with the rhizobacteria and Cd (0.5 mM).

Jézéquel and Lebeau (2008) showed that the beads made with alginate offer a lower mechanical resistance. These authors entrapped *Bacillus* sp. ZAN- 044 and *Streptomyces* sp. R25 in the beads and considered it as a transitional state to allow the adaptation of the bacterial cells to their new environment, before the cells are released from the beads. Van Elsaset al. (1992) noted that most of the immobilized rhizobacteria released endured in the root zone. Another work regarding the effect of immobilized rhizobacteria is the work of Aino et al. (1997) on the colonization of tomato roots with *Pseudomonas fluorescens* embedded in alginate beads. In this study, we suggest the colonization of roots of *A. affinis* plantlets by the rhizobacteria released from the alginate beds, as evident in the close interactions and adherence of alginate beads to the roots. This is shown in *Achromobacter* sp. strain C1 (Figure 4). It is important to mention that these evidences were a common

Figure 4. Details of *Axonopus affinis* plantlets inoculated with the immobilized rhizobacteria *Achromobacter* sp. strain C1. The arrows show the alginate bed are adhered to the roots, note the increase in the root length.

behavior found in all of the experiments inoculated with immobilized rhizobacteria.

Finally, the results obtained in this study show that the immobilized rhizobacteria *Achromobacter* sp. strain C1 and *Serratia* sp. strain 6 are suitable bioinoculants, because they maintain a higher cell density under this protected micro environment and growth-promoting response of *Axonopus affinis* plantlets against cadmium.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biotype, antibiotype, genotype and toxin gene tsst-1 in *Staphylococcus aureus* isolated from Cotija cheese in the state of Guerrero, México

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Milk and dairy products such as Cotija cheese are susceptible to contamination by *Staphylococcus aureus*. Some isolates of these bacteria carry the genes for enterotoxins and also the toxic shock syndrome toxin (TSST-1), additionally, many of these strains are potentially resistant to antibiotics. In the present study, a total of 50 samples of Cotija cheese sold at the Central Market "Baltazar R Leyva Mancilla" in the State of Guerrero were collected to determine the amount of bacteria growing in the cheese (expressed as CFU/g of cheese), the biotype, the antibiotype and the genotype. The results show that the amount of bacteria varies from $12x10^3$ to $3x10^6$CFU/g exceeding the permitted limits for ripened cheeses. Three biotypes were identified, 100% were susceptible to oxacylin, vancomycin, ciprofloxacin, trimethoprim-methoxazole, amikacin and clindamycin but tetracycline resistant. Only one of the four identified genotypes was positive to the TSST-1 gene. This is the first report of *S. aureus* isolated from Cotija cheese in the state of Guerrero, Mexico. Finally, our data evidenced cotija cheese as a vehicle carrying a large number of pathogenic *S. aureus* strains, suggesting that the public policy on food safety must be firmly reviewed.

Key words: *Staphylococcus aureus*, biotype, antibiotype, genotype, tsst-1, cheese.

INTRODUCTION

Staphylococcus aureus is a pathogenic bacterium in humans and animals, capable of producing a variety of toxins, as the enterotoxins A, B, C, D, E, G, H, I and J (also kwown as SEA , SEB , SEC, SED, SEE, SEG, SEH, SEI and SEJ, respectively) (Sospedra et al., 2012; Gouloumes et al., 1996; Morandiet al., 2007). It also produces the toxic shock syndrome toxin 1 (TSST- 1) which has a variety of effects on the host immune cells (Chapaval et al., 2006). As it is able to act as a super antigen, it leads to a massive release of cytokines

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including tumor necrosis factor alpha (TNF- alpha), interleukin 1 (IL -1) and IL- 6, thus causing capillary leak syndrome responsible of theTSST-1 symptoms (Sospedra et al., 2012). *S. aureus* has been isolated from clinical and environmental samples, in healthy carriers (nasal cavity, hands, etc.) and food. Symptoms associated with the consumption of food contaminated with *S. aureus* carrying the TSST-1 gene, usually present diarrhea, abdominal pain, cramps, exhaustion and in more severe cases include headache, muscle cramps, fever, vomiting, confusion, conjunctival hyperemia, hypotension, oliguria, renal damage, changes in blood pressure and pulse (Soriano et al., 2002; Sospedra et al., 2012), appearing from 1 to 6 h after ingesting contaminated food.

