

ADDIS ABABA UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY



Effect of Antibiotics in Eliminating Bacterial Wilt (*Ralstonia solanacearum*) from *In vitro* Propagated Ginger (*Zingiber officinale* Roscoe)

By:

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Chairman, Department's Graduate Committee

Declaration

I, hereby, declare that this thesis is my original work and its composition has never been submitted elsewhere for any other award. All sources of materials used for the thesis have been duly acknowledged.

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I confirm that the work reported in this thesis has been carried out under our supervision and submitted for examination with my approval as University Advisors.

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List of Abbreviations

ANOVA	Analysis of Variance
BAP	6-benzylaminopurine
BWG	Bacterial Wilt of Ginger
CFU	Colony Forming Unit
Hr	Hour
IZ	Inhibition Zone
MHA	Mueller HInternationalon Agar
ML	Milliliter
MS	Murashinge and Skoog
NAA	1-naphthaleneacetic acid
P	p-value (stastical Significance level)
RS	Ralastonia Solanacerum
SNNPRS	Southern Nations, Nationalities and Peoples Regional State
SE	Standard Error
UV	Ultraviolet
W/V	Wave Length

ABSTRACT

Ginger (Zingiber officinale Rosc.) is a perennial herb. It belongs to the family Zingiberaceae and commercially cultivated in most tropical regions of the world. The underground rhizomes are the planting materials in conventional propagation of ginger. However, it is affected by diseases that cause tissue senescence and degeneration. Heavy losses of rhizomes have been observed because of infection with Ralstonia solanacearum. A disease-free planting material is necessary in order to get a successful ginger cultivation. Plant tissue culture technology has been successfully used for the commercial production of pathogen-free plants and for germplasm conservation of rare and endangered species. In vitro culture techniques provide an alternative means of plant propagation and a tool for crop improvement. Ginger Multiplication media are often contaminated with Ralstonia solanacearum the bacteria may survive endophytically within these plantlets thereby making the plantlets unusable for in vitro propagation. An experiment was conducted to find out effectiveness of antibiotics against the growth of Ralstonia solanacearum under in vitro condition. Four antibiotics namely, Gentamicin, Tetracycline, Ampicillin, Streptomycin with four concentrations, 130 mg/l, 160 mg/l, 200 mg/l and 250 mg/l were used to test their effectiveness in eliminating bacteria from in vitro propagating ginger. Gentamicin was found to be superior to other treatments with highest inhibition (22 mm) at 250 mg/l followed by Tetracycline at 200 mg/l (18mm), Streptomycin 13 mm at 130mg/l and Ampicillin 12 mm at 130mg/l. Therefore, using antibiotics in ginger micro propagation medium can eliminate Ralstonia solanacearum from in vitro propagated ginger.

Key words: *Inhibition Zone, invitro susceptibility test, Isolation and identification.*

1. Introduction

Ginger is a herbaceous rhizomatous perennial plant belonging to the family *Zingiberaceae*, under the order Scitaminae. The genus *Zingiber* includes about 85 species of aromatic herbs. Edible ginger (*Z. officinale*) is one of the ear oriental spices and is being cultivated widely both as a fresh vegetable and dried spice (Sivarana, 2014). Ginger (Zingibil in Amharic), has been traded throughout history longer than most other spices and had perhaps been known since then in Ethiopia, and predominantly grown in the wetter parts of the Southern Nations, Nationalities and Peoples Regional State (SNNPRS). Ginger production, to a lesser extent, has been also extended to some parts of Western Oromia and Northern Amhara (Tadesse and Asfaw, 2013).

Ginger grows well in warm and humid climate and is cultivated from sea level to an altitude of 1500 meters above sea level. It can be grown both under rain fed and irrigated conditions. For successful cultivation of ginger, a moderate rainfall at sowing time till the rhizomes sprout, fairly heavy and well distributed showers during the growing period and dry weather for about a month before harvesting are necessary. Ginger thrives best in well drained soils like sandy loam, clay loam, red loam or lateritic loam. A friable loam rich in humus is an ideal soil for ginger cultivation. However, being an exhaustive crop it is not desirable to grow ginger in the same soil year after year (Ravindran and Nirmal, 2005). Ginger rhizomes produced in conventional propagation were infected by various pathogens such as *Fusarium oxysporum*, *Pseudomonas solanacmearum*, *Pythium* spp. and nematodes. Importation of fresh rhizomes for crop establishment either for research or commercial production seems hazardous as ginger is subject to infection by soil-borne diseases such as bacterial wilt, soft rot, and *Fusarium* wilt (Endrias *et al.*, (2011). In Ethiopia, currently ginger plants are attacked by pathogen and the Intensity of disease is increasing from time to time (Tariku *et al.*, 2016). This is due to Introduction of new strains of pathogens in to Ethiopia from other

parts of the world through latently infected planting materials (George and Sivarana, 2014). For routine vegetative propagation of ginger, many rhizomes are required because the efficiency of vegetative propagation is low. In addition, during storage and cultivation, rhizomes used for vegetative propagation are susceptible to diseases that cause tissue senescence and degeneration. Heavy ginger losses have been observed because of bacterial wilt caused by *Ralstonia solanacearum*. The disease is mainly transmitted by rhizomes. Production of disease-free clones is necessary in order to get a successful ginger cultivation. The most efficient practice to manage bacterial wilt is growing disease-free ginger starting with planting pathogen-free seed. *In vitro* culture techniques provide an alternative means of plant propagation and a tool for crop improvement (George and Michaeli, 2012). It becomes an imperative technique to alleviate the shortages of healthy planting material. Thus, there is a need to study methods that can provide disease free planting material. The most efficient practices to manage bacterial wilt are growing disease-free ginger. It needs to address effect of antibiotics in eliminating *Ralstonia solanacearum* from *in-vitro* propagated ginger plantlets.

2. Literature Review

2.1. Origin and distribution

Ginger (*Zingiber officinale* Roscoe) is a monocotyledon, slender herbaceous perennial herb belonging to *Zingiberaceae* family, which is a tropical group consisting of more than 1200 plant species in 53 genera (Shakel .2013). It is a tropical plant believed to have originated in South East Asia probably India or China (Bailey, 1949; Parry, 1969). Ginger was introduced into Europe in the ninth century AD and was brought to the Mediterranean region from India by traders during the 13th century (Archana and Geetha, 2013). It has a long and well - documented history of both culinary and medicinal use throughout world history, especially in Chinese, Indian and Japanese medicinal care

2.2. Production of ginger

The area under ginger cultivation in the world was estimated to be, 104, 3000 hectares in 2017, with a total production of 30,538 tones, out of which Ethiopia has a world share of 0.3%. The top five ginger producer countries are India, China, Nepal, Nigeria, and Thailand. India is the largest producer of ginger accounting for more than 35% of the world production in 2017 followed by China. Ethiopia stood 10th and 14th position in terms of area harvested and total production of ginger, respectively among the 36 countries engaged in ginger production globally (FAOSTAT, 2017).

