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Phytophthora infestans responses to stinging nettle extract, phosphoric acid and fungicides combination, in Kenya

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Research Paper

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ABSTRACT

Late blight is a significant disease of solanaceous crops worldwide. Knowledge on sensitivity of late blight pathogen (*Phytopthora infestans*) to metalaxyl and different fungicides is important for effective management of late blight. *In vitro* studies were carried out to evaluate the sensitivity of *P. infestans* to different levels of Metalaxyl Ridomil, Dithane M45, phosphate and stinging nettle. Sensitivity to metalaxyl and other different fungicides was determined by growing *P. infestans* isolates on 15% V8 medium amended with 0, 5 and 100 ppm metalaxyl and 20ppm of Ridomil, 25 ppm of Dithane M45, 20ppm of Phosphate and 1ml of stinging nettle extract in 1ml of water. The pathogen isolates were from the leaves collected from different potato growing areas of Kenya. Sensitivity was determined by measuring the colony diameter at 14 and 21 days, counting the numbers of spores and determining the weight of the mycelium.The result of the experiment indicated that there was intermediate resistance of *P. infestans* to metalaxyl in all the region as the growth was recorded as 57.8% in 5 ppm and 19.8 in 100 ppm in Tigoni station and 58.3 in 5 ppm and 19.7 in 100 ppm in Njambini while in Meru region the growth was 52.9 in 5 ppm and 25.9 in 100 ppm metalaxyl relative to the control. Njambini and Tigoni regions did not differ significantly in term of resistance. However both regions differed significantly with Meru region in terms of weight of mycelium and number of spores, Meru location having the highest number of spores and weight of mycelium compared to both Tigoni and Njambini locations. All the fungicides tested differed significantly in their effect on pathogen colony diameter, number of spores and weight of mycelium. The reduction of colony diameters of isolates collected from Tigoni of 41.7 and 80.3 in 5 and 100 ppm metalaxyl did not differ significantly with that from Njambini significantly of 42.2 mm and 80.2 mm in 5 and 100 ppm respectively. The pathogen was more sensitive to phosphoric acid and least sensitive to stinging nettle extract.

Key words: Plant extract; phosphoric acid; fungicide combinations; late blight; fungicide resistance.

INTRODUCTION

The management of late blight in Kenya remains a major production challenge because of the continuous potato cultivation which ensures abundant *P. infestans* inoculum for disease development throughout the year (Olanya *et al.,* 2001; Nyankanga *et al.,* 2007). It has been estimated that 30–60% of the crop is lost due to late blight annually in Kenya (Nyankanga et al. 2004). Metalaxyl and mefenoxam have been effective in controlling late blight previously, but metalaxyl-resistant populations of P. infestans have been reported in many parts of the world (Derie and Inglis 2001). Foliar blight and tuber blight have been frequently reported to occur and cause considerable losses in

the tropical highlands (Nyankanga *et al.,* 2007). Attempts to control late blight are almost entirely through the use of protectant or systemic fungicides with little regard to application strategy in addition to other disease management options (Nyankanga *et al.,* 2004, Nyankanga *et al.,* 2008; Ojiambo et al., 2001). Metalaxyl is very effective against sensitive strains and when applied early can suppress an epidemic (Fry *et al.,* 1979). The immediate effect on epidemic progress is achieved through suppression of lesion expansion (Bruck *et al.,* 1980) and possibly by prevention of latent infections. Effects that also contribute to suppression of established epidemics are reduced sporulation from lesions, and prevention of sporangium germination and subsequent infection (Bruck *et al.,* 1980). However, against resistant strains of *P. infestans*, metalaxyl alone has little or no detectable effect on epidemic development (Goodwin *et al.,* 1996). Until recently, populations of the late blight fungus, *P. infestans* world wide contained only the A1 mating type.