The probability of food poisoning by *S. aureus* (Necidova et al., 2009), is given by either the presence of only one of the enterotoxins or a microbial amount equal to or greater than 10^5 CFU/g (Kerouanton et al., 2007). Strains of *S. aureus* capable of producing enterotoxins isolated from cows, sheep, buffalo and other dairy products have been reported in Italy (Morandi et al., 2007). There have also been reports of outbreaks associated with the consumption of food in restaurants, buffets and cafeterias in USA, Japan, United Kingdom, Thailand, France, England and Mexico (Soriano et al., 2002). In France, *S. aureus* is the second microorganism associated with food borne infections after *Salmonella* sp. (Kerouanton et al., 2007). In the state of Guerrero, the Ministry of Health reported an outbreak caused by *S. aureus* and *Salmonella* sp. that led to 335 poisoned people, including 317 children and 18 adults. This happened during the celebration of the Children's Day at an elementary school in the village of Organs (http://prosaia.org). We were interested since it is of interest to study the food sources that may be contaminated with the bacteria. The strains of *S. aureus* producing the TSST-1 toxin isolated from food have been also associated with antibiotic resistance. Among the known cases are the isolates resistant to methicillin (MRSA) which confers resistance to all beta-lactamase antibiotics, making the treatment ineffective and being the cause of hospital outbreaks (Montesinos et al., 2002). The high health risks associated with MRSA infections in hospitals and in the community have prompted the government to implement surveillance and monitoring programs. It is important to note that in the city of Chilpancingo, Gro., Mexico, Cotija Cheese constitute an important part of the diary diet. The Cotija cheese is a Mexican handmade product with unique features in the world, which is derived from the coagulation of milk proteins, mainly casein, followed by a draining, salting and molding. The process of making cheese is completely handmade and begins with the milking of the cow to obtain milk then coagulant agent is added. This coagulation process removes water and the greater amount of lactose. Then, the required amount of salt is manually added. At least three months of maturing is required to ensure their sensorial and microbiological quality. Due to the existence of critical points or cross-contamination by the lack of good hygienic practices, Cotija cheese constitutes an important substrate for *S. aureus* growth. When temperature is above 40°C (normal room temperature in Guerrero), the genes for enterotoxins and toxic shock syndrome are activated in *S. aureus*. The aim of this study was to quantify the microbial load of *S. aureus* isolates in the Cotija cheese sold at the central market in the city of Chilpancingo, Guerrero, as well as to determine the phenotype and genotype of the isolated strains. The isolates were analyzed for biotype antibiotic resistance profiles and by PCR for the presence of TSST-1 gene. The genotype was determined by restriction pattern PFGE profile. This allowed us to compare and conclude the genetic relationship among our isolates and previously collected samples of Cotija cheese that were contaminated too.

MATERIALS AND METHODS

Food samples collection and processing

A total of 50 samples of Cotija cheese (250 g each) were collected from different spots at the central market Baltazar R Leyva Mancilla in the city of Chilpancingo. They were collected from August to October 2012 following the recommendations stated by NOM -109-SSA1-1994, which refers to the Decision Procedures for Handling and Transportation of Food Samples for Microbiological Analysis. All samples were analyzed at the Laboratory of Microbiology Research of Food and Beverages at the Autonomous University of Guerrero.

Isolation and identification of *S. aureus*

Isolation and identification of *S. aureus* from Cotija cheese samples were performed by using the guidelines of the NOM-115- SSA1-1994, specifying the Goods and Services Method for the determination of *S. aureus* in food. Colony forming units (CFU/g) were calculated per gram of Cotija cheese from each of the samples in the same way it was done previously for a common standard brand.

S. aureus was grown in Baird Parker agar with egg yolk emulsion potassium tellurite. 1% agar plates were incubated at 35°C for either 45 or 48 h. After this time, typical black, round, shiny, convex, smooth, diameter 1-2 mm colonies were observed. They showed opaque zones and clear halos surrounding the colonies. The biotype of the *S. aureus* isolates was confirmed by conventional biochemical tests that included: growth on Mannitol Salt Agar, Gram staining (where Gram positive cocci grouped in clusters were observed), the production of specific enzymes including coagulase, catalase, thermonuclease lecithinase and finally, the ability of fermenting carbohydrates such as glucose, mannitol, lactose, sucrose, maltose and trehalose.