2.3. Uses of Ginger

Ginger is used throughout the world as a spice or fresh herb for cooking and varieties of other value added products (Bartley and Jacobs, 2000). It is available in the form of oil, oleoresins, ground and fresh ginger. The crop has multiple advantages in that it is highly productive per unit area, tolerant to drought, can be stored for long period of time in dried form ,and can also be Intercropped with other crops like beans, maize and taro. Ginger is mainly used as a

flavoring agent. Especially, powdered dry ginger is an important component of curry powder. Dry ginger is used for the manufacture of several products, such as ginger oil, ginger essence, ginger oleoresin, and effervescent ginger powder used in soft drinks, local foods and drinks (Endrias *et al.*, 2010). It is widely used for culinary purposes in ginger bread, biscuits, sauces, and cakes. Besides, it is used in breweries for the preparation of ginger beer, ginger ale, and ginger wine in different countries. Ginger finds immense usage in many of the different medicinal systems of the world for a wide variety of disorders. It is used in traditional medicines to treat several ailments including nausea, diarrhea, motion sickness, migraine, dyspepsia, heart problems, arthritis, and to reduce flatulence and colic. Moreover, ginger is believed to relieve the common cold, flu-like symptoms, headaches, and even painful menstrual periods. Ginger is known to be effective as an appetite enhancer and an improver of digestive system (Wresdiyati *et al.*, 2007). Young rhizomes that are harvested early are also used in pickles and confectionery. The rhizome yields essential oil which is obtained as a result of steam distillation, a yellow liquid lacking the pungency. It is used in the manufacture of flavoring essences and perfumery. As a matter of fact, ginger contains a number of different pungent and active ingredients. For instance, the oleoresin contains the constituents that are responsible for pungency of ginger. The underground rhizome of this crop is also valued throughout the world as a spice of flavoring agent for its two major classes of constituents, such as essential oils and oleoresins (Baladin *et al.* 1998) .The more volatile essential oil consists of monoterpenes and sesquiterpenes, which contribute to the characteristics flavor of ginger, and oleoresins which is responsible for the pungent flavor, which is also a source of antioxidants (Sanwal *et al.*, 2010). The principal compounds responsible for the pungency of ginger are gingerols and shagols. Ginger is commercially available in various forms, such as green ginger, dry ginger, ginger powder, ginger oil, ginger oleoresin and preserved ginger (Kizhakkayi and Sasikumar, 2009).

Ginger contains an Oleoresin (6.5%) commercially known as “Gingeberene” which is obtained by solvent extraction from ginger rhizomes. The other components of dry ginger are moisture (10.85%), volatile oil (1.8%), water extract (19.6%), cold alcohol extract (6.0%), starch (53%), crude fiber (7.7%), crude protein (12.4%), total ash (6.64%), water soluble ash (5.48%) and acid-insoluble (0.14%). The volatile oil is a pale yellow liquid with warm, spicy, sweet aromatic odor, and a strong pungent flavored. The chief constituent is a sesquiterpine. It is reported to contain 200 different volatile substances. It is the oldest spice with a distinct flavor and pungency. Both dry and fresh ginger is used in cooking for its aroma, flavor, and pungency. The rhizome yields essential oil which is obtained as a result of steam distillation, a yellow liquid lacking the pungency. It is used in the manufacture of flavoring essences and perfumery. An oleoresin, which marks the full presence of spices is used for flavoring purposes, and also has medicinal values. As a matter of fact, ginger contains a number of different pungent and active ingredients.

2.4. Ginger Diseases and Economic Impact

The production of ginger, however, is largely affected by diseases caused by bacteria, fungi, viruses, mycoplasma and nematodes, the crop suffers from diseases like bacterial wilt caused by *Ralstonia solanacearum*, rhizome rot caused by *Pythium* species, *Fusarium* species, *Sclerotium* species, *Pseudomonas* species and others (Senapati and Ghose, 2005; Paret *et al.*, 2010; Sharma *et al.*, 2010; Kavyashree, 2011).

Ralstonia solanacearum is classified as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range and broad geographic distribution. It infects over 250 plant species in over 50 families at a time. It causes a wilt disease in several important agricultural crops such as potato, tomato, tobacco, banana, pepper and eggplant. Many more dicots suffer from the disease than do monocots. (Wicker *et al.*, 2007).

In Southern Nations, Nationalities, and Peoples' Region (SNNPR) of Ethiopia the prevalence of the disease was noted to begin with the over seasoned ginger plantation (Tariku *et al.*, 2016). The symptom of the disease on new plantation was observed 3-5 months of planting leading to a ceased development of rhizomes .This, in turn, resulted in a considerable yield reduction of the crop. Ginger was found devastated by the bacterial wilt and cause yield loss up to 98%. In Ethiopia, ginger has been used as a flavoring agent, carminative and stimulant and it has become the most important spice for the local as well as the export market. Ginger is one of the most important spices, largely for small scale farmer in Ethiopia. The crop is mainly grown in the southern part of the country and it is dominant in the cropping system. In the Southern Nations and Peoples' regional states (SNNPRS), 85% of the total arable land often allotted for ginger production and 35% of the farmers in the region produce ginger (Endrias Geta and Asfaw Kifle, 2011). The yield loss in monetary terms can be estimated to be about 20 million US Dollars. Moreover, an incredible sudden drop of a ginger price has occurred and seriously affected the economy of most ginger farmers to the extent of causing food insecurity. Above all, farmers are highly discouraged as they cannot get "seed" of a healthy ginger for the immediate future use for ginger production. In general, as the result of the epidemics of the disease, the socioeconomic conditions of the actors of the ginger value chain including farmers, traders, transporters, investors, exporters, processors, casual laborers etc., have been affected significantly. Although, ginger is a minor crop, its contribution to the national economic development is not negligible. Because it is one of the export commodities selected to play its important role in achieving the objectives of the Growth and Transformation Plan (GTP) of the government (GTP II.2015).

In order to meet the demands of an ever expanding world human population, global crop production needs to double by 2050. However, current estimates are far below what is needed. Plant diseases, insects, and weeds decrease the production of crops worldwide by

36%, and diseases alone have been shown to reduce crop yields. Thus, the control of plant diseases contributes to increased crop production (Yuliar *et al.*, 2015).

2.5. Bacterial wilt

The important factors which limit the production of ginger are the occurrence of diseases. Bacterial wilt, incited by *Ralstonia solanacearum* is at present considered as a very serious threat in most of the ginger growing areas of Ethiopia (Habtewold *et al.*, 2015). The pathogen infects over 450 plant species including many economically important crops other than ginger. *R. solanacearum* causes a vascular wilt disease and has been ranked as the second most important bacterial pathogen. Next to *Pseudomonas syringae* pathovars, it is a severe obstacle to the production of ginger plants in both tropical and temperate regions. Mansfield (2012). As a diverse species complex, *R. solanacearum* has developed an extremely broad host range throughout the world, including >450 host species representing 54 plant families (Wicker *et al.*, 2007). In Ethiopia bacterial wilt caused by *Ralstonia solanacearum* has been reported on potato, tomato, pepper, enset, banana and ginger (Tariku *et al.*, 2016). *Ralstonia solanacearum* is an aerobic non-spore-forming, Gram-negative plant pathogenic bacterium. *R. solanacearum* is soil-borne and motile with a polar flagellar tuft. It colonizes the xylem, causing bacterial wilt in a very wide range of potential host plants. Ginger wilt is the most limiting factor in the production of ginger. *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, (1995). Its Biotype III has been identified as the causal agent of the disease (Sarma *et al.*, 1978). It is a complex and difficult disease to control, infecting the ginger crop through all phases of a production cycle. It is present systemically in seed rhizomes as both an active and latent infection that contaminates seed-pieces when they are cut and prepared for field planting (Abedela, 2017).

