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Incidence and Occurrence of Latent *Ralstonia solanacearum* Infection in Seed Potato from Farmer Seed Grower Cooperatives in Southern and Central Ethiopia

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Abstract

Bacterial wilt (BW) of potato caused by *Ralstonia solanacearum* (*Rs*) has been and continues to be a devastating disease of potato and related crops worldwide particularly vegetatively propagated ones. In Ethiopia, potato BW has spread to many areas due to absence of an effective seed potato system with fair seed certification, disease monitoring, and containment. Seed potato in Ethiopia is generally visually inspected resulting in failure to detect *Rs* in latently infected stock assumed clean by visual assessments. Consequently, a study was conducted to assess the extent of *Rs* latent infection in seed potato in major seed potato producing cooperatives in southern, southwestern, and central highlands of Ethiopia using Nitrocellulose membrane ELISA. A total of 41,600 tuber samples were collected between 2015 and 2016 from 121 fields of 57 registered seed potato grower cooperatives representing 107.05 ha distributed in 14 districts. Results of latent *Rs* infection indexing indicated that 58.3% of analysed samples were infected, more so during the March–June 2015 cropping season. The prevalence of latent infection was not significantly ($P \geq 0.05$) influenced by potato variety or altitude at which the sample was collected but occurred randomly across altitude. The disease was evident even in samples collected at altitudes as high as 3000 m above sea level. Two samples collected at more than 3000 m above sea level were clean. However, these were too few to assume there is no BW above this altitude. In the absence of a reliable and sustainable seed potato testing for latent infection, farmers seed group cooperatives (FSGCs) who are the main producers of seed potato in Ethiopia as quality declared seed (QDS) will continue to disseminate *Rs* even in seed produced at extremely high altitude agro-ecologies. Consequently, it is recommended that QDS from FSGCs should be subjected to mandatory latent *Rs* infection indexing before they can be distributed as trusted potato planting stock despite the cost that would be involved. This should be complemented with BW infection containment effort combining biological, agronomic, policy, and social contexts.

Keywords Bacterial wilt · Nitrocellulose membrane ELISA · Quality declared seed · Seed potato certification · Seed potato system

Introduction

Bacterial wilt or brown rot caused by *Ralstonia solanacearum* is one of the most destructive plant diseases globally of many economically important crops like potato (Priou et al. 2010; Kurabachew and Ayana 2016; Okiro et al. 2016). It affects production of host crop plant species in tropical, sub-tropical, and temperate regions (Champoiseau et al. 2010). The pathogen is rapidly spreading from low to high altitude areas in many countries including Ethiopia (Bekele et al. 2011; Muthoni et al. 2012) and to colder, higher latitudes (Liu et al. 2017; Sharma et al. 2018). Its effects in potato farming systems are very large because potato is staple food for more than one billion people in the world (CIP 2019). Likewise, potato has an immense economic role in most countries in SSA including Ethiopia in highland agro-ecology as a principal source of food and income for most smallholder farmers (Wassihun et al. 2019). In Ethiopia, approximately five million households are directly engaged in potato production and millions more indirectly earn a living from this value chain (Labarta 2013; CSA (Central Statistics Agency), 2016). In most districts of Ethiopia, potato plays a vital food security role especially in the months when grain crops are not ready for harvesting. It therefore fills the food gap between planting and harvesting of major staples and is usually referred to as a hunger relief crop.

These attributes of potato notwithstanding, its production in Ethiopia is challenged by many diseases among which bacterial wilt is becoming top on the list (Gorfu et al. 2013; Sharma et al. 2018). It is becoming a key threat of potato production in Ethiopia because it is rapidly spreading to many potato-growing agro-ecologies where it was previously unknown (Bekele et al. 2011; Bekele 2016) since its first reported presence in the country in 1956 (Stewart 1956). The disease however was not considered a threat to potato production in Ethiopia, and consequently, it did not get full attention until mid-1990s because it was confined to very few areas in the country that were not considered important in the potato value chain (Bekele and Berga 1993; Bekele and Yayinu 1994). However, in the late 1990s, BW was identified as one of the key threats to the potato industry that was causing serious challenges to many potato growers in the country (Bekele et al. 2011; Gorfu et al. 2013).

