CONTROL OF BACTERIAL WILT (*Ralstoniasolanacearum)* IN POTATO (*Solanum tuberosum*) USING RHIZOBACTERIA AND ARBUSCULAR MYCORRHIZA FUNGI

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ABSTRACT

Bacterial wilt disease, race 3 biovar 2A, is a devastating disease of potato and other important solanaceous crops, with no chemical control method. The current studies were, therefore, undertaken to assess the efficacy of biocontrol agent (BCA) and organic amendments to manage bacterial wilt (BW) of potato caused by *Ralstonia solanacearum,* under controlled conditions. The present studies evaluated disease severity, latent infection, arbuscular mycorrhiza fungi (AMF) root colonization and tuber number and weight on two potato cultivars upon inoculation with AMF, rhizobacteria in various combinations and organic amendments which included poultry manure, cow manure and compost. Disease severity was more pronounced in CIP 381381.13 (Tigoni) than in CIP 387164.4 (Clone). *Glomus intradices + Bacillus* spp. and *G. etunicatum + Bacillus* spp. for both cultivars and for the Clone, *G. intradices + Pseudomonas* spp., *Pseudomonas* spp. + *Bacillus* spp. + *Azoctobacter* spp. had area under disease progress curve (AUDPC) of zero. These were also the treatments with the highest AMF root colonization ranging from 50-36 compared to the controls with zero AMF root colonization and also tested negative for latent infection test except for *G. etunicatum + Bacillus* spp. in Tigoni. Poultry manure had AUDPC of 33 and 42 in Tigoni and clone, respectively compared with cow manure with 56 and 43 and compost with 54 and 42 in Tigoni and clone. A repeat of the trial involving the promising treatments, however, had all treatments having BW infected tubers. There was no significant difference (P≤0.05) in tuber number and weight in the BCA treatments with half fertilizer application and complete fertilized control. Mycorrhizal root colonization ranged from 23-49% in AMF inoculated treatments while no colonization was observed in the controls. The BCAs were effective as biocontrols against bacterial wilt even in the susceptible cultivar. More studies especially under field conditions are needed to further determine the response of the BCAs and organic amendments under different soil conditions.

Key words: *Ralstonia solanacearum,* potato, *Arbuscular mycorrhiza* fungi, rhizobacteria, organic amendments

INTRODUCTION

Ralstonia solanacearum is an aerobic non-spore forming, Gram-negative, soil-borne pathogenic bacterium that is motile with a polar flagellar tuft. The pathogen causes bacterial wilt, which is a devastating vascular disease of potato that limits tuber production with negative social impact and economic losses [1]. It has strong adaptive potential and globally distributed, affecting over 200 plant species, including economically important crops such as potato [2]. Many weeds are hosts to the pathogen increasing the persistence of the pathogen in the field [1]. The disease is transmitted through use of infected seed tuber, soil, water and farm tools and implements [3]. The pathogen infects the plants' roots through wounds or natural openings and is more severe in the presence of root knot nematode species [4]. It multiplies rapidly within the host, attacking the vascular system of the plant, inhibiting nutrient and water translocation resulting to wilt, collapse and eventual death of plant and decay of harvested tubers [5].

Principal strategies of managing the disease are crop rotation, use of disease-free seed and planting disease-resistant varieties. Continuous cultivation by smallholder farmers in East Africa who recycle seeds from season to season without rotation result in disease build-up in soils [6]. Currently, there are no resistant potato cultivars while certified seeds are expensive and would take nearly half of the production cost thereby limiting their accessibility by most farmers [7]. Furthermore, the pathogen can survive in the soil for several years even in the absence of host plant [8]. The disease spreads undetected when latently infected tubers are used as seed [3]. Lack of effective chemical control has also made it difficult to management the disease. Integrated and sustainable disease management options are, therefore, needed to control this highly destructive and challenging disease.

Studies have shown that the use of biological control agents (BCA) is effective in reducing the effect of the disease [9,10]. Some of these BCAs with high efficacy against bacterial wilt are soil rhizobacteria namely: *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp.