In Ethiopia, the major bottlenecks of ginger have been low productivity and poor product quality resulting in low quality varieties, poor pre- and post-harvest handling practices, low product market prices, lack of access to markets, crucial shortage of planting material (Biruk

et al., 2012). Disease problems in ginger were reported at 2013/14 production year all in the country. In the history of the country, diseases, which were spreading unusually quickly and extensively, had occurred suddenly in southwestern zones of SNNRPS, Ethiopia including Sheka, Kefa and Bench Magi. In the following season, its rapid spread was extended to the central zones of the region, covering the areas of Wolaita, Kembata Tembaro and Dawro, causing almost complete crop loss irrespective of varietal, climatic and geographic variations. Correspondingly, farmers and even inventors engaged in ginger production in the region are compelled to shift from ginger to the production of other crops. Now a day, disease in ginger is becoming one of the most notable bottlenecks of ginger production in Ethiopia. In SNNPR, the prevalence of the disease was noted to begin with the over seasoned ginger plantation (Habtewold, *et al.*, 2015).

2.5.1. Taxonomy and Diversity of *Ralstonia solanacearum*

R. solanacearum has not always has been the name of this species. On the contrary, it has been classified in numerous genera since it was first described. Smith was the first to publish a scientific description and classify it in the genus *Bacillus* as *B. solanacearum* in 1896. However, afterwards it was moved to the genus *Bacterium*, and to the genus *Pseudomonas* with the name of *P. solanacearum* temporary reclassified in the genera *Phytomonas* and *Xanthomonas* and eventually transferred back to the genus *Pseudomonas* in 1948. In 1992, it was placed in the genus *Burkholderia*. But, more recent phylogenetic and polyphasic phenotypic analyses pointed out that it would rather be accommodated in the new established genus of *Ralstonia*, in 1995 (Yabuuchi *et al.*, 1995). Apart from races *R. solanacearum* strains have been traditionally classified into biovars, on the basis of the pathogen ability to utilize and/or oxidize several hexose alcohols and disaccharides. Five races and biovars have been described so far (Hayward, 1991; EPPO, 2004) and Africa is the continent where the highest diversity is found (Elphinstone, 2005). Both classification schemes constitute informal groupings at the infer sub specific level. The relationship between them is only

evident with race 3 strains, which generally correlate with biovar 2 phenotype (Hayward, 1991). It has been known that, biovar III of *solanacearum* oxidizes both disaccharides and hexose alcohols (Tariku *et al.*, 2016). Classifications based on molecular techniques have also been reported, as the phlotypes or monophyletic clusters of strains, which are determined after phylogenetic analysis of sequences of particular genes. Thus, the species is divided into four phlotypes corresponding to four broad genetic groups, each of them related to a geographic origin (Fegan and Prior, 2005). Phylotype I contain all strains belonging to biovars 3, 4 and 5, isolated primarily from Asia. Phylotype II includes biovar 1 and 2 strains, and 2T (a subgroup of biovar 2 for tropical areas) isolated from America, all race 3 strains pathogenic to potato and the race 2 banana pathogen. Phylotype III comprises strains belonging to biovars 1 and 2T from Africa and surrounding islands. Phylotype IV is more heterogeneous, with biovar 1, 2 and 2T strains from Indonesia, strains isolated in 11. The diverse classification schemes proposed for *R. solanacearum* reflect the great phenotypic and genotypic variation within the species, which has led to the term “*R. solanacearum* species complex” (Gillings and Fahy, 1993). *R. solanacearum* is a gram negative, rod shaped, strictly aerobic bacterium that is 0.5-0.7 x 1.5- 2.0 μm in size, with a single polar flagellum. Individual bacterial colonies are usually visible after 36 to 48 h of growth at 28°C and colonies of ginger strains were highly fluidal with characteristic spiral pink center whereas in the case of other strains fluidity and pink centre was less conspicuous (Kumar and Sarma, 2004; Sambasivam and Girija, 2006). Occasionally colonies of the mutant or non-virulent type appear uniformly round, smaller and butyrous or dry. Cultural characteristics of isolates on Tetrazolium Chloride (TZC) agar selective medium showed light to red color with the characteristic red center which resembles the pathogen *Ralstonia solanacearum*. Biochemical and physiological characteristic revealed that similarities among isolates and strains were grouped in biovar III and corresponds to race 4 of the pathogen. Strains of *R. solanacearum* have been classified Into five biovars (Kumar *et al.*, 1993) and

five races (Buddenhagen *et al.*, 1962; Bin Li *et al.*, 2010). The bacterium is a rod shaped, strictly aerobic, gram negative, oxidase positive and amphotrichously flagellated, but not utilized asparagines as a sole source of carbon and nitrogen. It was positive for catalase reaction utilized glucose, fructose, and sucrose for acid production, positive for liquefaction of gelatin and produced hydrogen sulphide and did not produce indole, in addition, the strain failed to reduce nitrate to nitrites (Abdella, 2017). Three biotypes of *R. solanacearum* have been described and out of these, biotype III causes wilt in India (Dake *et al.*, 1988). Biotype III of bacterium causes slow wilt, whereas biotype IV causes rapid wilting and death of the infected plants (Pegg *et al.*, 1974). Biotype III has been found restricted only to ginger and its common weed hosts, whereas biotype IV has a wide host range including tomato, potato, *Zinnia elegans*, *Capsicum frutescens*, *Physalisperuviana* and eggplant. Biotype II was also isolated from potato plants (Hayward *et al.*, 1967). Quinon *et al.* (1964) observed that the isolates different in their pathogenicity on Tomato, ginger, groundnut, plum and potato. The tomato and ginger biotypes grow best at 34 °C and were similar in their cultural and biochemical characteristics.

2.5.2. Symptomology

Bacterial wilt is caused by *Ralstonia solanacearum*. Water soaked patches or linear streaks on the collar region of the pseudo stem are observed. Later, leaves become flaccid with intense yellowish bronze color and droop. The leaves show roll up symptoms and the whole plant dry up. Pseudo stem comes off easily with a gentle pull. Such rhizomes when pressed exhibit oozing of bacterial exudates. The symptom of the disease on new plantation was observed 3-5 months of planting leading to a ceased development of rhizomes (Habtewold, *et al.*, 2016). Water soaked patches or linear streaks appear on the collar region of the pseudostem of ginger plants. These symptoms are followed by yellow or bronze coloration on margins of the lower most leaves which gradually progress upwards, at later stages the leaves become flaccid with intense yellow bronze color and droop exhibiting typical wilt

symptoms.(Sarma, 1994). Pseudostem can be easily separated from rhizome with a gentle pull. Plants which are infected by the disease stand persistently and do not collapse. If the affected rhizomes are pressed a milky bacterial exudate oozes out. When infected tissues are steeped in clear water for a while, the water turns cloudy and milky (Nelson, 2013). The characteristic symptoms of bacterial wilt of ginger include green leaves roll and curl due to water stress caused by bacteria blocking the water-conducting vascular system of the ginger stems, leaf yellowing and necrosis (White *et al.*, 2013).