Considerable evidence suggests that about 20 years ago new populations of the fungus containing both A1 and A2 mating types is spreading around the world (Fry *et al.,* 1992). These isolates are rapidly replacing the original lineages in many regions (Fry *et al.,* 1993). They carry the potential to complete their sexual life cycle, which includes formation of oospores that enable the fungus to survive for long, and appear to have higher levels of virulence and high frequencies of resistance against the widely used fungicide metalaxyl (Deahl *et al.,* 1995; Goodwin *et al.,* 1996).

Except for one report of the occurrence of the A2 mating type in an Egyptian population (Shaw *et al.,* 1985), there are no definite indications of occurrences of new biotypes in Africa. However, the increased occurrence of devastating late blight epidemics, coupled with reduced fungicide sensitivity (Fry *et al.,* 1993) suggest the possible existence of new *P. infestans* populations in the continent. A premix of Ridomil (a.i. metalaxyl and Mancozeb) and Dithane M-45 (a.i. Mancozeb) have been widely used by potato growers for the control of late blight in the highland tropics (Ojiambo *et al.,* 2001; Nyankanga *et al.,* 2004). In other situations, systemic fungicides have been used for blight control in excess of four times per cropping cycle (Nyankanga *et al.,* 2004).

In Kenya, there are no evidences for the presence of new lineages or genotypes of the pathogen and the *P. infestans* population consists of US-1 genotype, A1 mating type (Vega-Sanchez *et al.,* 2000; Olanya *et al.,* 2001). However, isolates of *P. infestans* collected from potato fields in Kenya have shown a high level of metalaxyl insensitivity in areas where growers routinely and excessively use the systemic fungicide for blight control on potato and tomato hosts (Hohl, 2000, Ojiambo *et al.,* 2001).

Metalaxyl insensitivity has also been reported among some isolates in production regions with similar agroecological conditions and production techniques as those used in the Kenyan highlands (Mukalazi *et al*., 2001). Fungicidal compounds with systemic mode of actions as well as chemicals with protective properties have often been used by potato farmers for late blight control in various application sequences, rates or combinations. Strategies to reduce the potential destructive effects of late blight on potato production must be developed. To do this it would be helpful to acquire more knowledge on the resistance of this fungus to metalaxyl in Kenya. Also studies on sensitivity of this pathogen to different fungicide may result in more effective late blight management and contribute to better tuber yield. This study was conducted to test the occurrence of pathogen resistance to metalaxyl, and evaluating the sensitivity of late blight pathogen to Ridomil, Dithane M45, Phosphate and stinging nettle extract compared to the untreated check on the solid media.

MATERIALS AND METHODS

Collection of leaf samples

Potato leaf samples infected with late blight were collected from three major potato- growing areas in Kenya, Meru Central, Njabini in Nyandarua District and Tigoni in Kiambu District. The leaf samples were collected randomly from 10 farmers fields within a radius of one kilometer between the farms in each district apart from Tigoni research centre where the leaves were collected from the field experiment. In each farm 20 leaf samples each containing 10 leaflets with single late blight lesion were collected along a transect (Forbes et al., 1997). The leaflets were kept in iced box until taken to the laboratory where they were maintained at 5^0C for 1-2 days before isolation.

Isolation of the *P. infestans*

Sporulating lesions on leaf tissue were washed in fresh water and placed in water agar plates in humid chambers (inverted petridish with water agar) with the leafs abaxial side up. Then these boxes were incubated at 15-18^0 C for 1-2 days and when sporuration appeared. Small pieces of infected tissue from the sporurating border of the lesion were cut and placed under potato slices in empty petri dishes and washed potato were incubated at 15-18^0 C for 1 week until there was abundant sporuration on the upper side of potatoes slice. The fungus was then transferred on to selective V8 based media (V8 juice 100ml, $CaCO_3$ 1g, B-sistosterol 0.05g, Agar 15g) by touching the surface of the mycelia with a sterile needle and placing pieces of mycelia on the selective media surface.

