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Identification of levels of resistance to cassava root rot disease (*Botryodiplodia theobromae*) in African landraces and improved germplasm using *in vitro* inoculation method

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Summary

Cassava root rot disease is an increasing problem in Africa where yield losses of about 80% have been recorded. We evaluated 290 African landraces and 306 improved genotypes from the germplasm collections of the International Institute of Tropical Agriculture (IITA), for sources of resistance using root slice laboratory assay. Disease severity was assessed quantitatively by direct percentage estimation (PS) and by use of a rating scale (RS). Both methods of assessment were compared for identification of variability in the germplasm, and genotypes were classified into response groups using an enlarged rank-sum method that combined the PS and RS assessments. The two scoring methods revealed continuous variation ($P < 0.001$) for resistance in the sets of germplasm. Disease assessments based on PS and RS were highly correlated in both the improved germplasm ($r = 0.75$) and the landraces ($r = 0.72$). Based on PS assessment, 50 improved genotypes (16.3%) and 53 landraces (18.3%) showed significantly lower disease scores than the resistant control. The rank-sum method separated each set of collections into highly resistant, resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible groups. Fifty-nine improved genotypes (16.4%) and 61 African landraces (16.9%) were identified as either highly resistant or resistant. Generally, these genotypes exhibited resistance by limiting the growth of the pathogen (reduced amount of invaded surface area). This type of rate-reducing resistance is highly heritable and a quantitative trait which can be harnessed in breeding. Genotypes subsets were identified for further studies into the genetic basis of resistance to root rot disease.

Introduction

Root rot disease of cassava is an emerging problem in many African countries, particularly in the sub-Saharan region where cassava accounts for approximately one-third of the total staple food production (FAO, 1993). This disease is caused by different root rot fungi, and has been reported to cause yield losses of up to 80% in farmers' fields (Msikita et al., 2005). Because the rot pathogens affect the underground tuberous roots of cassava, the magnitude of the damage cannot be quantified until harvest. Therefore, the nature and effects of the disease are poorly understood by the farmers and the disease remains a pressing concern in Africa.

Recent field surveys conducted in different countries in sub Saharan Africa identified *Botryodiplodia theobromae*, *Nattrassia mangiferae* and *Fusarium* spp. as the most important pathogens of this disease in the region (Msikita et al., 1998, 2005; Onyeka et al., 2004). In a comprehensive survey across cassava ecological zones in Nigeria, *B. theobromae* was observed in more than 70% of 115 farmers' fields (Onyeka, 2002). Pathogenicity and virulence studies identified *B. theobromae* as the most virulent pathogen isolate; cassava varieties that were resistant to *B. theobromae* were also resistant to other pathogen isolates.

Integrated pest management based on the planting of resistant cultivars is presently the most economical

and reliable approach to controlling cassava root rot disease. Genetic improvement and search for varieties that are resistant to the various pests and diseases of cassava have formed the main focus of cassava research in last three decades (Hahn et al., 1989; Ceballos et al., 2004). However, due to the difficulties and the high cost in collecting and maintaining cassava germplasm, the genetic variability within cassava has not been fully exploited. There have been efforts to characterise some of the African landraces and breeders' lines for resistance to other diseases of cassava (Fokunang et al., 2000), but the distribution of resistance to root rot disease in these collections remains unknown. Variations in the response of different cassava genotypes to root rot disease have been demonstrated in field studies (Onyeka et al., 2005a). However, the long growth cycle for cassava to develop storage roots for assessment and the non-uniform distribution of inoculum in the soil make field screening very difficult. Consequently, *in vitro* methods for the assessment of cassava root rot have been developed (Barragan & Alvarez, 1998; Onyeka et al., 2005b). These methods involve the inoculation of different plant parts, such as whole roots, root slices, stem cuttings and young rootlets, and disease intensity is estimated by rating the inoculated materials on a disease rating scale, or by actual calculation of the percentage of the diseased area.