In Ethiopia, there is currently no formal seed potato production system nor an organized approach to check seed potato for health. Hence, infected but symptomless seed tubers harbouring the pathogen transfer the bacterium to progeny crop and uninfected soil in infected tubers that are distributed as having been produced in healthy, BW-free areas (Gildemacher et al. 2009; Hirpa et al. 2010; Bekele et al. 2011; Gorfu et al. 2013; Abdurahman et al. 2017). Such latently infected seed tubers became a major cause for the rapid spread of *Rs* from one part of the country to others (Bekele et al. 2011; Gorfu et al. 2013; Abdurahman et al. 2017). In recent years, the disease prevalence has reached 80–90% in major potato-growing areas of Ethiopia (Sharma et al. 2018) further threatening the livelihoods of already vulnerable smallholder potato farmers. Bacterial wilt prevalence in Chencha district, southern Ethiopia in 2015 for instance, was reported to be 97% (Abdurahman et al. 2017). The high prevalence of BW in potato crops in recent times in most developing countries could be due to farmers lacking reliable sources of quality assured seed potato supply, relying on poorly monitored seed sources and home-saved planting material that may be potentially latently infected with BW (Abdurahman et al. 2017, Abdurahman et al. 2019; CIP 2020).

The nature of the BW pathogen hampers research efforts towards the full eradication of the disease since *Rs* is soil-borne (Kelman 1953; Gorfu et al. 2013), which can be disseminated through infected run-off water (Farag et al. 1999; Williamson et al. 2002; Hong et al. 2005), latently infected seed tubers (Williamson et al. 2002; Elphinstone 2005; Abdurahman et al. 2017), contaminated farm tools, and other unexpected means. Symptomless weeds and contaminated irrigation water, surface irrigation, and drainage water are also reported to be important means for cross-season survival and diffusion of *Rs* (Allen et al. 2001; Lin et al. 2009). The possible survival of long-starved pathogenic *Rs* in fresh aquatic environments may raise new fears about the epidemiology of bacterial wilt (Alvarez et al. 2008).

Increasing potato production and productivity while protecting potato producers, consumers, and the environment requires an integrated approach encompassing many policies (CIP 2020). Global, regional, national, and local BW disease monitoring, identification, and formulation of appropriate control methods are important to reduce the risk to the potato value chain of BW constraints. Development and implementation of effective BW disease management tactics is crucial to safeguard crop losses that are worth billions of dollars worldwide and to sustain food security (Le and Vu 2017).

Sensitive and user-friendly serological disease diagnostic techniques are available from the International Potato Centre (CIP) and can be used for pathogen detection in latently infected stocks to prevent their use (Priou et al. 2001, 2010). Nitrocellulose membrane (NCM) ELISA, for example, is one of the appropriate tools for seed testing in most developing countries for seed potato certification programs (Elphinstone et al. 1996; Priou et al. 2010; Mihovilovich et al. 2017). Currently, DNA-based and serological methods provide essential tools for precise plant disease diagnosis (Martinelli et al. 2015). Identification of areas that are free from *Rs* for quality seed potato production, probably in the highland districts in Ethiopia, should be given research priority to reduce the risk of the *Rs* build-up and dissemination in the entire potato agro-ecology of the country (Abdurahman et al. 2017). The objectives of the present study were therefore to (i) assess the level of latent infection in the seed stocks of different potato varieties showing no visible *Rs* infection symptoms in the field and (ii) assess the status of latent BW infection and distribution in major seed potato producing cooperatives in Ethiopia.