Identification of potential indigenous BCA and biofertilizer involved vigorous *in-vitro* screening of rhizobacteria isolated from soil and testing against bacterial wilt and for plant growth, which were studied by Chen *et al.* [11] and Zeinab and Behrouz [12]. Effective antagonistic isolates were further tested under greenhouse using tomato and potato to determine and select the efficacy of the various strains. There were 51.50% and 38.58% disease reduction in potato treated with *Pseudomonas putida* and *Pseudomonas fluorescens,* respectively [12]. *Pseudomonas fluorescens* and *Bacillus subtilis* reduced bacterial wilt severity by 55% and 46%, respectively, in tomato study conducted by Seleim *et al.*[13] while Tahat *et al.*[14] found no disease symptoms of bacterial wilt (race 2 biovar 3) in tomato inoculated with *Glomus mosseae*.

Such promising results justified the current research where indigenous rhizobacteria isolates combined with commercial arbuscular mycorrhiza fungi (AMF) inoculum were evaluated under controlled conditions to identify promising combinations for crop improvement and disease management on potato challenged with bacterial wilt.

MATERIALS AND METHODS

Isolation of Bacterial wilt pathogen and inoculum preparation

From an infested potato field, symptomatic infected plants were identified and uprooted together with the tubers. The plant roots were separated from the stem and washed thoroughly with water. Ooze test was done to confirm bacterial wilt as the cause of infection using the stem vascular flow system as described by Martin and French [15].

The bacterial ooze was streaked on Semi-selective media, South Africa (SMSA) [16]. After three days the petri dishes were examined for distinct bacterial wilt colonies which were further multiplied in Casamino acid-Peptone-Glucose medium. After three days, bacterial wilt colonies were suspended into two litres of distilled water and concentration determined using standard decimal serial dilution on SMSA and incubated at 30°C for 48hrs. Colonies that showed typical *R. solanacearum* characteristics (fluidal and irregular with red or pinkish red centres and whitish periphery) were counted and bacterial count of 10^{10} CFU/ml was observed.

Soil preparation and inoculation of bacterial wilt

The experiment was conducted in a screen-house at Upper Kabete field station, University of Nairobi. Soil used was sampled from a 15-year fallow plot at the Kenya Agriculture and Livestock Research Institute (KALRO)-Tigoni in Limuru. Nutrient analysis of the soil showed the following results: $pH(CaCl_2)$ 6.0, available phosphorus (Olsen) 12.4mg/kg, exchangeable cations 16.0 cmol/kg, and particle size distribution of 11.8%, 54% and 34.1% for silt, sand and clay, respectively. Large soil clods and debris were removed by sieving the soil through 5mm mesh sieve, solarized by covering with clear polythene sheet and left exposed to the sun for thirty days [17]. Sterilized coco peat was mixed with the soil in a ratio of 1:1 and used as the growing media at a rate of 22 kg per crate. Potato varieties; Tigoni that is highly susceptible to bacterial wilt (BW), and CIP clone 387164.4 that is considered less susceptible to BW were used in this experiment. Well-sprouted seed potatoes from International Potato Center were planted in bread crates (9'H*21'L*16'W) lined with polythene bag. Ten milliliter (10ml) of bacterial wilt inoculum was applied in each crate and mixed evenly in 22kg of soil-cocopeat mixture, watered regularly and left for two weeks before planting with 4 well sprouted seed potato.

The treatments that were applied included:

1. *Glomus intradices+Bacillus* spp.
2. *G.mossea+Bacillus* spp.
3. *G.etunicatum+Bacillus* spp.
4. *G.intradices+Pseudomonas* spp.
5. *Bacillus*spp.+*Azoctobacter* spp.
6. *Pseudomonas*spp.+*Bacillus* spp.
7. *Pseudomonas* spp.+*Bacillus*spp.+*Azotobacter* spp.
8. Poultry manure
9. Fertilized control
10. Unfertilized control

Microbial inoculants were applied in four rows on the surface and approximately 5cm deep at the time of planting. For the rhizobacteria 5µl (10^8cfu/ml) of *Pseudomonas* spp., *Bacillus* spp. and *Azoctobacter* spp. and 25g of AMF supplied by Dudu-tech division of

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Finlays Horticulture, *G. intraradices, G. mosseae,* and *G. etunicatum* were applied in the rows according to the respective treatment except for the controls which were not inoculated with the microbial inoculants. This experiment was arranged in randomized complete block design (RCBD) with four replicates. Diammonium Phosphate fertilizer (18-46-0) was applied at half the recommended rate of 25 kg/ha in each case except for the fertilized control that received the full fertilizer dose while the poultry manure was applied in the planting at the rate of 5 tonnes/ha. The experiment was repeated again in the screen house to determine the consistency of the treatments in the management of bacterial wilt.