2.5. 3.Management Strategies of Bacterial Wilt of Ginger

Bacterial wilt caused by *R. solanacearum* is a disease difficult to control in field production on crops, since pathogen's properties as a soil borne bacterium, their broad host range and the genetic variation level within the strains; they have not allowed implementing a universal control measure for this disease (Saddler, 2005 and Denny, 2006).It is complex and difficult disease to control, infecting the ginger crop through all phases of a production cycle. It is present systemically in seed rhizomes as both an active and latent infection that contaminates seed-pieces when they are cut and prepared for field planting. The most efficient practices to manage bacterial wilt are growing disease-free ginger starts with planting pathogen-free seed. To get pathogen free planting material, there is a need to investigate the effect of antibiotics to eliminate *Ralstonia solanacearum* from *in-vitro* propagated ginger plantlets.

The general strategies for management of bacterial wilt are selection of healthy rhizome material from a disease-free area; Selection of field with no previous history of bacterial wilt; pre plant treatment of rhizomes by application of heat or chemicals; strict phyto sanitation in the field, including restrictions on movement of farm workers and irrigation water across the field; clean cultivation and minimum tillage; crop rotation with non host plants such as paddy and maize; insect pest and nematode control in the field; and soil amendments, including biological control agents.

2.5.4. Resistance cultivars

The best strategy to control bacterial wilt caused by *R. solanacearum* is breeding for resistance cultivar (Persley, 1992). A virulent mutant of *R. solanacearum* has been used as a bio-control agent for the virulent pathogen (Trigalet and Trigalet-Demery, 1990). Abdalla and Abdulla (1998) have found that the degree of susceptibility to bacterial wilt is significantly different among six tomato cultivars which were tested and this indicated that the additive genes were more important than the non-additive genes. Thus, in breeding programs, selection for disease resistant plants (genotype) after each generation is recommended. The infection by *R. solanacearum* can be controlled by the use of hot pepper accessions (*Capsicum annuum* L.), sweet pepper (*Capsicum annuum* L.) in Japan. Bacterial multiplication in stems of resistant tomato plants was suppressed owing to the limitation of pathogen movement from the proto xylem or the primary xylem to other xylem tissues (Nakaho *et al.*, 2004). The limitation was most conspicuous in Hawaii 7996. Grafting experiments indicated that the percentage of wilting of 21 Ponderosa scions was less on Hawaii 7996 rootstocks than that on the most resistant rootstock (LS-89) used in Japan. Hawaii 7996 could be an alternative genetic source for breeding for resistance to bacterial wilt (Nakaho *et al.*, 2004).

2.5.5. Antibiotics

Antibiotics are chemical substances (natural or synthetic) that are able to destroy or kill bacterial cells, or inhibit their growth. Antibiotics are grouped by their mode of action, i.e. inhibitors of bacterial cell wall synthesis, inhibitors of bacterial protein synthesis and DNA replication blockers, or by their chemical structure, i.e. β -lactams, amino glycosides, quinolones, glycopeptides, polymixins, macrolides and lincos amides (Quesnel and Russell, 1983; Falkiner, 1990). Ideally, antibiotics used in plant tissue culture should be soluble, stable, unaffected by

the components or pH of the medium, lack side effects, broadly active, non-resistance inducing, inexpensive and non-toxic to humans (Falkiner, 1990). Many attempts have been made to suppress/eliminate endogenous bacteria from plant cultures with antibiotics with varying degrees of success (Falkiner, 1990). Often, bacterial growth is only suppressed (bacterio static effect) by antimicrobial treatments and when chemicals are removed, the bacteria resume growth (Falkiner, 1990, 1997; Barret and Cassells, 1994). In other instances, antibiotics effective on isolated organisms cannot be used for treating contaminated plants, due to phytotoxicity or poor penetration into tissues (Reed *et al.*, 1995). Although phytotoxicity and development of antibiotic-resistant bacterial populations have restricted the use of antibiotics, these side effects can be taken care of by the use of combination of antibiotics at relatively lower concentrations.

Several antibiotics are frequently used in plant biotechnology to eliminate endogenous bacteria in plant tissue culture, although knowledge of the effect of antibiotics on both bacteria and plant is important for the elimination of contaminants and the recovery of the plants (Cassel and Liferat, 2001). Surface sterilization eliminates most epiphytic contaminants except endophytic ones (Habiba *et al.*, 2007). Commercial bleach (Clorox™) is often used as disinfectant for surface sterilization of plant materials and also it is not hazardous. The disinfection method for emerging buds of ginger is soaked in 0.2% Tetracycline and 0.2% Metalaxyl for one and half hours before being treated with 10% Clorox for 15 min followed by 5% Clorox for 10 min .The optimum regime for decontamination of ginger is sterilizing rhizome buds using 20% Clorox for 20 min (Hamirah *et al.*, 2007). However, bacterial contamination is a common problem in ginger but it can be controlled by incorporation of antibiotics to initial culture medium. In general, lack of browning and absence of contamination on *invitro* explants are as the criteria for survival of explants for subsequent shoot multiplication. Mbah *et al.* (2012) reported that antibiotics for the elimination of bacteria from *in vitro* yam plantlets by using tetracycline combined with

rifampicin (TR), streptomycin plus gentamycin (SG), rifampicin (Rn), vancomycin plus streptomycin (VS) at $\geq 125 \mu\text{g/ml}$ and TVSGR (tetracycline + vancomycin + streptomycin + gentamycin + rifampicin) at $\geq 100 \mu\text{g/ml}$ were highly bactericidal. Nadha *et al* (2012). Also identified kanamycin and streptomycin sulfate as potentially useful antibiotics in eliminating the contaminating bacteria *in vitro* cultures of Guadua. It grew shoots on multiplication medium containing BAP (2 mg/l) and adenine sulfate (10 mg/l) supplemented with kanamycin (10 $\mu\text{g/ml}$) for 10 days and transferred them to fresh medium without antibiotics and found that bacterial growth was inhibited. Antibiotics were evaluated to determine their potentials to inhibit different bacterial species.

Antibiotics such as Gentamycin and Ampicillin are best supported inhibition of mycelial growths with 90% each. These were followed by Streptomycin with 80%, Rifampicin (80%), Tetracycline (60%), Cefotaxime (55%), Penicillin (45%) and Sterile distilled water (10%) ten days after incubation in antibiotics nutrient agar medium. Janiffe *et al.* (2016) and Eziashi *et al.* (2014). Reported alternatively, an incorporation of antibiotics and antifungal agents into the growth media of plant cultures eliminate microbial contaminants. Singh *et al.* (2017) have found effective antibacterial chemicals and bio agents against the growth of *Ralstonia solanacearum* under *in vitro* conditions. Average inhibition was ranged from 6.2 mm (Copper hydroxide) to 20.05 mm (Streptocycline). However, significantly highest average inhibition was recorded in the antibiotic Streptocycline (20.05 mm). This was followed by the antibiotics viz., Gentamycin (17.5 mm), Tetracycline (16.5 mm) and Copper oxychloride + Streptocycline (11.95 mm). Morgan and Goodman (1955) also have found the *in- vitro* sensitivity of plant bacterial pathogens to antibiotics. It was found that the antibiotics varied considerably in their capacity to inhibit the entire spectrum of the test organisms and none was consistently superior to the others. *R. solanacearum* was effectively inhibited by lower concentration of aureomycin and Terramycin and relatively insensitive to the other two antibiotics, streptomycin and polymixin.