The objectives of this study are to evaluate relative resistance of genotypes in the African landraces and the improved cassava germplasm collections of IITA, to compare disease assessment based on a rating scale and assessment based on estimation of percentage diseased area, and to identify study subsets and new sources of resistance to root rot disease using root slice inoculation assay.

Materials and methods

Plant and pathogen materials

The plant material used in this study consisted of 290 African landraces and 306 improved genotypes (breeders' lines at advance selection stage) from the cassava germplasm collection of the International Institute of Tropical Agriculture (IITA), Nigeria. The evaluation was carried out twice in 1998/1999 and 1999/2000 planting seasons. The genotypes were planted each season in single rows in the field at a spacing of 1 m × 1 m. Tuberous roots were harvested from the plants at 12 months after planting and used in the laboratory assay. The screening was carried out in independent batches with internal checks which were included in all the batches. Improved genotype 30572 was used as resistant control and TME-1 (a popular African landrace) as susceptible control. Both genotypes have been widely used as resistant and susceptible references to other pest and diseases of cassava.

Isolates of *B. theobromae* were obtained from root rot samples. To isolate the pathogen from diseased plants, root samples showing typical root rot symptoms were cut into small segments (approx. 1 cm), surface sterilized for 3 min in 10% sodium hypochlorite solution, and rinsed in five changes of sterile distilled water. The root segments were dried on sterilized filter paper, placed on acidified potato dextrose agar (PDA) and incubated at 26 °C for 5–7 days. A single isolate of *B. theobromae* recovered during a survey of farmers' field in 1998 was used in the screening. This isolate was maintained as conidia suspension in sterile distilled water at 4 °C.

Inoculation and disease assessment

The inoculation procedure followed the root slice laboratory method previously described (Onyeka et al., 2005b). Medium sized (4–6 cm diameter) tuberous roots of the 12-month-old plants were washed, surface sterilized in 1% sodium hypochlorite, rinsed in distilled water, and dried under a laminar flow hood. Root slices, 10 mm thick, were cut and each was placed in a Pyrex glass Petri dish. Inoculum discs (2 mm in diameter) were cut with a sterile cork borer from the margins of 7-day-old *B. theobromae* cultures grown at 26 °C on PDA. An inoculum disc was placed mycelia-side down on a root slice surface and the Petri dish cover was replaced. Four slices were inoculated to provide replication for each genotype. The Petri dishes were placed in a Gallenkamp incubator maintained at 26–27 °C for four days.

At the end of the incubation period, each root slice was evaluated quantitatively by taking the radial spread of the pathogen on the slice surface as the average of two diametric measurements taken in perpendicular directions, and calculating the percentage surface area of the root slice invaded by the pathogen. Also each root slice was evaluated by visual observation. The extent of mycelia formation on the slice was rated on a scale of 1–5, where 1 = no mycelia formation and 5 = densely packed mycelia covering the surface of the slice.

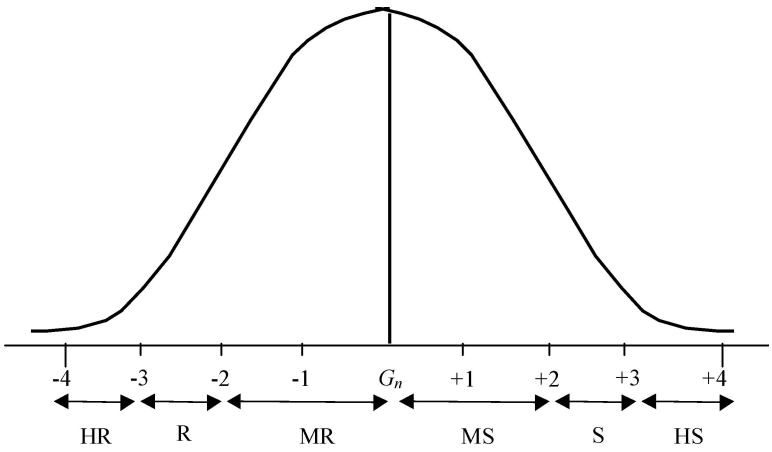

Figure 1. Mean distribution curve for evaluating resistance status. G_n: rank-sums grand mean, HR: highly resistant, R: resistant, MR: moderately resistant, MS: moderately susceptible, S: susceptible, HS: highly susceptible.