DNA extraction

The DNA from the *S. aureus* isolates was extracted as previously described by Morandi et al. (2007).

Table 1. Phenotypic characteristics of *S. aureus* in Cotija cheese sold at the market of Chilpancingo city, Guerrero, Mexico.

Biotype	Lec	Term	No. (%) of isolates	SyM	Carbohydrates				
					Glu	Lac	Sac	Mal	Tre
1	-	-	8 (23)	+	+	+	+	+	+
2	+	-	18(51)	+	+	+	+	+	+
3	+	+	9 (26)	+	+	+	+	+	+

Lec: lecitine, Term: termonuclease, SyM: mannitol salt agar, Glu: glucose, Lac: lactose, Sac: sacarose, Mal: maltose and Tre: Trehalose.

PCR detection of the tsst-1 gene

Purified DNA was used for PCR detection of the TSST-1 gene using the following oligonucleotides; GTSSTR -1 5'-ACCCCTGTTCCCTTATCATC -3´ and GTSSTR -2, 5'-TTTTCAGTATTTGTAACGCC -3' (Chapaval et al., 2006) which amplify a product of approximately 326 bp. PCR was performed with a 50 µl reaction mixture containing 1 µl (10 ng) of DNA as template, each primer at a concentration of 5 µM, 2.5 mM $MgCl_2$ and dNTP´s at a concentration of 2.5 µM, as well as, 2 U of Taq DNA polymerase (Platinum® Taq DNA polymerase Invitrogen, Carlsbad, CA, USA) using the amplification protocol described by Chapaval et al. (2006). DNA of *S. aureus* ATCC 25923 was used as negative control in each round of PCR. All PCR reactions were carried out in a Mastercycler gradient Cycler® Ep (Eppendorf, Hamburg, Germany). PCR products were visualized on 2% agarose gels stained with ethidium bromide and visualized in the UV transiluminator (Bio-Rad system).

Antibiotic resistance assay

Both, the antibiotic resistance assay and the bacterial identification were performed by Post Combo Panel Type 41 and read on a WalkAway System (Dade Bering Inc., West Sacramento, Calif.). An inoculation control (0.5 McFarland units) was used for all the assays. The antibiotics tested were oxacylin (OXA), tetracyclin (TET), chloramphenicol (CHL), clindamycin (CLI), trimethroprim-sulfamethoxazole (SXT), gentamicyn (GEN), erythromycin (ERY), fosfomicyn (FOF), amikacin (AMK), rifampicin (RIF), ciprofloxacin (CIP), vancomycin (VAN) and teicoplanin (TEC). Bacterial isolates growing in at least one different antibiotic from the rest was considered to belong to a different antibiotype.

Pulsed field gel electrophoresis (PFGE)

Molecular typing of the whole genomic DNA was done as previously described (Chung et al., 2000). After digestion with *SmaI* endonuclease, DNA was separated in a CHEF-DRII apparatus (Bio-Rad, Birmingham, UK) (Chung et al., 2000). We used the Tenover criteria for the interpretation of the PFGE patterns were the profiles were compared by visual inspection (Tenover et al., 1995).

RESULTS

Identification of *S. aureus* in the Cotija cheese samples

Fifty four percent (27 out of 50) of the collected samples was found to be positive for *S. aureus*. The morphology of the colonies grown on Baird Parker agar plates with egg emulsion and potassium tellurite 1%, was typical for *S. aureus*. The colonies were black, circular, shiny, convex, smooth and had diameters from1 to 2 mm. They showed opaque areas and clear halos surrounding the colonies. From the 27 samples that resulted positive, 35 different isolates of *S. aureus* were obtained, 77% of these (27 out of 35) were found to be lecithinase positive.

S. aureus counts in the 27 contaminated samples increased from $12x10^3$ to $3x10^6$ CFU/g. The maximum allowable limit (CFU/g) of *S. aureus* in mature cheese (such as Cotija) is 100 CFU/ g as specified by NOM-121-SSA1-1994 (Goods and Services Cheese; Fresh, ripened and processed sanitary specifications). So, all the cheese samples analyzed exceeded the maximum permissible limits.