2.5.6. Plant Tissue Culture

Plant biotechnology is the agglomeration of several activities such as tissue culture, genetic Engineering and/or molecular biology technologies. These have been developed for several plant species and varieties. The last two activities of plant biotechnology depend on the first one (i.e. tissue culture). Plant tissue culture is the art of growing plant cells, tissues, or organs on artificial media by isolating them from the donor plant. This is based on the doctrine that states a cell is capable of autonomy and potentially totipotent i.e. a cell can start expressing it's switched off genes provided that the necessary environmental conditions are fulfilled, and grow to a whole plant. Plant tissue culture techniques can provide us with complete new plants through direct or indirect morphogenesis, and somatic embryogenesis. Direct morphogenesis is the production of shoots & roots from explants without passing through callus (unorganized tissue). Indirect morphogenesis is the proliferation of shoots or roots through callus phase. Somatic embryogenesis involves the production of embryos similar to zygotic embryos from somatic cells directly from explants, or from callus. Such embryos can germinate and grow into a whole plant. Plant tissue culture technology is being widely used for a large scale plant multiplication. The primary advantage of micro propagation or tissue culture is rapid production of high quality, disease-free and uniform planting materials. The plants can be multiplied under controlled environment, anywhere, irrespective of the season of the reason and weather, on a year round basis (Kadinna *et al.* ,2014). Plant tissue culture techniques proffer a substitute method of vegetative propagation of horticultural crops (Alizadeh *et al.*, 2010). The ability to establish and grow plant cell, organ, and tissue cultures has been widely exploited for basic and applied research, and for the commercial production of plants (micro propagation). Regardless of whether the application is for research or commerce; it is essential that the cultures be established *in vitro* free of biological contamination and be maintained as aseptic cultures during manipulation, growth, and storage (lifert *et al.*, 2001).

3. Objectives of the study

3.1. General objective

The general objective of this study was to evaluate the effects of antibiotics in eliminating *Ralstonia solanacearum* from ginger plants through *in vitro* propagation.

3.2 . Specific objectives

- To screen broad spectrum antibiotics that to eliminate *Ralstonia solanacearum* through *in vitro* propagation of ginger.
- To determine optimum concentration of antibiotics to eliminate *Ralstonia solanacearum* from *in vitro* propagating plantlets of ginger.
- Isolation and identification of bacterial contaminants.
- *Invitro* propagation disease free ginger.

4. Materials and Methods

4.1. Preparation of donor plant and stock solutions

Donor plant preparation: The ginger rhizomes were collected from experimental site of Areka Agricultural Research Center, which is located in Wolayita Zone of the Southern Nations Nationalities and Peoples Regional state 300 km Southwest of Addis Ababa, Ethiopia. The fresh rhizomes were thoroughly washed with water then retained in with sterile sand in greenhouse at temperature of 20 to 25°C. The rhizomes were watered regularly using distilled water for 15 to 20 days to initiate sprouting. Sprouted shoot tips and auxiliary buds from these rhizomes were used as a source of explants for subsequent experiments.

MS stock preparation: Murashinge and Skoog (MS) (1962) medium with its full macro and micro nutrients, vitamins, sucrose and agar were used as the basic components of the medium. All components of MS stock solution were prepared by weighing and dissolving the powder in double distilled water (Table.1.).

Table .1. Composition of MS medium.

Composition of Constituent	Final concentration (mg/l)
Major salt	
NH ₄ NO ₃	1650
KNO ₃	1900
CaCl ₂ 7H ₂ O	440
MgSO ₄ 7H ₂ O	370
KH ₂ PO ₄	170
MINOR	
KI	0.83
H ₃ BO ₃	6.2
MnSO ₄ 7H ₂ O	22.3
ZnSO ₄	8.6
NaMoO ₄	0.25
CuSO ₄	0.025
CoCl ₂	0.025
Iron Source	
FeSO ₄ 7H ₂ O	27.8
Na-EDTA 2H ₂ O	37.3
Sucrose	30000

Plant growth regulators stock preparation: The stock solutions of plant growth regulators (2.0 mg/l BAP and 1.0 mg/l kinetin) were prepared by weighing and dissolving the powder in double distilled water at by dissolving in 3-4 drops of 1N HCl.

4.2. Culture medium preparation and culture condition

Culture medium was prepared by taking proper amount of MS stock solutions and 3% (w/v) sucrose. Plant growth regulator was added and pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Finally, 0.6% (w/v) agar was added and dissolved by using micro-oven. The medium was sterilized by autoclaving at a temperature of 121°C at a pressure of 105 Kpa for 15 min. For shoot initiation, shoot multiplication and rooting, 40 ml of the medium was poured into baby jar culture vessel. Culture vessel and its cap were flamed prior to closing and sealed with a strip of Para film. The vessels were clearly labeled. Cultures were maintained under 12 h photoperiod at light Intensity of 3000 Lux provided by cool white fluorescent lamps at a temperature of $25 \pm 2^\circ\text{C}$ for 30 days.

4.3. Surface sterilization of explants and shoot initiation

After fresh rhizomes were kept in the greenhouse for 15 to 20 days at temperature of 20 - 25°C, the sprouted shoot tips and auxiliary buds were used as a source of explants. The excised shoot tip and auxiliary bud explants were washed with sterile distilled water followed by a brief immersion in 70% ethanol. Then the explants were treated with 5% active chlorine concentration local bleach (Clorox) containing two drops of Tween-20 for 15 min under aseptic condition. Then, explants were thoroughly rinsed (3 to 4 times) with sterile distilled water. About 0.7-1.5 cm long shoot tips were used for shoot initiation. The explants were cultured on MS medium supplemented with 2.0 mg/l BAP in combination with 1.0 mg/l Kin.

4.4. Shoot multiplication, rooting and acclimatization

The initiated shoots were transferred to MS medium supplemented with 4.0 mg/l BAP for 30 days. The multiplied shoots were grown on MS Full strength medium containing 1.0 mg/l NAA for root development. Then the plantlets were planted in sterilized standard potting mixture with soil, sand and coffee husk in 3:1:2 ratio and number of survived plants was recorded.

4.5. Isolation of bacterial contaminants and identification of the selected bacterial isolates

In order to isolate, detectable bacterial contaminants (*Ralstonia solanacearum*), well regenerated plantlets were cut down at lower parts and the upper parts were suspended in the five-milliliter sterile distilled for ten minutes. After the water in test tube became turbid due to oozing of bacterial cells from cut ends of diseased tissue, the bacterial suspension was serially diluted in 9.0 ml sterile water. Bacterial ooze obtained from the infected discolored pseudo stem of the plants by serially diluting the bacterial suspension in sterile distilled water and culturing on TZC media (Kelman, 1954). One hundred micro liter of the bacterial suspension was poured onto the surface of the Nutrient Agar (NA) both serial dilution and streak plate method. The plates were then incubated at 28 °C for at 24 hrs. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TZC) medium the plates were incubated at 30°C for 36 hours as described by Kelman (1954).