Statistical analysis

To compensate for variation between the batches, a correction of the scores of each genotype was made based on the reaction of the internal checks as follows: $DS_{cor} = DS - (CHK_n - CHK_N)$, where DS represents either the percentage scores or rating scores of the genotype, CHK_n the average of the checks in the particular batch, and CHK_N the total mean of the checks in all the batches.

The data for the percentage scores (hereafter referred to as PS) and the rating scores (hereafter referred to as RS) were subjected to one-way analysis of variance using the NPAR1WAY procedure of SAS programme version 8 (SAS, 2000) with the Kruskal–Wallis and Van der Waerden options for multiple comparison test. Relative resistance of the genotypes in comparison to the resistant control was determined, based on the standard error of the mean. Relationship between the PS and RS was tested by simple correlation coefficient.

Genotype classification

Genotypes were classified into different response groups using a modified rank-sum method (Ariyo et al., 2002) based on the means of the PS and the RS for each genotype across the two assessment periods. To calculate the rank-sum, the mean PS and RS for the genotypes were assigned ranks from the smallest to the largest using RANK procedure of the SAS programme (SAS, 2000) with option average for handling ties. The sum of the ranks (X_n) was computed for each of the genotype and compared to the grand mean of the rank-sums across all the genotypes (G_n). Deviation of each genotype from the grand mean was calculated as $[(X_n - G_n)/\text{standard deviation}] \times 2$. Deviations to the right (positive) of the grand mean on the mean distribution curve were rated susceptible while deviations to the left (negative) of the grand mean were rated resistant (Figure 1).

Results

Variability in root rot resistance among genotypes and correlation of the two disease assessment methods

The data from each set of germplasm (improved genotypes and African landraces) were analysed separately. The results showed high continuous variation ($P = 0.0001$) in the resistance response of the genotypes in each set of germplasm based on both the PS and the RS assessment methods. The Kruskal–Wallis statistics and the Van der Wearden's statistics were highly significant, indicating that there are different genotype groups with varying resistance within each set of germplasm (Table 1).

Based on the PS method, 50 improved genotypes (16.34%) had significantly lower disease scores than the resistant control (30572) with a score of 53.52%. The mean score for the 306 genotypes was 65.78% with values ranging from 22% to 100%. The susceptible control (TME-1) had a score of 68.89%. Among the African landraces, the mean disease score for the

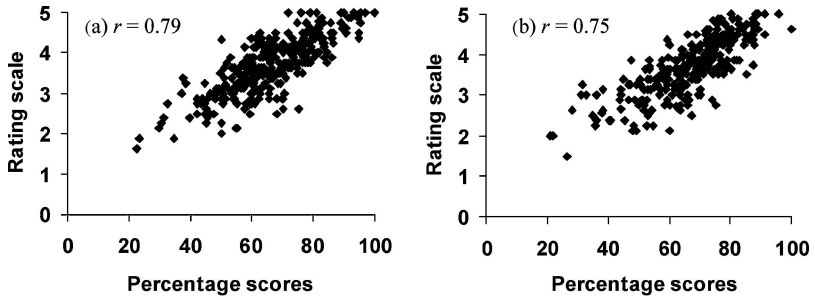

Figure 2. Correlation of percentage area and rating scale scoring methods for resistance to cassava root rot disease assessed by root slice inoculation assay in improved germplasm (a) and African landraces (b).