Materials and Methods

Sample Collection and Study Area

Tuber samples were collected from mature potato crops previously inspected visually and passed as fit for use as future quality planting material to assess latent infection with *Rs* from major seed producing cooperatives in Ethiopia (Tessema et al. 2020). A sample size of 200–600 tubers per field of 0.5 to 2 ha was taken and analysed in 8–24 sub-samples of 25 tubers each based on sample size following CIPs' instruction manual for the detection of latent *Rs* in potato (Priou et al. 2001). The samples were collected from 14 districts (Fig. 1) in southern, southwestern, and central highlands of Ethiopia (Wolmera-Jeldu belt) that constitute the major sources of seed potato in the country (Abdurahman et al. 2017). Two most dominant potato varieties of Belete and Gudene

were emphasized as sample sources in this study. During sample collection, data related to crop production and geographical position using the geographical positioning system (GPS) were recorded (Fig. 1), including sample collection location elevation.

Sample tubers were collected from fields that had been previously tested with pocket, *Rs* ELISA lateral flow device and confirmed as BW-free during visual field assessments. Most samples were collected from lots before harvesting or from heaps at harvest or seed potato stores within 3–4 weeks after harvesting. A total of 57 seed grower cooperatives from 14 districts were sampled during the study where 41,600 tubers were collected from 121 potato fields representing 107.05 hectares in 2015/2016 cropping season (Table 1). The composite sample from each seed lot was labelled and delivered in crates to the Microbiology Research Laboratory in the National Agricultural Biotechnology Research Centre (NABRC) at Holetta for latent *Rs* infection analysis using post-enrichment NCM-ELISA.

Sample Preparation

The collected samples were kept at room temperature until extraction of sample tuber fragments for pathogen culturing and enrichment. Tubers in each sample were divided into 25-tuber sub-samples and accordingly labelled with a code corresponding to field sample. The recoded samples were washed in tap water, disinfected in 5% sodium hypochlorite (v/v) for 5–10 min, rinsed in clean tap water, and allowed to drip dry on clean nets or tissue papers.

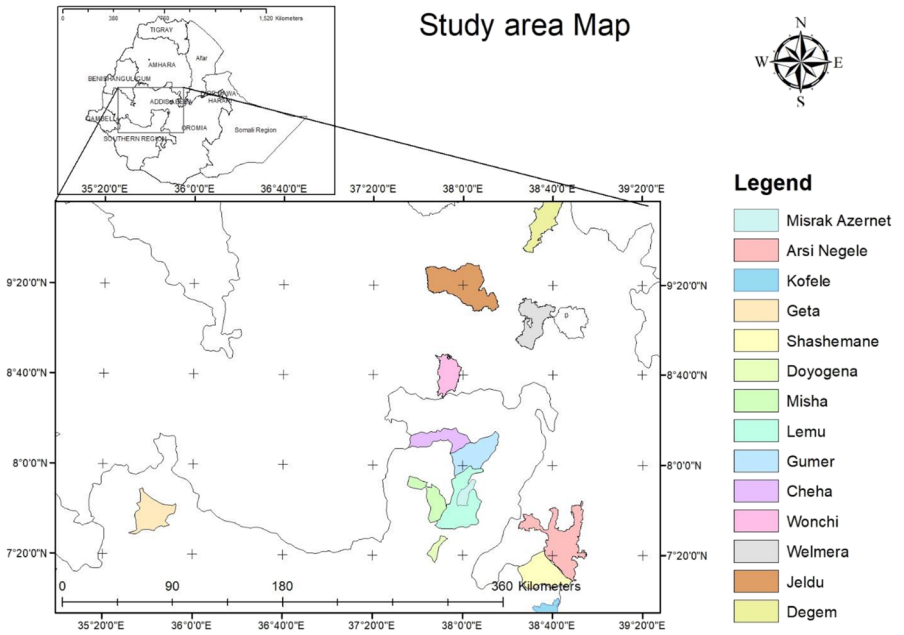

Fig. 1 Location map of districts in southern and central Ethiopia where samples for latent *Ralstonia solanacearum* analysis were collected in 2015 and 2016