Observation of Bacterial wilt symptoms on potato
The plants were left for natural disease infestation and regularly watered to ensure that the soil was moist. Upon emergence, the plants were rated visually for bacterial wilt severity every week from the third week to the ninth week of the experiment. A scale of 0-5 (from wilted leaves to death) was used where, 0 = no symptoms, 1 = 1 leaf wilted, 2 = 2 or 3 leaves wilted, 3 = all the leaves wilted except the top 2 or 3 leaves, 4 = all leaves wilted and 5 = plant dead, [18]. Percent severity index (PSI) was calculated using the method described by Cooke [19].

PSI=∑ *(scores × 100) / (number of plants rated × maximum scale of the scores)* for each scoring date.

From the PSI, reaction to bacterial wilt was then calculated using the area under disease progress curve (AUDPC) as per the formula according to Campbell and Madden [20]:

$$AUDPC = \sum_{j=1}^{N_{j-1}} \left(\frac{y_j + y_{j+1}}{2}\right)(t_{j+1} - t_j)$$

where,
y_j = percentage (%) of bacterial wilt on the jth observation,
t_j= the date of observation in days after planting and
N = the number of disease severity readings.

Harvesting was done when 75% of the plants had reached senescence and the tuber number and weight taken. Tuber grading was based on weight; <20g for small tubers and >20g for medium tubers. Tuber phosphorus content was determined using microwave-assisted acid extraction/dissolution of plant material [21]. Tubers were sampled for latent infection test using enzyme linked immunosorbent assay on nitrocellulose membrane (NCM-ELISA) according to Priou *et al.* [22].

Assessment of mycorrhizal root colonization
This was done for the AMF applied treatments and the controls. Fine roots were washed free of adhering soil and used for analysis of mycorrhizal colonization. They were chopped into fragments, one centimeter long and cleaned using 10% KOH, bleached in ammonium and hydrogen peroxide solution, neutralized in 1% HCl and stained with

0.05% trypan blue in acid glycerol according to method described by Koske and Gemma [23]. The stained roots fragments were mounted on slides in polyvinyl alcohol-lactic acid-glycerol solution and examined under a microscope at magnification power of x40 to obtain the percentage of roots colonized by mycorrhizal fungi. Percentage root colonization was calculated using the following formula:

$$Root\ colonization\ (\%) = \frac{No.of\ colonized\ segments}{Total\ No.of\ segments\ examined} \times 100$$

Mycorrhizal root colonization and Tuber P content was not determined in the repeat experiment.

Preliminary trial of the Bacterial wilt experiment
The trial also included 16 treatments that were not repeated. The methodology and data collected are as described earlier. Cattle and compost manure in this case were applied at 10 tonnes/ha. These treatments included:

1. *G. etunicatum*
2. *G. mosseae*
3. *G. intradices*
4. *G. etunicatum+ Pseudomonas* spp.
5. *G. mosseae +Pseudomonas* spp.
6. *G. etunicatum + Azoctobacter* spp.
7. *G. mosseae + Azoctobacter* spp.
8. *G. intradices+ Azoctobacter* spp.
9. *Pseudomonas* spp.
10. *Bacillus* spp.
11. *Azoctobacter* spp.
12. *Pseudomonas* spp.*+Azoctobacter* spp.
13. Cattle manure
14. Compost
15. Fertilizer treatment with no microbial inoculant
16. No fertilizer treatment & microbial inoculant

Nutrient composition was tested for each of the organic amendments as indicated in Table 1.