Table 1. Analysis of variance for resistance to root rot disease in 306 improved genotypes and 290 African landraces based on percentage scores (PS) and rating scores (RS) in root slice screening method

	Improved germplasm		African landraces	
	PS	RS	PS	RS
Between group MS	1587.09	3.94	1566.43	3.97
Within group MS	313.48	0.81	403.58	0.95
F-ratio	5.07	4.86	3.89	4.03
$P >$ value	0.0001	0.0001	0.0001	0.0001
Kruskal Wallis test	981.39**	989.89**	824.05**	872.86**
Van der Waerden test	1041.5**	998.66**	883.21**	880.86**

**Kruskal–Wallis and Van der Waerden statistics. ($P \leq 0.001$).

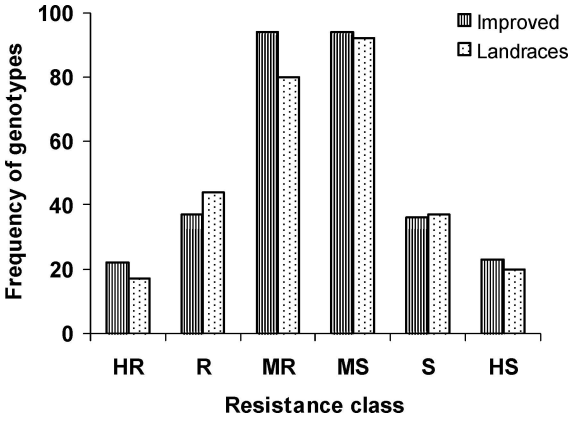

Figure 3. Distribution of 306 improved genotypes and 290 African landraces of cassava for resistance to root rot disease (*B. theobromae*) determined by rank-sum classification method.

290 genotypes was 66.71%; the resistant check had a score of 55.21% and the susceptible control had a score of 71.79%. A total of 53 genotypes (18.27%) had disease scores lower than 30572 and 231 genotypes (79.66%) showed disease scores higher than 30572.

Assessment based on RS, showed that 91 improved genotypes performed better than the resistant control; and 64 African landraces were rated better than the resistant control. For each set of germplasm, the results obtained from PS and the results obtained from RS were highly correlated, with correlation coefficient (r) = 0.79 for the improved germplasm and $r = 0.75$ for the African landraces (Figure 2).

Classification of genotypes based on rank-sum method

Classification of the 306 improved genotypes based on rank-sum method showed that 22 genotypes (6.11%) were highly resistant, 37 (10.28%) were resistant and 94 (26.11%) were moderately resistant. Twenty-three genotypes (6.39%) were highly susceptible, 36 (10%) were susceptible and 94 (26.11%) were moderately susceptible.

Among the landraces, 17 genotypes (4.72%) were highly resistant, 44 (12.22%) were resistant and 80 (22.22%) were moderately resistant. Ninety-two genotypes (25.56%) were moderately susceptible, 37 (10.28%) were susceptible and 20 (5.56%) were highly susceptible (Figure 3). The reference genotype for resistance (30572) was found to be moderately resistant.

The rank-sum method identified a total of 59 improved genotypes and 61 African landraces (highly resistant and resistant groups) that are more resistant than the resistance control. The highly resistant and highly susceptible genotypes from each set of germplasm are shown in Tables 2 and 3.

Table 2. Standardized average root rot rating of the highly resistant and the highly susceptible improved cassava genotypes identified by rank-sum classification method