Table 1 Prevalence of latent *Ralstonia solanacearum* infection in planting materials among major potato producing districts in central and southern Ethiopian highlands in 2015 and 2016 cropping seasons

District	Collected samples	Number of infected samples by season				Latent infection %
		March–June 2015	June–Sep 2015	March–June 2016	Total	
Doyogena	5	0	2	2	4	80.0
Lemu	10	0	0	7	7	70.0
M/Azernet	6	0	4	0	4	66.7
Jeldu	42	24	0	1	25	59.5
Gumer	15	2	6	0	8	53.3
Wonchi	17	8	0	1	9	52.9
Wolmera	13	6	0	0	6	46.2
Total	108	40	12	11	63	58.3
Infected %		37.0	11.1	10.2	58.3	

Sample Testing

All races, biovars, and serotypes of *Rs* can be detected with the *Rs*-specific antibodies provided in the CIP NCM-ELISA kits. Sample tubers and working benches were surface sterilized with 70% ethanol. A thin slice of approximately 0.5 g per tuber from each sample was taken using a flame sterilized blade (cuticle remover) from the stolon end of vascular ring and put in a labelled plastic bag. The tuber fragments from each sub-sample of 25 tubers were weighed. Sterile citrate extraction buffer (pH 5.6) at 2 mL per gram of tuber tissue was added to tuber fragments and homogenized with a pestle or a conical flask edge. The homogenized vascular ring fragments were placed vertically in crushed ice before drawing homogenate supernatant to prevent oxidation of the phenols.

From each sub-sample, 500 μL of the supernatant of tuber extracts was taken carefully without sediments using a 1000-mL micropipette with a sterile tip. The drawn supernatant tuber extracts were incubated in an equal volume of SMSA 1X in sterile 1.5-mL Eppendorf tubes for 48 h at 30 °C with constant agitation in an incubator shaker. After incubation, the samples were kept at −20 °C until performing the NCM-ELISA. After the enrichment procedure, 20 μL of clear tuber extract from each sample was taken with a sterile micropipette and slowly released onto the NCM by holding the pipette vertically and allowing a slight contact of the open end of the pipette tip onto the membrane. Dot-blotting of the samples on NCM was performed on a bed of sterile filter papers moistened with the extraction buffer (Priou 2001). The loaded membranes were left to dry at room temperature embedded between two dry filter papers.

The serological test was done at room temperature, with a rotary shaker at 50 rpm for incubation and 100 rpm during the washing steps. Blocking of the unoccupied areas of NCM was done by incubating the membranes in a blocking buffer including positive and negative controls that come with the kit. The blocked membranes were incubated with the *Rs*-specific antibodies for 2 h with gentle agitation. The membranes were then washed at 100 rpm three times to remove excess antibodies. The loaded membranes

were thereafter incubated with *Rs*-goat-anti rabbit antibody enzyme conjugate solution for 1 h with gentle agitation before conducting the final *Rs* detection step. Finally, 25 mL per membrane of colour development solution was added and the reaction allowed to take place for 5 to 20 min at room temperature without agitation. The results were interpreted based on the intensity of purple colour development per sample compared to the positive controls included in the kit. Any sample in which any of the sub-samples tested positive to presence of *Rs* was considered to be wholly infected.

Probability of detection and data analysis

The number of positive samples from each sample field was recorded and used to calculate the percentage of infection in each sampled field. The probabilities of detection by the serological technique were calculated using a binomial distribution model. For the detection of symptomless fields using NCM-ELISA, a sample size of 350 tubers was recommended to obtain a probability of 99% for the detection of at least one positive sample (Priou et al. 2001; Priou 2001). The percentage of latently infected tubers was calculated from Mihovilovich et al. (2017).

$$Percent\ latent\ infection = (number\ of\ positive)/(total\ samples) \times 100 \quad (1)$$

Results

The results of latent *Rs* infection in seed potato collected from seed farmer group cooperatives in central and southern highlands of Ethiopia indicated that 58.3% of all the samples tested positive to latent *Rs* infection (Table 1). The highest *Rs* latent infection was obtained in Doyogena district and least in Wolmera (Table 1). Data also showed that more than 50% of the samples from all the districts except Wolmera tested positive to latent *Rs* infection (Table 1). Data further revealed that the March–June 2015 cropping season had more latent *Rs* infection than the other two seasons (Table 1), and all exceeded 10% disease attack.