Data analysis and management
Data was subjected to analysis of variance (ANOVA) using proc GLM and least significant difference test for the variables conducted using Statistical Analysis System (SAS version 9). Differences at P≤0.05 were considered significant.

RESULTS

Area under disease progress curve (AUDPC) and latent infection
The microbial inoculants and poultry manure effectively suppressed bacterial wilt compared to the controls with the unfertilized control being the most infected with the disease (Table 2).

There were no disease symptoms in both Clone and Tigoni cultivars in duo application of *G. etunicatum+Bacillus* spp. and *G. intradices+Bacillus* spp. This was also observed in Clone in treatments having consortium mixture of *Pseudomonas* spp.*+Bacillus* spp.*+Azoctobacter* spp. and *G. intradices+ Bacillus* spp.

For these asymptomatic (symptomless) treatments, latent infection test was negative for all the treatments except for *G. etunicatum+ Bacillus* in Tigoni that tested positive.

Tuber number and weight
The effect of the treatments when compared with the controls was not pronounced in relation to tuber number and weight. It is only *G. etunicatum+Bacillus* spp. that had the highest values of 20 tubers weighing 448g compared with pathogen infected unfertilized control as shown in Table3. Though this treatment performed similar to the fertilized control treatment, this was achieved at half recommended fertilizer rate. The least number of tubers was in the poultry manure, which was less than the controls.

Mycorrhizal root colonization
The cultivars responded differently to mycorrhizal root colonization with the Clone having generally higher colonization than Tigoni cultivar. The Clone treated with *G. intradices+Pseudomonas* spp. had 13 and 7 higher root colonization than in Tigoni with similar treatment and in *G. etunicatum+Bacillus* spp., respectively (Figure 1). The controls did not have AMF colonization.
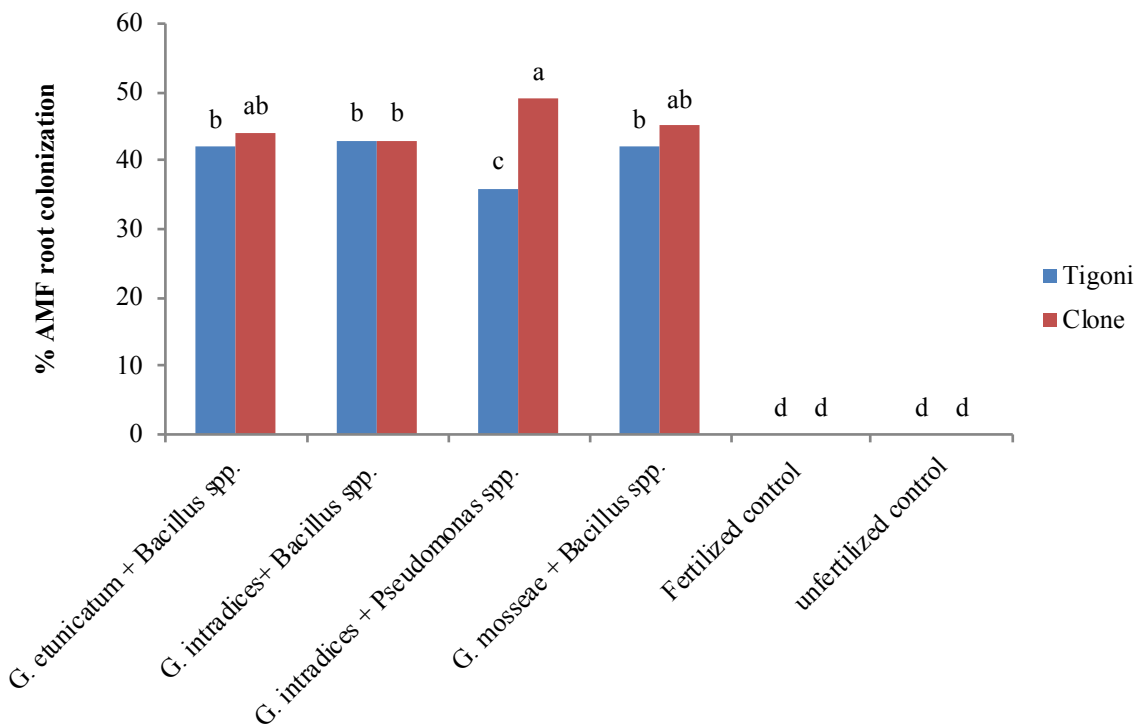
Figure 1: Percentage Mycorrhizal root colonization of BCAs on potato; Values with the same letter are not significantly different at P≤0.05

The BCA treatments had no effect on tuber phosphorus content; this was only observed at the variety level with Tigoni recording 0.18% and the Clone having 0.15%.