Production of *P. infestans* Inoculum

Sporulating lesion on the leaf tissue from the field were

washed in sterile distilled water and placed in a humid chamber (inverted petri dish with water agar) with abaxial side up. The plates were then incubated at 15-18^0 C for one week when sporulation occurred.

Tubers from Tigoni potato variety which tubers are known to be susceptible to late blight were washed in sterile distilled water and then allowed to dry. The tubers were dowsed in alcohol and then flamed off. Using sterile forceps to hold the tubers, 0.5cm thick tuber slices were cut using sterile scalpel and placed in sterile plates. Small pieces of infected potato leaf tissues from the sporulating border of the lesion were cut and placed under the potato slices in empty plates. The Petri dishes were incubated at 15-18^{0}C at 90% RH for 1 week until abundant sporulating occurred on the upper surface of potato slices, then the *P. infestans* was sub cultured in a V8 based media to produce pure cultures which were used in subsequent experiments. Isolates were assessed for growth on V8 agar (V8 juice 100 ml, $CaCo_3$ 1g, B-sistosterol 0.05g, Agar 15g) amended with different levels of metalaxyl and then Phosphite, stinging nettle extract, Dithane M45 and Ridomil after the media was autoclaved and cooled to between 35 and 40 ^{O}C.

In vitro Screening for sensitivity of *P. infestans* isolates to metalaxyl, phosphoric acid and stinging nettle extract in solid media

In vitro screening was done on V8 selective media whose ingredients included V8 juice 100ml, $CaCO_3$ 1g, B-sistosterol 0.05g, and Agar 15 g. V8 Juice and distilled water were mixed to bring up to 1litre, $CaCO_3$ and glycerol were added and mixed well. Then agar was added and the mixture was autoclaved at a temperature of 70^{0}C and 15 psi for 20 minutes. After cooling, antibiotics which included penicillin, tetramycin and streptomycin were added each at the rate of 20 ppm in 100 ml of media. After cooling the media to a temperature of 40 ^{O}C in a laminar flow hood and then the media was amended with two different concentration of Metalaxyl (5 pm and100 ppm) and different fungicides using one concentration of each fungicide (20 ppm of Ridomil, 25 ppm of Dithane M45, 20 ppm of phosphite and 1ml of stinging nettle extract in 1ml of water) at the same temperature of 40 ^{O}C. Control consisted of the selective media without any fungicide. Each treatment was replicated three times in a complete randomized design.

Fifteen ml of medium which had been amended with different fungicides was dispensed into 8.5 cm –diameter plastic petri dishes. A 0.5 mm-diameter agar disk was taken using a sterilized cork borer from an actively growing colony of *P. infestans* grown on V8 based media and was placed with the fungal side downward in the centre of each plate. After 14 and 21 days of incubation, radial growth was determined by measuring colony diameter at two points on isolates using a divider which was then fitted into a ruler and the average measurement of all the isolates taken. The spores from each isolates were harvested and counted using a haemocytometer and average spores in each treatment was recorded. After 21 days the mycelium of each isolate from each treatment was harvested. During harvesting 10 ml of distilled water was added in each petri dish and then the mycelium was scrapped from the V8 based media using a sterile slide and sieved through cheese cloth and then dried in an oven at controlled temperature of 70 ^{O}C to obtain a dry weight.

Based on the results of radial growth, sporulation and mycelial dry weight, isolates were classified as susceptible, moderately resistant, or resistant depending on sensitivity to metalaxyl following the procedures by the International Potato Center (Forbes, 1997). Susceptible isolates were those isolates with less than 40% growth relative to control on both concentrations of metalaxyl (5 and 100 ppm) while intermediate were those with growth greater than 40% at 5 ppm but less than 40% at 100 ppm of metalaxyl and resistant growth greater than 40% on both concentrations of metalaxy.