Assessment of latent infection by potato variety in each season showed that variety Gudene was more latently infected than Belete in each of the three cropping seasons of the study (Fig. 2). This might be due most to the fact that most fields of variety Belete were found with visible BW disease symptoms and were not sampled compared to variety Gudene where most crops were not showing signs of *Rs* attacks. Consequently, probably more healthy fields planted with variety *Belete* were sampled than fields planted with variety Gudene that visually looked healthy but was largely latently infected with *Rs* when serologically tested. From this result, assessment of seed potato health with serology or by other means in laboratory is more dependable than visual field observations that are routinely used in seed potato quality assurance in Ethiopia.

Analysis of variance to test the significance of potato variety on latent infection and altitude at which the samples were collected and by district showed that *Rs* latent infection was not significantly ($P \geq 0.05$) influenced by the potato variety or altitude at which a sample was collected.

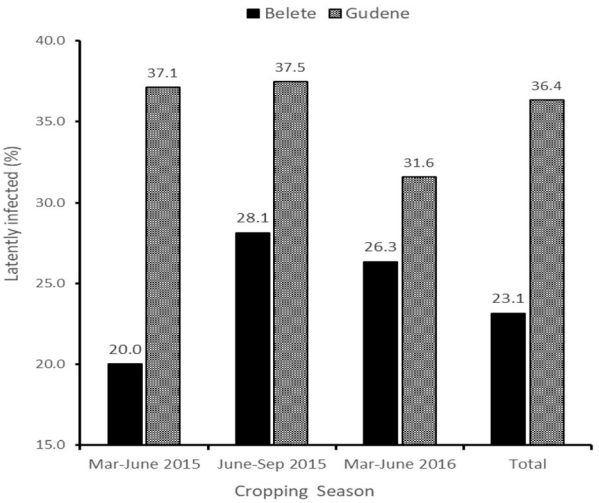

Fig. 2 Variation in latent *Ralstonia solanacearum* infection in seed tubers of varieties Belete and Gudene in central highlands of Ethiopian in 2015 and 2016

Therefore, the *Rs* latent infection detected serologically was not influenced by potato variety or the altitude at which the sample was collected but probably by other factors that could not be revealed by this kind of study. Thus, similar results would be obtained without disaggregating sample collection by variety and district within the geographical areas where these samples were collected.

Assessment of *Rs* latent infection in seed potato by district showed that Lemu district had the highest infection while Wolmera had the least. Nevertheless, the level of infection was above 45% across a group of seven districts that had at least ≥ 10 samples collected (Fig. 3).

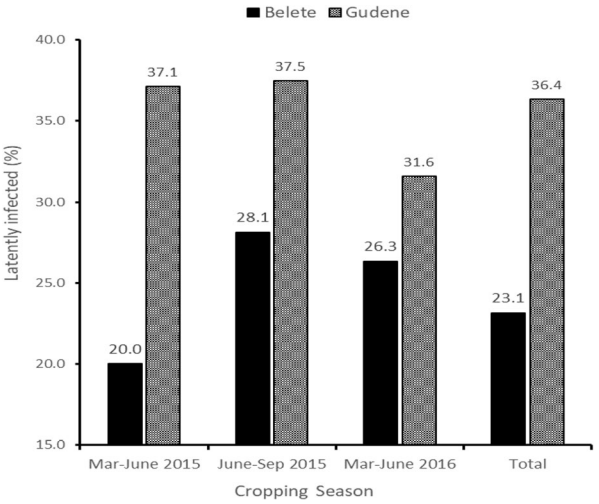

Fig. 3 Incidence of latent infection in seed potato samples from selected districts in central and southern Ethiopia in 2015 and 2016 cropping seasons