Repeat of Bacterial wilt main trial experiment

AUDPC and latent infection
All the BCA inoculated treatments exhibited antagonistic activity against bacterial wilt. There was suppressive effect in treatments of triple rhizobacteria, *G. etunicatum+Bacillus* spp. and *G. intradices +Pseudomonas* spp. with AUDPC of zero (Table 2). Fertilized control and poultry manure showed disease symptoms with AUDPC of 625 and 530, respectively compared to the unfertilized control with AUDPC of 1911.The plants treated with BCA were taller and more vigorous than untreated controls. During harvesting, however, all the treatments had tubers infected with bacterial wilt.

Seemingly, healthy tubers from the asymptomatic treatments were selected and further tested for latent infection and all treatments tested positive except for *G.mossea+Bacillus* spp. that tested negative for latent infection.

Tuber number and weight
The microbial treatments with half fertilizer application and poultry manure had higher tuber number and weight as the fully fertilized control, while the unfertilized control had the lowest levels (Table3).

Pretrial experiment

Area under disease progress curve of different microbial inoculants
All the treatments had bacterial wilt symptoms however; compared to the controls the microbial inoculated treatments and the organic amendments were effective in reducing bacterial wilt disease (Figure 2).
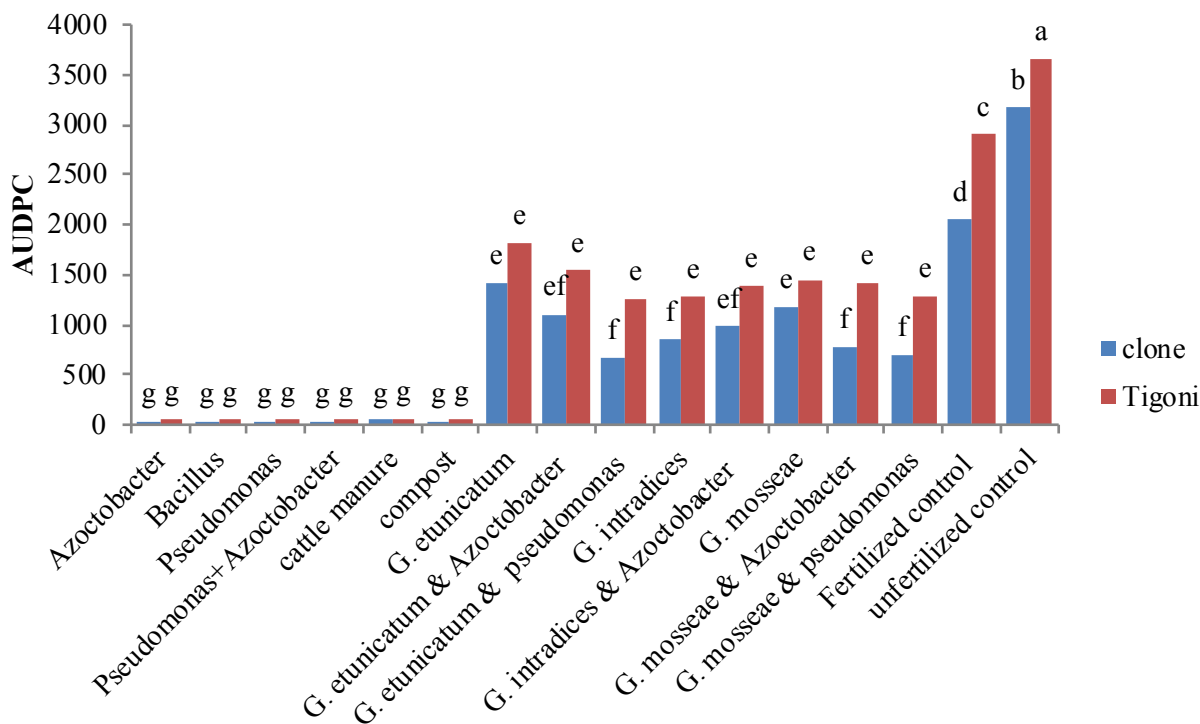
Figure 2: Area under disease progress curve for variety Tigoni and Clone; Values with the same letter are not significantly different at P≤0.05