Data analysis

All data was subjected to analysis of variance (ANOVA) using the PROC ANOVA procedure of Genstat (Lawes Agricultural Trust Rothamsted Experimental station 2006,Version 9) and difference among the treatment means were compared using the Fishers protected LSD test at 5% probability level.

RESULTS

Sensitivity of *P. infestans* isolates to different concentration of metalaxyl

Differences in mean growth rates of colony diameter for the different metalaxyl concentrations were highly significant *(P* = .05) .The addition of metalaxyl to culture medium significantly reduced growth rate of the *P. infestans* isolate collected from the two regions and Tigoni Research Station (Table 1). The reduction of colony diameter of isolates collected from Tigoni of 41.7 mm and 80.3 mm in 5 and 100 ppm did not differ significantly with that from Tigoni of 42.2 mm and 80.2 mm in 5 and 100 ppm respectively while this differed significantly from colony diameter of those isolates collected from Marimba of 41.7 mm and 74.1 mm in 5 and 100 ppm metalaxyl respectively (Table 1) For all the regions. *P. infestans* isolates was not considered resistant but intermediate in resistance to metalaxyl.The result indicated that all the tested rates differed significantly amongest themselves and the control.

There was a significant difference in the weight of mycelium and numbers of spores produced after amending the media with different concentrations of metalaxyl. However there was no significant difference between Tigoni and Njambini location while the two locations differed signifiantly with

Table 1. Mean colony diameters (mm) of *P. infestans* isolates collected from different region measured at 14 and 21 days after incubation at different metalaxyl concentrations

Location	Metalaxyl concentration (ppm)	14 days	21 days	Mean	%,colony
Tigoni	0	32.6a	70.6a	52.6	0
	5	20.3b	40.5b	30.4	42.2
	100	10.3c	10.5c	10.4	80.2
	LSD(*P=. 05*) Treatment	4.8	6.2		
	CV (%)	19.9	32.8		
Niambini	0	29.2a	71.5a	50.3	0
	5	19.1b	39.6b	29.3	41.7
	100	9.3c	10.5c	9.9	80.3
	LSD(*P=. 05*) Treatment	4.8	5.2		
	CV (%)	24.6	27.5		
Marimba	0	55.5a	76.5a	66	0
	5	23.3b	46.5b	34.9	47.1
	100	14.3c	19.9c	17.1	74.1
	LSD(*P=. 05*) Treatment	5.9	6.2		
	CV (%)	31.8	24.9		

*Value followed by same letter along the column in the same location are not significantly different while those followed by different letter are significantly different.

Table 2. Mean mycelia weight (g) and number of spores of *P. infestans* isolates collected from different regions measured at 14 and 21 days after incubation at different levels of metalaxyl concentrations.

Locations	Metalaxyl concentration ppm	Mean mycelia weight (g)	Number of spores
Tigoni	0	0.96a	116a
	5	0.85a	96a
	100	0.56b	34.6b
	LSD(P=.05) Treatment	0.15	24
	CV (%)	29.3	27.9
Njambini	0	0.93a	126a
	5	0.73a	92.4b
	100	0.4b	56.7c
	LSD(P=.05) Treatment	0.16	21.7
	CV (%)	35.7	16.8
Marimba (Meru)	0	1.12a	178a
	5	0.91b	116.4b
	100	0.67c	76.5b
	LSD(*P=05*) Treatment	0.13	46.6
	CV (%)	23.7	29.4

*Mean followed by same letter along the column in the same location are not significantly different while those followed by different letter are significantly different.

meru location (Table 2). Metalaxyl concentration of 100 ppm resulted in the lowest number of spores of 34.6, 21.7 and 76.5 in Tigoni, Njambini and Marimba respectively, as well as the biomass of 0.56 g, 0.4g and 0.67g in Tigoni, Njambini and Marimba respectively.

Media amended with metalaxyl at 5 ppm did not differ significantly in term of number of spores with control plates in Tigoni location. However the two differed significantly with100 ppm in the same location (Table 2) while in Njambini the two tested rates differed significantly between themselves and the control. In Meru location the rates of 5 ppm differed significantly with the control, however this did not differ significantly with the higher concentration of 100 ppm (Table 2).