Examination of prevalence of *Rs* latent infection in seed tuber samples in representative sample districts across increasing altitude showed a random distribution of *Rs* disease in seed over altitude from 2100 to 3200 metres above sea level (Fig. 4). Latent *Rs* infection was as likely at lower as at high altitudes (Fig. 4). In some districts such as Wolmera and Wenchi, there was even more latent *Rs* infection at higher than lower altitudes (Fig. 4 B and D).

The variation in the prevalence of latent *Rs* across the sampled districts was further explored using correlation analysis between percentage prevalence of latent *Rs* infection and altitude. The correlation coefficient between altitude and latent infection was rather low but negative ($r = -0.4738$). The negative correlation coefficient means that latent *Rs* infection would decrease with increasing altitude albeit a low value. Regression analysis between altitude and percent latent infection was not significant and had a low coefficient of determination ($R^2 = 0.160$). However, it still has a negative gradient depicting that latent *Rs* infection decreases with increased altitude (Fig. 5). This

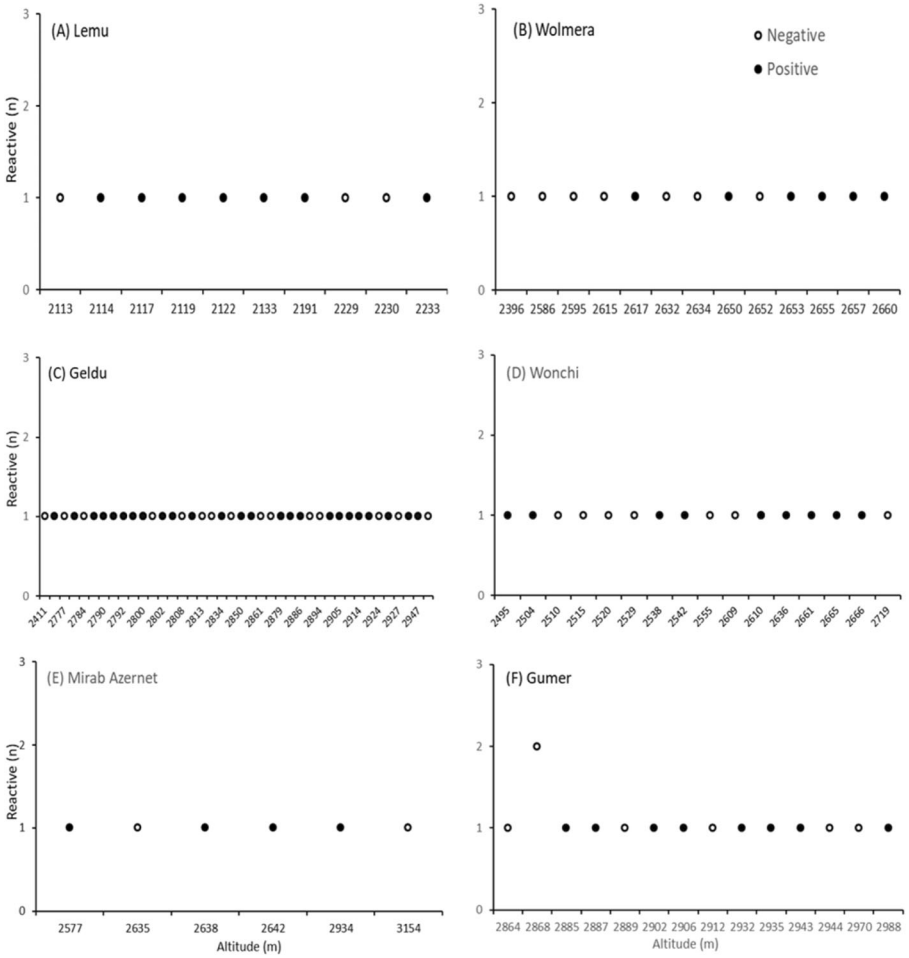

Fig. 4 Distribution of latent *Ralstonia solanacearum* infection as number of reactive samples across altitude in selected districts in central and southern highlands of Ethiopia