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Among the BCA treatments, the disease was more pronounced in single AMF and duo inoculants of AMF and rhizobacteria ranging from 670-1800 than in single and duo inoculates of rhizobacteria, which ranged from 32-57.

Tuber number and weight
G intradices treatment had almost twice the number of tubers as the unfertilized control. Compost treatment, which had the lowest tuber number, also had the least tuber weight in all the treatments including the controls (Table 4).

Mycorrhizal root colonization
Root systems of potato were colonized in all the AMF treatments, while there was no colonization in the controls (Figure3). Among the AMF applied treatments, *G. mosseae+Pseudomonas* spp. had the highest root colonization with 39% and 41% while the lowest was observed in *G. etunicatum* with 24% and 23% for Clone and Tigoni cultivar, respectively.
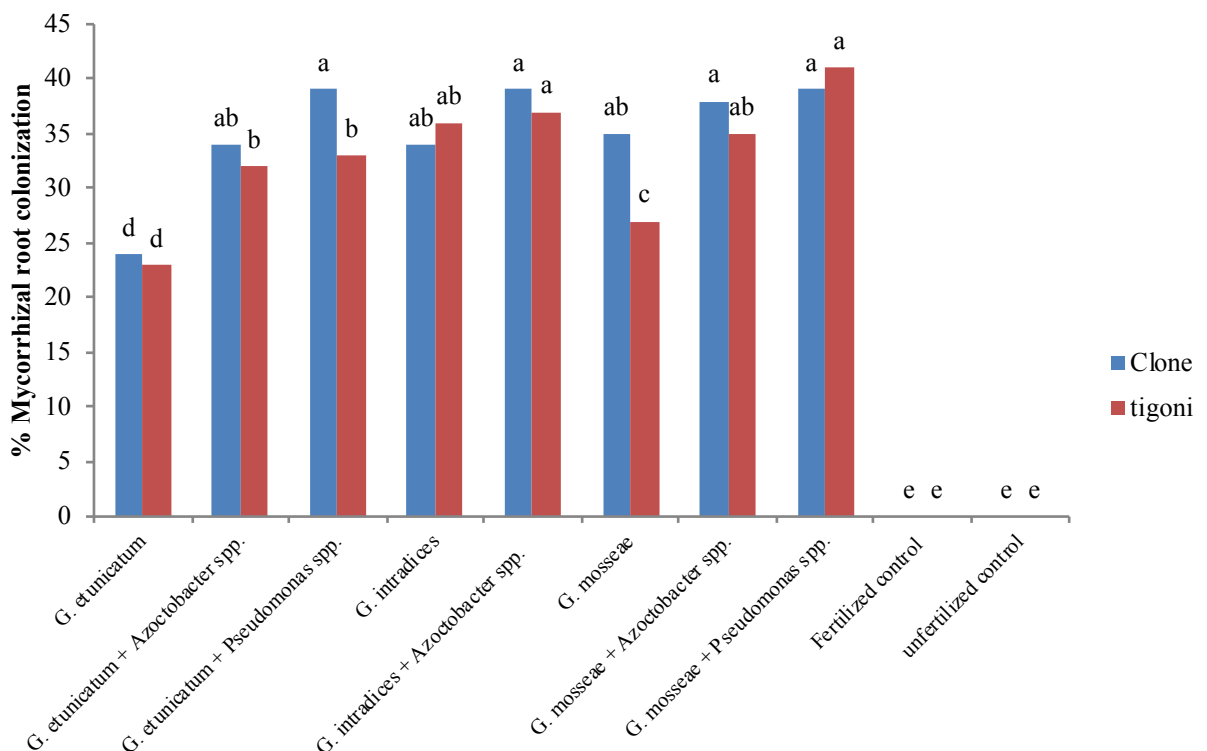
Figure 3: Percentage mycorrhizal root colonization on different AMF treatments on variety Tigoni and Clone; Values with the same letter are not significantly different at P≤0.05