Three *S. aureus* biotypes identified

The biochemical characteristics of the *S. aureus* isolates are as follows: Biotype 1 representing 22.85% of the isolates (8 out of 35) was negative to lecithinase and thermonuclease but positive to coagulase and catalase (data no show). This biotype was found to ferment manitol, glucose, lactose, sucrose, maltose and trehalose. Biotype 2, which represents the largest number of isolates, covering 51.42% (18 out of 35), included isolates positives to the lecithinase assay and negative to the thermonuclease one or vice versa. The rest of the tests were positive in this group. Biotype number 3 includes 25.71% of the samples (10 out of35), positive phenotypes were observed for all the tests included in the study (Table 1).

S. aureus growing in the Cotija cheese samples was resistant to antibiotics

S. aureus was resistant to four different antibiotics when tried on a panel of 12 different antibiotics (Table 2). 43% of the isolates were resistant to ERY, TET, CHL and RIF, this group was named as Ant 2. 28% was resistant to

Table 2. Antibiotic susceptibility of isolates in this study.

Antibiotype	Susceptibility to:													No. (%) of isolates
	ERY	FOF	GEN	TET	SXT	AMK	CLI	CHL	RIF	CIP	VAN	TEC	OXA	
Ant1	R	S	S	R	S	S	S	R	R	S	S	S	S	8 (23)
Ant2	R	R	R	R	S	S	S	S	S	S	S	S	S	15 (43)
Ant3	S	S	S	R	S	S	S	S	S	S	S	S	S	10(28)
Ant4	S	S	R	R	S	S	S	R	R	S	S	S	S	2 (6)

R, resistant; S, susceptible. ERY, erythromycin; FOF, fosfomycin; GEN, gentamicin; STX, trimethoprim-sulfamethoxazole; AMK, amikacin; CLI, clindamycin; CHL, chloramphenicol; RIF, rifampin; CIP, ciprofloxacin; VAN, vancomycin; TEC, teicoplanin; OXA, oxacylin.

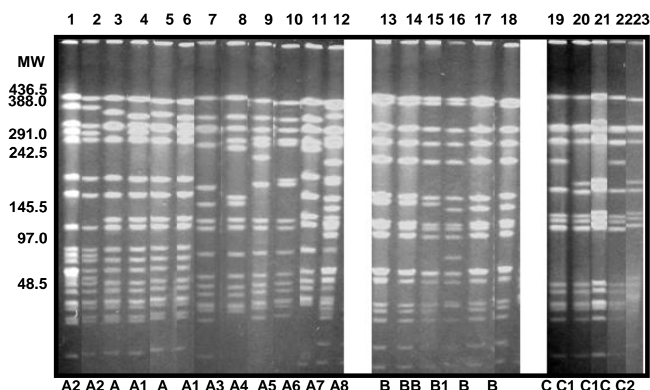

Figure 1. Examples of pulsed-field gel electrophoresis profiles obtained for *S. aureus* isolates from the Cotija Cheese, Guerrero, Mexico. Lanes: 1, lambda ladder used as molecular size (MW) marker; 2 to 23, isolates in this study.

TET (Ant 3) (Table 2). All the isolates were sensitive to OXA, VAN, TEC, STX, AMK, CLI and CIP.

The TSST-1 gene was present in one of the isolates

As for PCR detection of the TSST-1 gene, only the M9 strain proved to be positive for the presence of the TSST-1 gene that represents 2.85% of the total collection (data not show).

Genome profiles of the isolates are different

Twenty three isolates were classified in three PFGE patterns. Pattern A include 8 subtypes (A1 to A8), pattern B only one subtype (B1) and finally the profile C had two subtypes (C1 and C2). The patterns were different in up to six bands (Figure 1).