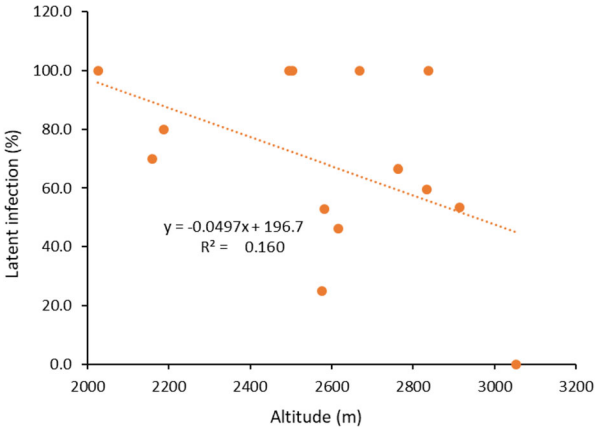

Fig. 5 Relationship between prevalence of *Ralstonia solanacearum* latent infection (%) and altitude (m) in the central and southern highlands of Ethiopia in 2015 and 2016

therefore does not contradict the concept of low *Rs* infection at high altitudes despite the low strength of the linear relationship.

Discussion

Assessment of latent infection in seed potato samples revealed an unacceptably high prevalence of BW in purportedly quality seed produced by registered farmer seed group cooperatives. This is worrisome considering that BW is an international and national quarantine disease with zero tolerance in seed potato. Latent BW infection was evident in very high proportions in seed potato which had been passed for seed generation using visual assessments. Most of these seed fields would not qualify for generation of future potato planting material if they were to be tested with sensitive laboratory procedures rather than the current practice of visual assessment for seed potato certification. The high BW infection in samples from most seed cooperatives and districts could be due to failure to implement BW disease management practices required of seed producers, potato farmers, or other responsible seed regulatory bodies (Kago et al. 2016). In Ethiopia, distribution of latently infected seed potato was identified as a major pathway for the spread of bacterial wilt in Ethiopia (Abdurahman et al. 2017).

Assessment of latent BW infection did not reveal any significant ($P \geq 0.05$) difference between Belete and Gudene, the two popular varieties grown by farmers. This means both varieties are equally susceptible to BW, and it would not make any difference in latent *Rs* infection in segregating samples by potato variety. It is known that neither resistance to BW in potato nor tolerance is helpful in containing bacterial wilt. Cultivars that appear resistant or tolerant to the BW only help to spread the disease if seed potato is not appropriately indexed. What would be probably helpful is full *Rs* infection immunity which has not been achieved despite many years of breeding and research for commercial potato varieties. Therefore, in future similar work with the currently released potato varieties in Ethiopia, assessment for latent infection based on variety

difference may not improve the quality of result and the information that is already known. Emphasis should be put in general disease monitoring irrespective of the potato variety being grown.

The prevalence of latent *Rs* infection was postulated to be influenced by the altitude and district where the sample was collected. Analysis of variance showed that the prevalence of latent *Rs* infection was not significantly *($P \geq 0.05$)* influenced by the altitude at which the potato sample was collected. Correlation analysis between average altitude at which the samples were collected and prevalence of latent *Rs* infection showed a low but negative correlation coefficient. The negative correlation coefficient indicates that latent *Rs* infection would decrease with increasing altitude, and this is a long-held view (Bekele and Yayinu 1994). Based on this result, one would anticipate high BW latent *Rs* infection at lower rather than higher altitudes. The regular occurrence of BW at high altitudes where it was formerly unknown reveals the spread of the disease where it was formerly absent, probably due to unsustainable human or climate-related changes (Priou et al. 2010).