DISCUSSION

Results from this study indicate that microbial inoculants of AMF and rhizobacteria have great potential in suppression of potato bacterial wilt disease. A mixture of BCAs tested here, exceeded the effectiveness of single strains and this was mostly in the co-inoculants of AMF and rhizobacteria and mixed rhizobacteria whose consistency was confirmed in the repeat experiment. This can be attributed to the synergistic effect of the antagonistic mixed application of the BCAs resulting into a complex interaction, which reduced the damage that might have been caused by the pathogen. Combining antagonists with different mode of action can therefore be effective in disease control [24]. Organic amendments especially poultry manure also reduced the severity of the disease. This can be attributed to its high nutrients content and high microbial biomass that increase the biocontrol activity [25].

Positive latent infection test of the symptomless treatments shows that a plant may appear healthy since it is able to tolerate development of visible bacterial wilt symptoms but is a carrier of the disease, which can only be detected and confirmed through use of diagnostic tools. The varied response of these microbial treatments shows the technical difficulties associated with coming up with promising BCA. This is because according to Azcon-Aguilar and Barea [26], the biocontrol effectiveness of these beneficial microorganisms is difficult to conclude due to complexity of the microbe, soil, plant system and environmental condition influence. It is, therefore, critical to identify the right combination factors so as to fully exploit their effectiveness.

Productivity in terms of tuber number and weight was rather low for the microbial treatments even for those that were disease free. This study indicates that in Bacterial wilt challenged condition, application of BCAs may not necessarily result in increased yield. The *Ralstonia solanacearum* bacterium may have been highly virulent which the BCA were fighting to counteract with advanced effect on potato productivity. This goes contrary to the expectation that reduction in disease severity would result in increased plant productivity [27]. In this study, enhanced tuber phosphorus content due to the microbial treatments was not observed. This may also be due to AMF inability to build a strong hyphal network to increase nutrient absorption as for the case of AMF treatments that was achieved by Taha and Sijam [28], with AMF root colonization as high as 70% with enhanced N, P, K uptake in bacterial wilt challenged tomato plant which is of the same family as potato. This possibly shows that the effectiveness of microbial inoculants depends on the time and method of inoculation. For instance, inoculation of the pathogen one month after the AMF application and evenly mixed in the soil resulted to low disease severity in microbial applied treatments as observed by Taha and Sijam [28]. This is in contrast with this experiment where the microbial inoculants were applied two weeks after the bacterial wilt inoculum. Furthermore, *R. solanacearum* has the potential of producing bacteriocins that can inhibit AMF spore germination [14]. The strategic means of controlling bacterial wilt is preventing entry of the pathogen in the plant by conferring biofilm protection and/ or boost immunity by activating the disease-resistance genes in plants. The mode of action by antagonistic microorganisms should therefore, begin at the early stage of host-pathogen interaction showing the importance of early application of beneficial microbes on potato seeds prior to inoculation with pathogen. This ensures

competitive colonization and establishment of microbial inoculants in the root zone, a prerequisite for effective biocontrol, considering that the first step in controlling pathogenic soil borne microorganisms is efficient rhizosphere colonization by the applied beneficial microbes.

Both cultivars of potato had AMF root colonization, which varied with the Clone having higher colonization than Tigoni in the presence of *R. solanacearum*. This shows that different plant cultivar responds differently to microbial inoculates. Single AMF had high root colonization but this was further improved in the presence of rhizobacteria signifying that rhizobacteria do not inhibit but enhance mycorrhizal colonization of the roots and are best referred to as mycorrhizal helpers confirming the findings by Azcon *et al.* [29]. This also links the bioprotection effect of AMF on the level of root colonization [30], implying that a high degree of AM roots colonization leads to high localized bioprotective effect.