	Percentage score		Rating score		Genotype ranking		
Genotype	PS	*a*	RS	*b*	*c*	*d*	Class
I87/01004	22.50	1.00	1.63	1.00	2.00	−3.66	HR
I88/00188	23.61	2.00	1.88	2.50	4.50	−3.63	HR
I84/00460	29.86	3.00	2.13	6.00	9.00	−3.57	HR
I92/0211	34.55	7.00	1.88	2.50	9.50	−3.57	HR
I81/01610	30.73	4.00	2.25	9.00	13.00	−3.53	HR
O82/00438	31.39	5.00	2.38	12.00	17.00	−3.48	HR
I88/02561	39.93	11.00	2.38	12.00	23.00	−3.41	HR
I91/00417	42.26	14.00	2.50	16.50	30.50	−3.32	HR
I4(2)1425PUB	45.21	22.00	2.25	9.00	31.00	−3.31	HR
I85/01887	45.49	23.00	2.38	12.00	35.00	−3.26	HR
I84537	32.81	6.00	2.75	29.50	35.50	−3.26	HR
I90853	44.76	19.00	2.50	16.50	35.50	−3.26	HR
O87/00395	42.19	12.00	2.75	29.50	41.50	−3.18	HR
O88/00417	45.07	20.00	2.63	22.00	42.00	−3.18	HR
I82/01438	46.63	26.00	2.50	16.50	42.50	−3.17	HR
I89/01327	50.24	38.50	2.00	4.00	42.50	−3.17	HR
O87/00613	45.14	21.00	2.63	22.00	43.00	−3.17	HR
I88/02343	47.57	27.00	2.50	16.50	43.50	−3.16	HR
I91/02312	43.40	15.00	2.75	29.50	44.50	−3.15	HR
I81983	44.72	18.00	2.75	29.50	47.50	−3.11	HR
O82/00333	50.35	41.00	2.25	9.00	50.00	−3.08	HR
O85/00119	42.22	13.00	2.88	42.00	55.00	−3.02	HR
O87/00762	95.00	301.00	4.38	256.50	557.50	3.00	HS
I88/00108	86.42	288.00	4.50	271.50	559.50	3.03	HS
M83/00001	82.04	271.00	4.79	291.00	562.00	3.06	HS
I88/02614	83.40	278.00	4.75	287.50	565.50	3.10	HS
O85/00034	90.97	295.00	4.50	271.50	566.50	3.11	HS
I87/00503	81.08	266.00	5.00	301.00	567.00	3.12	HS
I88/00367	82.81	275.00	4.88	293.50	568.50	3.14	HS
O87/00375	94.83	300.00	4.50	271.50	571.50	3.17	HS
I30001	85.72	285.00	4.75	287.50	572.50	3.18	HS
I90/00350	89.69	292.00	4.63	282.00	574.00	3.20	HS
I91/01216	90.63	294.00	4.63	282.00	576.00	3.23	HS
I88/00159	89.06	290.50	4.75	287.50	578.00	3.25	HS
I89/02778	91.56	296.00	4.88	293.50	589.50	3.39	HS
M86/00083	92.47	297.00	4.88	293.50	590.50	3.40	HS
O86/00603	95.31	303.00	4.75	287.50	590.50	3.40	HS
O87/00611	89.06	290.50	5.00	301.00	591.50	3.41	HS
I89/01017	94.72	299.00	4.88	293.50	592.50	3.42	HS
I80/00086	90.00	293.00	5.00	301.00	594.00	3.44	HS
M82/00126	93.13	298.00	5.00	301.00	599.00	3.50	HS
M6298	95.31	303.00	5.00	301.00	604.00	3.56	HS
O87/00183	95.31	303.00	5.00	301.00	604.00	3.56	HS
O71762	96.88	305.00	5.00	301.00	606.00	3.59	HS
O87/00412	100.00	306.00	5.00	301.00	607.00	3.60	HS
30572	53.52	53	3.38	107	160	−1.54	MR
TME-1	68.89	180	4.11	210	390	0.76	MS
Mean	65.78		3.68		307†		

†Grand mean of the rank-sums (G); PS: Percentage score. RS: Rating scale score; *a*: genotype ranking on the basis of the PS; *b*: genotype ranking on the basis of RS; c = Rank-sum ($a + b$) for each genotype. d: deviation from the grand mean (G) of the rank-sums [$(d = (c - G)/\text{standard deviation}) \times 2$]. HR: highly resistant; HS: highly susceptible; MR: moderately resistant and MS: moderately susceptible.

Table 3. Standardized average root rot rating of the highly resistant and the highly susceptible African landraces identified by rank-sum classification method