4.5.1. Preparation of inoculums and Pathogenicity test

A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected and adjusted to 3.2×10^8 cfu ml⁻¹ for inoculation. Tissue cultured Ginger plantlets at eight leaf stage were inoculated by stem puncture and leaf infection pin pricks. The bacterial suspension was inoculated in to the two leaves of each test

plant (Stromberg *et al.*, 2004). Virulence character of the *R. solanacearum* isolates were carried out on healthy tissue culture plantlets in the greenhouse. Plantlets were transplanted in plastic pots (9 cm in diameter) containing sterilized soil and sand and coffee husk in the ratio 1:1:2 and then grown in the greenhouse. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected and adjusted to 3.2×10^8 cfu ml⁻¹ for inoculation. One milliliter of inoculum per plant was inoculated into ginger seedling by stem puncture and leaf infection pin pricks. Plants at the eighth leaf stage were inoculated one time by puncturing the basal part of the stem with a needle dipped in inoculum. Each experiment was repeated three times. The day and night temperatures varied between 25-35°C with 12 h light and 12 h dark. Symptoms of disease were observed for one week after inoculation. If the plant showed typical wilt symptoms, the interaction was considered as pathogenic.

4.6. Bacterial Isolates in Antibiotics Medium

In vitro* evaluation of antibacterial chemicals on the growth of *R. solanacearum

Antibiotic sensitivity testing was performed on the bacterial isolates in Muller Hinton medium containing (Acid Hydrolysate of Casein, 17.5gm, Beef Extract, 2.0gm Starch 1.5gm, Agar, 17.0gm).

Four antibiotics namely, Gentamicin, Tetracycline, Ampicillin and Streptomycin with different concentrations (130mg/l, 160mg/l, 200 mg/l, and 250mg/l) respectively were used. The filter paper discs (Whatman no-44) measuring 6mm in diameter were soaked in the respective chemical concentrations for 5 min and transferred onto the surface of seeded Muller Hinton medium in the Petri-plates. The inoculated plates were kept in refrigerator at 4 °C for 4 h to allow the diffusion of chemicals into the medium. The plates were incubated for 24 h at 25°C in the dark. The diameters of the inhibition zones around the disks were measured using ruler and recorded.

4.7. Antibiotics treatment of plant material

Four antibiotics Gentamicin, Tetracycline, Ampicillin and Streptomycin were used to test their effectiveness in eliminating bacterial contamination. Fresh filter sterilized 0.20 micro meter membrane. Antibiotics were added to multiplication medium supplemented with 4.0 mg/l BAP. Growing shoots were carefully excised together with the lower sheath base part and transferred to multiplication medium supplemented with 4.0 mg/l BAP and respective antibiotics which was then incubated for 10 days under 12 h photoperiod at light Intensity of 3000 Lux provided by cool white fluorescent lamps at a temperature of $25\pm 2^{\circ}\text{C}$.

4.8. Data Analysis

The experimental data was analyzed by using one way analysis of variance and comparison of means at 0.05 level by GLM. Mean and standard deviation and standard error of the mean were analyzed by using Minitab 17.0 software. Growth components and elimination of contaminants status was analyzed with comparison to the average number of shoots grown in in-vitro.

Design: The experiment lays on CRD design where one variety of ginger (boziab) and four different antibiotics with four concentration level was used for treatments replicated four times.

5. Results

Initial surface sterilization experiment was effective when 0.7-1.5 cm shoot tips were treated with 70% ethanol for 5 min followed by double sterilization with 5% active chlorine concentration of local bleach (Clorox), for 15 min under aseptic condition .This treatment resulted in 95% contamination free explants and only 2% dead explants until 10 days of inoculation in shoot induction MS media. However, later when shoots started to be multiplied, started to show wilting symptom. Shoot tips were initiated using plant material from the infected field and usually became contaminated after few subcultures.

The contamination commonly appeared as a white clouding yellowish colour or restricted strictly to the medium around the bottom of plant and on the surface MS medium (Fig. 1). However, bacteria may remain undetected for long time since the growth medium used for tissue culture may not be optimal for bacteria or may even inhibit their growth (Leifert *et al.* 1994, Isenegger *et al.* 2003).



Fig.1.Diseased ginger plantlets *in vitro*

Cultural characteristics of isolates on Tetrazolium Chloride (TZC) Agar selective medium showed light to red color with the characteristic red center which resembles the pathogen *Ralstonia solanacearum*. All the isolates of *R. solanacearum* were found virulent producing pink or light red color or characteristic red center and whitish margin on TZC medium after 48 hours of incubation. The virulent colonies appeared well-separated, irregular fluidal, dull white colored with slight pink centre (Fig.2A and B) and non-virulent colonies appeared dark red on TZC media (Fig. 2C).

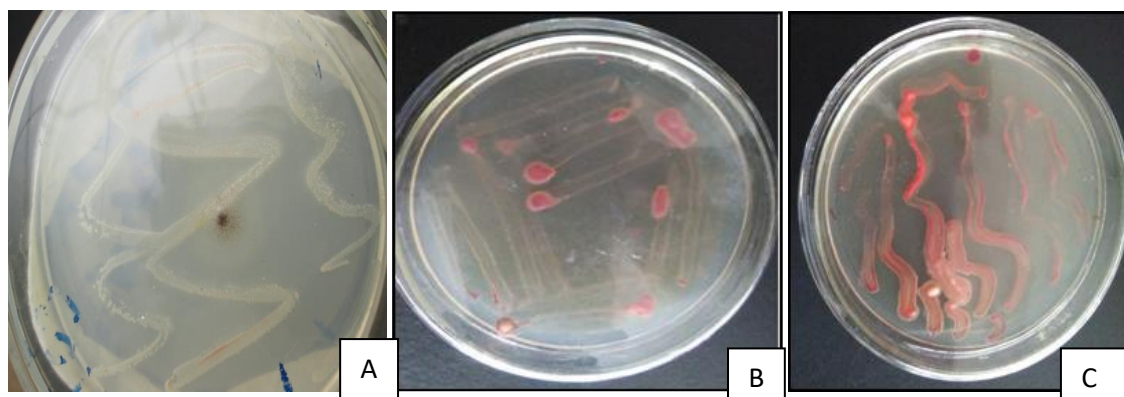


Fig.2. *R. solanacearum* isolates on Tetrazolium Chloride (TZC) medium. Virulent colonies (A and B) and non-virulent colonies (C).

Pathogenicity for *Ralstonia solanacearum*

Result of pathogenicity test of *R. solanacearum* isolate under artificial stem inoculation method revealed that wilt of ginger occur within 5 to 15 days after inoculation (Fig, a & b). The results were found in range of pathogenicity study in India which is wilt of ginger occurring within 5-7 days after inoculation (Kumar and Sarma, 2004). Symptoms were observed as small water soaked lesions with rolling up of the leaves. The plants initially showed wilting during day and recovered during night, but after 4 – 5 days, the plants collapsed suddenly. The wilt symptom on the above ground ginger plant part were found the first wilt symptoms were a slight yellowing and wilting of the lower leaves. The wilt progresses upward, affecting the younger leaves, followed by a complete yellowing and browning of the entire pseudo-stem. However, the plant dries very rapidly and the foliage becomes yellow-brown in 5 to 10 days.