A random scatter plot of *Rs* latent infection prevalence against altitude showed a negative gradient with a probable linear relationship. However, fitting the data with a simple linear regression showed that the model and its coefficients were not significant ($P \geq 0.05$). However, the slope of the simple linear equation explaining latent infection prevalence (Y) as a function of altitude (X), $Y = 196.7 - 0.0497X$, was negative. However, such response would be logically fitted with curve-linear decay functions where extreme low values of X would theoretically have 100% of the seed tubers latently infected with *Rs*. Conversely, extremely high altitude values (X) would predict absence of latent *Rs* infection in the samples. This is ecologically possible considering that at low altitudes, aggressive, more pathogenically fit, warm environment and multi-host adapted races of *Rs* become dominant in limiting potato or seed potato production (Martin and French 1985). Such low altitude environments additionally would be less suitable for potato production due to unsuitability of eco-climate and a multiplicity of other potato diseases, especially viruses.

Further examination of the prevalence of latent infection across altitudes at which the samples were collected in the representative districts indicated a random incidence of both clean and latently infected seed samples. Bacterial wilt was evident as high as 2988 m and as low as 2100 m above sea level. The disease was not detected in any sample collected at more than 3000 m above sea level in Mirab Azernet and Degam. However, this may not confirm absence of *Rs* at this altitude because only one sample was collected in each of the two districts and may not fully represent the entire districts. The presence of latent infection from 2100 to about 3000 m above sea level in the sampled seed potato fields indicates that BW disease is widespread in the potato agro-ecology and needs attention. It needs to be further proved if growing seed potato at higher altitudes than those that were sampled would reduce incidence of latent *Rs* in the crop planting material.

Conclusion and Recommendations

Results from this study have re-confirmed widespread presence of latent *Rs* infection in seed potato produced and distributed by farmer seed group

cooperatives in Ethiopia. The study revealed that the prevalence and distribution of the disease was not influenced by potato variety, district where the sample was collected, or the altitude at which the seed potato was produced except at two sites located at more than 3000 m above sea level. However, these sites were not a fair representative of this eco-ecology since only two samples were collected from each of the two district sites at this altitude. This study though relevant was not able to pin-point the possible sources of latent infection because it missed recording the source of the seed that was used to plant the sampled crops, the number years since the field site was last used for potato growing, or the frequency of seed replacement by the farmer. It is known that most of the seed potato crop is grown as quality declared seed that is visually inspected for quality assurance. This means most of the crops that were passed as good for seed generation were actually infected with *Rs* and such planting material will inherently spread the disease to more areas. The samples used in this study were purposively collected from farmer seed group cooperatives whose potato crop revealed no symptoms of bacterial wilt infection. The presented results therefore may be a low estimate of the actual BW infestation of more representative ware potato fields.

It appears that BW will continue to threaten potato production in Ethiopia unless there is deliberate effort to introduce and institutionalize routine seed potato indexing using sensitive and specific techniques such NCM-ELISA, or other more advanced alternatives such as real-time, field adapted LAMP assay, than the current practice of visual-based certification of both certified and quality declared seed, the cost of such testing notwithstanding. Additionally, efforts should be made to implement a BW clean-up and containment strategies before the few remaining BW-free areas are contaminated. This is possible based on existing knowledge and recent research in Ethiopia where race 3 of *Rs* is likely the most predominant biotype in this agro-ecology that can be easily eradicated from infected soils compared to other races.

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Author Contribution KS, GW, and LT contributed to study idea development, methodology, and investigation; LT and ES designed and executed sample collection; LT, ES, AM, MW, and KN executed the laboratory diagnosis; LT conducted data analysis and result interpretation and wrote the first draft. All other authors edited the subsequent versions of the manuscript.

Declarations

Conflict of Interest The authors declare no competing interests.

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