CONCLUSION

Co-inoculation is more effective biocontrol than single microbial inoculation in bacterial wilt management. The low yields show how damaging the bacterial wilt disease is to potato production. Even when plants appear symptomless latent infection test especially for seed tuber is critical to minimize the spread of the disease. Understanding of the BCA mechanisms of disease control is also important to be able to determine the rate of BCA application and if multiple inoculations at different plant stages are required. Field trials should be undertaken especially under field conditions to confirm their effectiveness considering variability in the native soil fauna, flora and microorganisms that may militate against the success of the inoculants and reveal different responses on the efficiency of AMF and rhizobacteria inoculants in control of diseases especially bacterial wilt.

ACKNOWLEDGEMENTS

Table 1: Organic amendments nutrient composition

Nutrient element	Compost manure	Cattle manure	Poultry manure
pH	6.3	8.3	8.7
C/N ratio	18.3	16	14.4
Phosphorus %	0.41	0.22	1.64
Potassium %	0.63	1.54	2.74
Calcium %	1.46	1.28	6.16
Magnesium ppm	0.28	0.32	0.94

Table 2: Area under disease progress curve of different BCA treatments and organic amendment

Treatment	Main trial		Repeat of Main trial
	Tigoni	Clone	
Bacillus spp.+*Azoctobacter* spp.	40f	26f	9c
Pseudomonas spp. +*Bacillus* spp.	32f	25f	4c
Pseudomonas spp.+*Bacillus* spp.+*Azoctobacter* spp.	22f	0g	0d
G. etunicatum + *Bacillus* spp.	0g	0g	0d
G. intradices+*Bacillus* spp.	0g	0g	4c
G. intradices + *Pseudomonas* spp.	871c	0g	0d
G. mosseae + *Bacillus* spp.	521d	237e	4c
poultry manure	42f	33f	531b
Fertilized control	2901a	2052b	1912a
unfertilized control	3658a	3168a	626b
Means	809a	554b	296
Standard Error	39		118

Values are means of four determinations
Values with similar letters are not significantly different at 5% level of significance

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Table 3: Effect of the treatments on potato tuber number and weight

Treatment	Main trial		Repeat of Main trial	
	no. tubers	tuber weight(g)	no. tubers	tuber weight(g)
G. etunicatum + Bacillus spp.	20a	448a	31ab	447ab
G. intradices + Pseudomonas spp.	18ab	413ab	31ab	681a
G. mosseae + Bacillus spp.	17ab	395ab	30ab	641a
G. intradices+ Bacillus spp.	15abc	418ab	38a	719a
*Pseudomonas*spp.+*Bacillus*spp.+*Azoctobacter* spp.	15abc	362ab	36ab	644a
Pseudomonas spp. +*Bacillus* spp.	13bcd	328ab	37a	630a
*Bacillus*spp.+*Azoctobacter* spp.	10cd	322ab	40a	698a
poultry manure	8d	266ab	27ab	570ab
Fertilized control	15abc	268ab	38a	696a
unfertilized control	10cd	235b	18c	220c
Means	14	346	33	595
Standard Error	1.4	42.7	4	74

Values with similar letters within a column are not significantly different at 5% level of significance

Table 4: Tuber number and weight as influenced by AMF and rhizobacteria inoculants

Treatments	tuber number	tuber weight
G. intradices	19a	415a
*G. etunicatum+ Pseudomonas*spp.	18a	412a
G. etunicatum	18a	356a
G. etunicatum+Azoctobacter spp.	17a	329a
*G. mosseae+Pseudomonas*spp.	16a	341a
G. intradices+Azoctobacter spp.	15a	302a
G. mosseae+Azoctobacter spp.	15a	291a
G. mosseae	14a	312a
cattle manure	11a	334a
*Pseudomonas*spp.+*Azoctobacter* spp.	11a	384a
Bacillus spp.	11a	297a
Azoctobacter spp.	11a	323a
*Pseudomonas*spp.	10a	272a
Compost	5b	144b
Fertilized control	15a	235a
unfertilized control	10b	268a
Means	33	313
Standard Error	1.2	35.2

Values with the same letter in the column are not significantly different at P≤0.05

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