Sensitivity of *P. infestans* isolates to stinging nettle extract, phosphoric acid and different fungicides

There was a significant difference in late blight pathogen sensitivity due to different fungicides in all regions as shown by mean colony diameters measured at 14 and 21 days, among the different fungicide tested (Table 3). Phosphoric resulted in the least diameter size and this differed significantly with other tested fungicide. There was no significant difference in colony diameter in plates which were amended with either stinging nettle or Dithane M45 after seven days, however after 14 days the two fungicides differed significantly with Dithane M45 having less colony diameter compared to stinging nettle extract. The highest

Table 3. Mean colony diameter (mm) of *P. infestans* isolates Collected from different locations measured at 14 and 21 days after incubation from stinging nettle extract, phosphoric acid and different fungicides.

Location	Treatments	14 Days	21	Mean diameter	Percentage reduction
Tigoni	(Ridomil)Metalaxyl + Mancozeb	9.6a	12.3a	10.5	80.5
	Phosphoric acid (Phosphite)	4.4b	6.8b	5.7	89.4
	Dithane M45 (Mancozeb)	19.6c	39.1c	29.4	45.3
	Stinging nettle extact	20.3d	51.9d	36.6	31.8
	Control	53.7e	74.7e	53.7	0
	LSD(*P* = .05) treatment	5.1	5.2		
	CV (%)		29.6		
Njambini	(Ridomil)Metalaxyl + Mancozeb	10.3a	12.5a	11.4	79.4
	Phosphoric acid (Phosphite)	4.9b	7.7a	6.3	88.6
	Dithane M45 (Mancozeb)	20.3c	39.1b	29.7	46.2
	Stinging nettle extract	20.3c	48.9c	34.6	37.3
	Control	42.6d	67.7d	55.2	0
	LSD(*P* = .05) treatment	4.6	4.8		
	CV (%)		19.3		
Marimba	Ridomil)Metalaxyl + Mancozeb	12.9a	18.3a	15.6	76.1
	Phosphoric acid (Phosphite)	5.4b	7.9b	6.7	89.7
	Dithane M45 (Mancozeb)	25.3c	45.4c	35.3	45.9
	Stinging nettle extact	23.8c	59.7d	41.7	36.1
	Control	55.5d	75.7e	65.3	0
	LSD(*P* = .05) treatment	5.8	6.2		
	CV (%)		30.1		

*Values followed by same letter along the column in the same location are not significantly different while those followed by different letter are significantly different.

colony diameter was recorded in untreated plates in all the readings which differed significantly with all the fungicide tested (Table 3).

Different fungicides resulted to a significant difference (*P=.05)* on both the weight of mycelium and the number of spores. Phosphate resulted in the least weight of mycelium and the numbers of spores and this was observed in all the regions and it did not differ significantly with the Ridomil in Njambini and Tigoni though the two differed significantly (Table 3). Stinging nettle extract and Dithane M45 differed significantly with other pathogens in all the regions but the two did not differ significantly apart from Tigoni region.

The result indicated that control plates resulted with the highest number of spores which differed significantly with other fungicides (*P=.05)*. Among the fungicides tested Dithane M45 produced the highest number of spores in all the location while Phosphate resulted with the least number of spores (Table 4). Spore production was observed to differ significantly across the different region with isolates from Meru region producing the highest number of spores.