	Percentage score		Rating score		Genotype ranking		
Genotype	PS	*a*	RS	*b*	*c*	*d*	Class
TME-139	20.84	1.00	2.00	2.50	3.50	−3.62	HR
TME-163	26.67	3.00	1.50	1.00	4.00	−3.61	HR
TME-514	21.77	2.00	2.00	2.50	4.50	−3.61	HR
TME-166	35.80	9.00	2.25	8.50	17.50	−3.44	HR
TME-136	36.11	10.00	2.38	12.50	22.50	−3.38	HR
TME-146	34.93	8.00	2.50	16.00	24.00	−3.36	HR
TME-119	28.02	4.00	2.63	22.00	26.00	−3.34	HR
TME-447	40.45	15.00	2.38	12.50	27.50	−3.32	HR
TME-3	40.84	16.00	2.38	12.50	28.50	−3.30	HR
TME-62	37.94	12.00	2.57	18.50	30.50	−3.28	HR
TME-502	47.74	27.00	2.13	5.00	32.00	−3.26	HR
TME-179	45.42	22.00	2.38	12.50	34.50	−3.23	HR
TME-262	49.13	30.00	2.13	5.00	35.00	−3.22	HR
TME-92	38.19	13.00	2.63	22.00	35.00	−3.22	HR
TME-423	48.34	28.00	2.25	8.50	36.50	−3.20	HR
TME-168	44.29	20.00	2.67	25.00	45.00	−3.10	HR
TME-424	52.43	44.00	2.25	8.50	52.50	−3.00	HR
TME-591	82.92	264.00	4.63	265.50	529.50	3.00	HS
TME-485	84.20	266.00	4.63	265.50	531.50	3.03	HS
TME-49	80.52	255.00	4.88	281.50	536.50	3.09	HS
TME-71	91.25	286.50	4.50	250.00	536.50	3.09	HS
TME-38	82.74	263.00	4.75	274.00	537.00	3.10	HS
TME-95	80.14	253.00	5.00	287.50	540.50	3.14	HS
TME-443	82.47	261.00	4.88	281.50	542.50	3.17	HS
TME-590	85.44	271.00	4.75	274.00	545.00	3.20	HS
TME-118	88.02	281.00	4.63	265.50	546.50	3.22	HS
TME-412	86.88	275.00	4.75	274.00	549.00	3.25	HS
TME-564	89.20	285.00	4.63	265.50	550.50	3.27	HS
TME-162	100.00	290.00	4.63	265.50	555.50	3.33	HS
TME-198	88.26	283.00	4.75	274.00	557.00	3.35	HS
TME-539	87.19	276.50	4.88	281.50	558.00	3.36	HS
TME-414	87.60	280.00	4.88	281.50	561.50	3.40	HS
TME-241	88.13	282.00	5.00	287.50	569.50	3.51	HS
TME-394	89.06	284.00	5.00	287.50	571.50	3.53	HS
TME-69	91.25	286.50	5.00	287.50	574.00	3.56	HS
TME-451	95.66	288.00	5.00	287.50	575.50	3.58	HS
TME-75	95.76	289.00	5.00	287.50	576.50	3.59	HS
30572	55.21	55	3.20	65	120	−1.77	MR
TME-1	71.79	171	4.60	260	431	1.34	MS
Mean	66.71		3.71		291[†]		

[†]Grand mean of the rank-sums (G); PS: Percentage score. RS: Rating scale score; *a*: genotype ranking on the basis of the PS; *b*: genotype ranking on the basis of RS; *c* = Rank-sum (a + b) for each genotype. *d*: deviation from the grand mean (G) of the rank-sums [$(d = (c - G)/\text{standarddeviation}) \times 2$]. HR: highly resistant; HS: highly susceptible; MR: moderately resistant and MS: moderately susceptible.

Discussion

Continuous and intense evaluation of cassava germplasm for disease resistance is one of the basic requirements for effective and sustained implementation of integrated disease management programme (Fokunang et al., 2000). Identification of disease resistance depends greatly on adequate assessment and

disease evaluation methods. Because cassava root rot pathogens affect mostly the underground portion of the plant, field assessment necessitates harvesting the plants to quantify disease severity. This method is very rigorous and time consuming, and it is subject to variation because of the non-uniform distribution of pathogen inoculum in the soil. *In-vitro* screening of cassava varieties is required to help establish the resistant or susceptibility status of the different varieties (Onyeka et al., 2005b).