Wilt of the pseudo-stem advances make young succulent shoots frequently become soft and completely rotted and the diseased shoots break off easily from the underground rhizome at the soil line (fig 3, A, B , and C ,). The same ginger bacterial wilt symptom was described by Kumar and Sarma (2004). The inoculated plant lost turgidity; leaves started dropping and plant wilted suddenly (Fig.3C). The symptoms observed on initial developmental stage on leaves, on pseudo-stems and rhizomes of infected ginger plants were identical to the symptom descriptions by (Trujillo, 1964). In vascular tissue, fine milky white strands, compound of mass of bacteria in extra cellular slime, stream down from the cut ends of xylem vessels. This bacterial exudates combined with symptoms observed on leaves distinguishes this wilt from fungus wilt (Hayward, 1964).

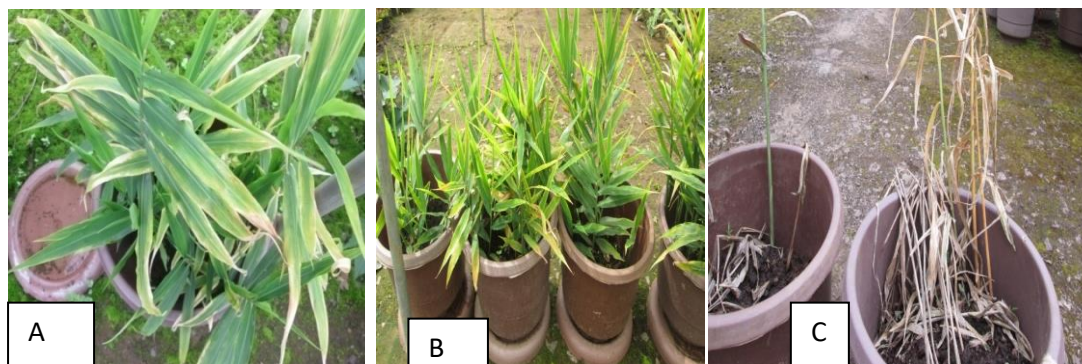


Fig.3.Plants inoculated with *R. solanacearum*, after 6 – 8 days of inoculation (A and B) the inoculated plant showed wilting symptoms 15 days after the inoculation(c)

In vitro* evaluation of antibacterial chemicals on the growth of *R. solanacearum

Present investigation was carried out to evaluate commercially available antibacterial chemicals to find out their effectiveness against the growth of *R. solanacearum* under *in vitro* condition. Susceptibility test results for the antibiotics were obtained from the data generated by the disc diffusion method. The disc diffusion method for antibacterial activity showed significant reduction in bacterial growth in terms of zones of inhibition around the discs.

Results of the activities of the four-antibiotics on each of the bacteria revealed that the zones of inhibition increased with increase in the antibiotic concentrations, thus, exhibiting concentration dependent activity. From this study, the results of the antibiotic susceptibility screening of the single antibiotics treatments showed that *R. solanacaerum* isolates were very susceptible to tetracycline, streptomycin, gentamycin and ampicillin. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics placed on the surface of Mueller-Hinton agar (MHA) medium inoculated with the *Ralstonia solanacearum*.

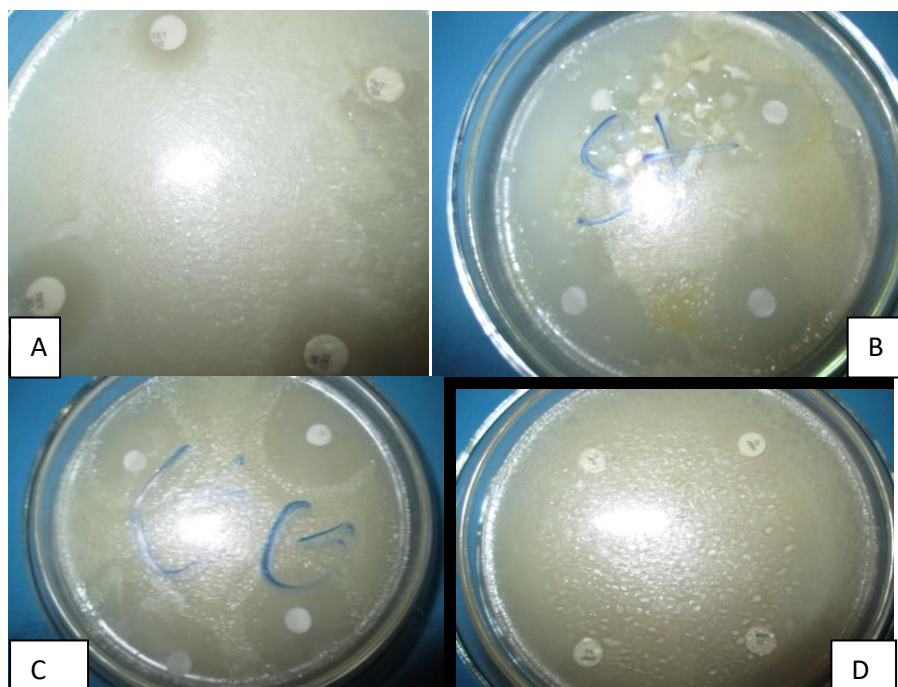


Fig.4. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics placed on the surface of Mueller-HInternationalon agar (MHA) medium inoculated with the *Ralstonia solanacearum*. Discs containing Gentamicin (C and D), Discs containing Streptomycin (A and B).

Table.2. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics placed on the surface of Mueller-Hinton agar (MHA) medium inoculated with the *Ralstonia solanacearum*.

Anti-biotics	Concentration mg/l	Inhibitory zones (mm)
Ampicillin		
	130 mg/l	10mm
	160 mg/l	12mm
	200 mg/l	5mm
	250 mg/l	6mm
Tetracycline		
	130 mg/l	12mm
	160 mg/l	10mm
	200 mg/l	10mm
	250 mg/l	15mm
Gentamicin		
	130 mg/l	13mm
	160 mg/l	15mm
	200 mg/l	18mm
	250 mg/l	22mm
Streptomycin		
	130 mg/l	6mm
	160 mg/l	14mm
	200 mg/l	12mm
	250	11mm
Antibiotics free	00 mg/l	00mm

Antibiotics treatment of plant material

Table.3. Antibiotics treatment of plant material results shoots free from *R. solanacearum*

Antibiotics	Antibiotics Concentration (mg/l)	Shoot number Mean±SD
Control	00 mg/l	8.00±2.71abc
Ampicillin	130 mg/l	11.51±1.29a
Ampicillin	160 mg/l	8.75±1.70abc
Ampicillin	200 mg/l	6.74±1.70bc
Ampicillin	250 mg/l	7.00±1.40bc
Gentamaycine	130 mg/l	6.00±1.40c
Gentamaycine	160 mg/l	5.70±1.50c
Gentamaycine	200 mg/l	5.75±1.70c
Gentamaycine	250 mg/l	6.75±0.50bc
Streptomycin	130 mg/l	6.75±1.25bc
Streptomycin	160 mg/l	7.00±0.00bc
Streptomycin	200 mg/l	6.60±0.57bc
Streptomycin	250 mg/l	6.50±0.57bc
Tetracycline	130 mg/	8.00±1.5bc
Tetracycline	160 mg/l	7.70±0.81bc
Tetracycline	200 mg/l	7.20±0.50bc
Tetracycline	250 mg/l	6.00±1.15c

Numbers are mean and S.D of Four replicates (four plant in each culture jars)

Means followed by the same letter with in a column are not significantly different by Tukey's test at $\alpha = 0.05\%$.