DISCUSSION

The result of this study revealed the intermediate metalaxyl resistance by the *P. infestans* isolate isolated from leaf samples collected from Njambini, Meru and Tigoni Research station. Mean colony diameter, number of spores and dry mycelia weight were reduced in all the isolates by different treatment compared to control. All the measured parameter were significantly reduced using phosphoric acids, although other fungicide also proved effective in reducing the number of spores, colony diameter and mycelia weight.These results are in agreement with Jonson *et al* (1994) who found that phosphoric acid reduced lesion development and sporuration when applied as a foliar spray in the field than other fungicides. In this country there is only moderate Ridomil (56% Metalaxyl and 7.5% Mancozeb) application to control late blight, largely due to economic restriction. Resistance to metalaxyl and other related compounds has developed rapidly in populations of *P. infestans* after the use of metalaxyl (Deahl *et al*., 1995; Dunstan et al.,1990) this should be arrested by using of alternative compound to prevent over use of metalaxyl as indicated by the finding of this study.

A greater frequency of resistance to metalaxyl occurs among the new biotypes having both A1 and A2 mating types (Fry *et al.,* 1993; Erwin and Ribeiro, 1996) This is in agreement with result found in this study. It was observed that Low metalaxyl dosage also tend to result in high frequencies of metalaxylresistant *P. infestans* isolates as indicated by (Dolan and Coffey 1988).

The presence of intermediate level of metalaxyl-resistant *P. infestans* in these locations is not necessarily a strong indicator of the presence of the A2 mating type as there is no

Table 4. Mean mycelia weight (g) and number of spores of *P. infestans* isolates collected from different region measured at 14 and 21 days after incubation from stinging nettle extract, phosphoric acid and different fungicides.

Location	Treatments	Mean mycelia weight	Mean number of spores
Tigoni	Ridomil (Metalaxyl + Mancozeb)	0.41a	12a
	Phosphoric acid (Phosphite)	0.35a	8a
	Dithane M45 (Mancozeb)	0.96b	52b
	Stinging nettle extact	0.73c	40c
	Control	1.1d	124d
	LSD(*P* = .05)	0.12	5.6
	Treatment		
	CV (%)	27.9	17.2
Njambini	Ridomil (Metalaxyl + Mancozeb)	0.38a	22a
	Phosphoric acid (Phosphite)	0.34a	9b
	Dithane M45 (Mancozeb)	0.74bc	62c
	Stinging nettle extact	0.69b	48d
	Control	0.85c	142e
	Lsd (*P* = .05) Treat	0.15	11.6
	Cv %	37.7	26.5
Marimba	Ridomil (Metalaxyl + Mancozeb)	0.5a	46a
	Phosphoric acid (Phosphite)	0.37b	12.4b
	Dithane M45 (Mancozeb)	0.85cd	69c
	Stinging nettle extact	0.78c	62c
	Control	0.97d	166d
	Lsd (*P* = .05Treat	0.12	19.8
	Cv %	27.2	11.4

*Mean followed by same letter along the column in the same location are not significantly different while those followed by different letter are significantly different.

genetic correlation between resistance and mating type and this agree with the finding that resistance to phenylamide became established in A1 populations before the appearance of the A2 type (Dunstan et al.,1990).

Thus, the metalaxyl-intermediate resistant isolates in Meru, Njambini and Tigoni locations may still be of the A1 mating type. However, the level of intermediate resistance to metalaxyl suggests that metalaxyl use should be planned carefully, as it could increase management costs (Bruck et al.,1981). Since Ridomil contains both Mancozeb and Metalaxyl, it is recommended to use Ridomil sparingly, and partially substitute it with other alternative fungicides. The results of these experiments provide information essential for the development of effective disease control strategies.The presence of *P. infestans* isolate with intermediate resistance requires that integrated management rather than depending on one fungicide on late blight management strategies be adopted. This is supported by the fact that the pathogen was able to grow for over 50% in media amended with 5 ppm metalaxyl and this also suggests that use of under dose may result in pathogen developing resistance to metalaxyl. None of the fungicide tested was effective in total control of the pathogen however result indicated that phosphoric acid and stinging nettle might have some useful effect in controlling late blight pathogen. Certainly, to minimize further development of fungicide resistant strains, application of fungicides should be combined with the use of host resistance.

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