Using the root slice *in-vitro* assay, both the PS and the RS used in this study identified continuous variation for resistance in the sets of germplasm. Assessment of disease severity based on visual rating has been described for several plant diseases (Campbell & Madden, 1990; Hau & Kranz, 1990). It is relatively easy to use compared to quantitative procedures that are normally too labour-intensive for large-scale evaluation. Forbes and Korva (1994) concluded that direct percentage estimation is more accurate than assessment based on a disease rating scale because visual assessments are often subjective, leading to variation between different users or different studies. The results of this study, however, showed a good correlation between percentage estimation and the assessment based on a rating scale. Also both methods produced consistent results across the 2 years of evaluation. The relatively simple rating scale is faster than the direct percentage estimation method. However, due to the quantitative nature of the resistance observed in this study, the use of a percentage estimation which gives more detailed evaluation of continuous variation will be more effective in studying the genetic nature of the resistance.

In plant breeding, different classification techniques can be used to group accessions and genotypes into homogenous groups for various traits and environment. However the best classification method should be able to produce compact and well separated groups without compromising the desired objective (Crossa & Franco, 2004). The desired objective in assessment of germplasm for disease response is to differentiate genotypes into different levels of resistance or susceptibility. The separation of genotypes based on their disease reaction relative to a known resistant has been widely used (Happstadius et al., 2003). This approach for evaluating disease resistance is limited by requiring prior knowledge and the availability of the resistant controls. It is, therefore, not appropriate in a situation where there is relatively little information on the disease, as is presently the case with cassava root rot problem.

The resistance reference (30572) was classified as moderately resistant in this study, this genotype however, showed a good level of field resistance to root rot disease in our previous study (Onyeka et al., 2005a). The performance of 30572 necessitates the need for a classification method that is independent of the control for separation of the genotypes into levels of resistance. Ariyo et al. (2002) used the rank-sum method to evaluate relative resistance of 25 newly improved cassava cultivars to African cassava mosaic disease (ACMD). The enlarged rank-sum method used in this study does not require prior knowledge of the structure of the germplasm and is able to use information available in continuous variable (percentage data) as well as in categorical variable (rating scale). By using the continuous variable and categorical variable, 22 improved genotypes and 17 landraces were identified as highly resistant, but when the percentage data were converted into categorical scale, the rank-sum method was able to identify only 6 of the improved and 3 of the landraces as highly resistant (Onyeka, 2002). Generally, because the rank-sum test makes use of ranks instead of the original observed data, it is less sensitive to outliers and noise which are almost inevitable in large data sets. The rank-sum method is, therefore, a valuable technique for classification of germplasm, especially where the shape and structure of the germplasm is not known or not adequately defined.

From the results obtained in this study, 17% of the 290 African landraces and 16% of the 306 improved genotypes showed appreciable levels of resistance (highly resistant and resistant groups). This is similar to the work of Barragan and Alvarez (1998) which showed that 14% of the 420 cassava genotypes screened at the Centro Internacional de Agricultura Tropical (CIAT) for resistance to root rot (*Phytophthora drechsleri*), were tolerant to the disease.

Root slice inoculation method involves wounding, and consequently does not take into account any resistance that may be associated with the cortical tissue (Onyeka et al., 2005b). Thus, the resistant genotypes in this study exhibited resistance by restricting the spread of the pathogen within the plant tissue. Under field conditions, cassava root rot pathogens penetrate the host roots either through damage caused by pests and farming tools or by piercing the roots themselves. Therefore, a resistance factor associated with preventing or reducing the spread of the pathogen within the host tissue will be more desirable.

The amount of tissue affected is an indication of the level of partial resistance of the host cultivar (Tooley &

Grau, 1984; Parlevliet, 1993; Dorrance & St. Martin, 2000). Rate-reducing resistance or partial resistance is believed to be effective against a large number of pathogen genotypes and more durable since it is non-race specific (Peever et al., 2000). Rate-reducing resistance is highly heritable and a quantitative trait (Walker & Schmitthenner, 1984). Therefore, the sources of resistance identified in this study will be useful to a cassava breeding programme. Also, this study separated the sets of germplasm into six response groups (highly resistant, resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible). These different genotype groups can be used as study subsets in further studies on the genetic basis of host resistance to cassava root rot disease.

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