Rooting and acclimatization

Data from the greenhouse experiment showed that only 10% of plants survived where ginger plantlets are not treated with antibiotics. But ginger plantlets treated with the antibiotics (prior to acclimatization antibiotics are added in rooting medium) suppressed the incidence of bacteria wilt and also increased its survival rate by 94 % (Fig.5.)



Fig.5. Acclimatized ginger plantlets treated with antibiotics (A), Control (B).

6. Discussion

In the present study, diseased samples were collected to know the pathogens associated with disease in *in-vitro* propagated ginger plantlets. The causal agent *Ralstonia solanacearum* was isolated from pseudo stem of *invitro* propagated plantlets of ginger showing typical characteristics symptom of the disease. After isolation, *R. solanacearum* isolates purified by streaking a single colony of each isolate on Tetrazolium chloride agar (TZC) medium .a single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center. The virulent isolates of *R. solanacearum* were observed in *vitro* propagated ginger plantlets. Isolates of *R. solanacearum* isolated from *in vitro* propagated ginger produced pink or light red color colonies or colonies with characteristic red centre and whitish margin on TZC medium. *R. solanacearum* produced fluidal colonies with pink or light red color TZC on media after 24 hours of incubation. These results are in agreement with the findings of Kelman (1954), Schaad (1980), Suslow *et al.* (1982), French *et al.* (1995) and Melo *et al.* (1999), Rahman *et al.*(2010).Microscopic observations clearly indicated that the organism was gram negative and thus the preliminary identification of *R. solanacearum* was confirmed that all isolates did not retain violet colour i.e. the isolates retained counter stain (pink color). These results are similar with the findings of Suslow *et al.* (1982).who observed that the isolates of *R. solanacearum* did not retain violet color i.e. the isolates retained counter stain (pink color).

The biochemical characterization revealed that the disease that threat ginger under *in vitro* conditions is caused by *Ralstonia solanacearum* and the diseases were found in tissue of ginger plantlets. The syptomology and pathogenicity test confirmed that *R. solanacearum* causing bacterial wilt of ginger.

The result of pathogenicity test of *R. solanacearum* isolate under artificial stem inoculation method revealed that it produced wilt symptom in ginger plants within 7 to 15 days after inoculation. Pathogenicity of *R. solanacearum* causing bacterial wilt was proved earlier by several workers (Winstead and Kelman, 1952; Schell, 2000; Williamson *et al.*, 2002; Rajan *et al.*, 2002; Kumar and Sarma, 2004; Umesha *et al.*, 2005; Hikichi, 2007; Rashmi *et al.*, 2012; Artal, 2012; Thomas and Upret, 2014; Zulperi *et al.*, 2014).

The Knowledge of the effect of the antibiotics on both bacteria and the plants is essential for the recovery of healthy plants. Thus, knowledge of the effect of the selected antibiotics against *R. solanacearum* isolates was achieved by determining their minimal inhibitory concentration (MIC) and subsequently the minimal bactericidal concentration (MBC) of all the stable antibiotics after 24 h of incubation succeeding the antibiotic susceptibility test. MIC indicates the inhibitory potential, while MBC shows the cidal potential of the antibiotics on the isolates. According to Bonev *et al.* (2008), the effectiveness of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal bactericidal concentration (MBC) Reed and Tanprasert (1995).

Antibiotic sensitivity testing revealed that the antibiotics most effective against the growth of *R. solanacearum* under *invitro* condition were gentamicin, tetracycline, streptomycin and ampicillin. When shoot tips were grown for three weeks on multiplication medium supplemented with antibiotics, bacterial growth was inhibited. Intensive formation of high-quality shoots also took place. Shoot in cultures grown on antibiotics was actually free from bacterial contamination and cultures have grown on MS without antibiotics medium. Thus, all the antibiotics tested were found effective against *R. solanacearum*. These results are in conformity with the findings of those reported earlier by several workers (Hidaka and Murano, 1956; Dutta and Verma, 1969; Indersenan *et al.*, 1981; Khan *et al.*, 1997; Singh *et al.*, 2000; Devnath *et al.*, 2002; Dubey, 2005; Sunder *et al.*, 2011; Gupta and Razdan, 2013;

Owoseni and Sangoyomi, 2014. Vijayaraghavan (2011) Reported The different concentrations of streptomycin restricted the growth of the pathogen, exhibiting an inhibition zone (IZ) of above 25 mm with the maximum at 400 ppm concentration. The inhibitory effect of streptomycin and streptomycin on *Ralstonia solanacearum*, the causal agent of bacterial wilt in ginger has been observed by Paul (1998) observed that 250 and 500 ppm concentration of streptomycin, oxytetracycline and streptomycin inhibited *R. solanacearum* under *in vitro* conditions. Roop *et al.* (2017) have also reported effective antibacterial chemicals and bio agents against the growth of *R. solanacearum* under *in vitro* conditions. Average inhibition ranged from 6.2 mm (Copper hydroxide) to 20.05 mm (Streptomycin). However, significantly highest average inhibition was recorded in streptomycin (20.05 mm). This was followed by the antibiotics viz., gentamycin (17.5 mm), tetracycline (16.5 mm) and Streptomycin (11.95 mm).

7. Conclusion and Recommendations

7.1. Conclusion

The study has shown that after the surface sterilization of ginger explants before being used in tissue culture laboratory, *Ralstonia solanacearum* survived the sterilization techniques. With the rising rates of *Ralstonia solanacearum* causing high mortality in plant tissue culture, antibiotics will reduce the contaminants there by increase survival rate of the plantlets. *R. solanacearum* remains a continuing threat to plant tissue culture, but techniques for reducing contaminants using antibiotic revealed that Gentamicin was found to be significantly superior over other treatments with highest inhibition followed by tetracycline and streptomycin. Plant treatment of with antibiotics could be used to eliminate bacterial contamination from contaminated *in vitro* plantlets.

Therefore, the evidence presented here is suggestive of the possibility of chemical control for bacterial wilt of ginger. In this particular experiment, the antibiotics were capable of eliminating the pathogen under laboratory conditions.

7.2. Recommendations

Looking at the seriousness of the disease the following recommendations were made:

Further studies are required on the antibiotic doses to test their effectiveness in eliminating *Ralstonia solanacearum* from *in vitro* plantlets of ginger.

it need developing molecular method for detecting contaminants of *in vitro* propagated ginger particularly in detecting latent and invisible bacteria.

Pytotoxicity study is required to determine the effect of the sterilizing agents and antibiotics on the explants before being used in tissue culture.

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