DISCUSSION

The dominant populations of microorganisms in the Cotija cheese are lactic acid bacteria that help to maintain shelf life for extended periods of time, preventing the growth of pathogenic microorganisms capable of causing disease (Campos et al., 2008). The contamination of food by *S. aureus* is given by strains that have and express genes for enterotoxins and/or the toxic shock syndrome toxin 1 (Gouloumes et al., 1996). Coupled with the presence and expression of genes encoding the enzymes described above, the susceptibility to antibiotics has been associated with strains of *S. aureus* isolated from clinical and food samples. One of the sources of contamination causing infection by strains producing enterotoxins and TSST-1 is precisely contaminated food (Sospedra et al., 2012), among which are the dairy products, such as cheese which has a high risk of bacterial contamination. Cotija cheese is sold in creameries Market Baltazar R Leyva Mancilla in the city of Chilpancingo, most exceed the permissible limits of the bacterial load of $12x10^3$ to $3x10^6$ CFU/g, given that, the maximum allowable limit (CFU/g) of *S. aureus* in mature cheese is 100 CFU/g as specified in NOM-121- SSA1- 1994. These data differ in some cases with those reported by Okineden et al. (2008), a study carried out in Germany where they worked on Curd (cream) cheese, soft cheese, semi-hard cheese and hard cheese prepared from pasteurized raw milk and in all cases the bacterial load ranged between $3x10^1$ - $3.1x10^3$, 5.3 $x10^1$ to $1.3x10^5$, $8x10^1$ to $5x10^1$ to $3x10^4$ and $8.6x10^4$, respectively.

Cotija cheese contamination can be due to many factors which include manipulation while obtaining milk, no milk pasteurization, inadequate hygiene of milk handling, contamination of containers where the product is prepared, storage of cheese during the ripening process in the stand and transport chain from retail outlets to the consumer. All this can lead to contamination of the product, which is why good hygiene practices are important for decreasing *S. aureus* contamination in food as described by Soriano et al. (2002). When the bacteria reach the product, they are able to multiply and cause infection in people who consume them, coupled with contamination by pathogenic bacteria, the ability to produce bacteria toxins, which include enterotoxins A, B, C, D and E, and the toxic shock syndrome toxin 1. In this study, we determine the microbial load in all the samples collected and it is the first report of *S. aureus* growingin Cotija cheese in the State of Guerrero. The results shown are alarming because the Cotija cheese is a staple in Guerrero and may cause a public health problem. The three biotypes highlight

toxin production as lecithinase and termocucleasa which turns them into a more pathogenic *S. aureus*. 100% of the strains were sensitive to oxacylin, vancomycin, teicoplanin, ciprofloxacin, amikacin, these findings is consistent with that of Tsen et al. (1998) which reported sensitivity to penicillin, oxaciylin, vancomycin and others. We detected the gene encoding the toxin (TSST-1) in 2.8% of the samples (only strain M9), these data is consistent with Sospedra et al. (2012), where 0.1% (1/53) of the isolates collected from food handlers and food service establishments in Spain was shown to have the gene. Furthermore, around the world, Oh et al. (2007), detected TSST-1 in13.5% of food samples in Korea. Rapine et al. (2005) found that 4.4% of the *S. aureus* isolates from goat's cheese handlers produced TSST-1. In another study conducted by Abdulmula El-Ghodban et al. (2006), the TSST-1 gene was detected by PCR in three strains (all from clinical sources). Zschock et al. (2000) detected it in 19 strains of *S. aureus* isolated from bovine mastitis in one case in Germany. All studies show the high frequency of *S. aureus* in foods derived from dairy products, clinical samples and mastitis carriers with the capacity to express TSST-1. Our results demonstrate the genotype of three clones of *S. aureus* isolated from Cotija cheese matches the antibiotype and the biotype so our findings are consistent with that of Peles et al. (2007) which showed genetic relation between the *S. aureus* strains recovered from quearter milk and bulk milk in two large farms, implying that farms have a high number of mastitic cows, etc.

In conclusion, we reported three clones (based on their biotype, antibiotyping and genotype) of *S. aureus* isolated from Cotija cheese from the central market in the city of Chilpancingo, Guerrero, Mexico. All samples exceeded the maximum permitted levels for human consumption in mature cheese, thus representing an important source and a risk factor for food borne infection which represents a serious public health problem in the exposed population. It should be noticed that just the M9 strain has the TSST-1 gene which encodes for the toxic shock syndrome toxin, so it is the first case reported in Mexico. All strains were sensitive to oxacylin and vancomycin which is noteworthy because although this bacterium is present and exceeds the maximum, it may be limited by antibiotics. Further studies are needed to determine the presence of genes coding for enterotoxins A, B, C, D and E in *S. aureus* isolated from foods in the State of Guerrero and recommend sanitation measures employed in the processing of cheese and food in general.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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