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Research Paper

Eleven years of breeding efforts to combat cassava brown streak disease

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Cassava (*Manihot esculenta* Crantz) production is currently under threat from cassava brown streak disease (CBSD), a disease that is among the seven most serious obstacles to world's food security. Three issues are of significance for CBSD. Firstly, the virus associated with CBSD, has co-evolved with cassava outside its center of origin for at least 90 years. Secondly, that for the last 74 years, CBSD was only limited to the low lands. Thirdly, that most research has largely focused on CBSD epidemiology and virus diversity. Accordingly, this paper focuses on CBSD genetics and/or breeding and hence, presents empirical data generated in the past 11 years of cassava breeding in Uganda. Specifically, this paper provides: 1) empirical data on CBSD resistance screening efforts to identify sources of resistance and/or tolerance; 2) an update on CBSD resistance population development comprising of full-sibs, half-sibs and S_1 families and their respective field performances; and 3) insights into chromosomal regions and genes involved in CBSD resistance based on genome wide association analysis. It is expected that this information will provide a foundation for harmonizing on-going CBSD breeding efforts and consequently, inform the future breeding interventions aimed at combating CBSD.

Key Words: BLUPs, CBSD genetics, incidence, resistance genes, root necrosis, severity.

Introduction

Cassava (*Manihot esculenta* Crantz) production, which currently supports livelihoods of more than 800 million people worldwide, is under a threat of cassava brown streak disease (CBSD). This disease has been identified among the seven most serious threats to world's food security (Pennisi 2010). CBSD was thought to be caused by two distinct virus species; Uganda cassava brown streak virus (UCBSV) and Cassava brown streak virus (CBSV), both (+) ssRNA viruses belonging to genus *Ipomovirus*, family *Potyviridae* (Mbanzibwa *et al.* 2009, Winter *et al.* 2010). However, recent findings based on analysis of 470 symptomatic leaf samples collected from Tanzania seem to suggest that up to four different species of cassava brown streak viruses (CBSVs) could be associated with CBSD (Ndunguru *et al.* 2015).

Historically, CBSD was first reported from northern Tanzania in the 1930s (Jennings 1957). Since then, the disease has been reported in coastal areas of Kenya, northern Mozambique, Zanzibar and areas close to the shores of Lake Malawi (Hillocks *et al.* 2002). Recently, reports have underlined the presence of CBSD especially that caused by UCBSV, in the Democratic Republic of Congo, Western Kenya, Burundi and Lake Victoria region of Tanzania (Bigirimana *et al.* 2011, Legg *et al.* 2011). In Uganda, CBSD was first observed in the 1940s on cassava genotypes introduced from Tanzania. The disease was, however, eradicated through implementation of phytosanitary measures (Jameson 1964), but re-emerged in 2005 and attained epidemic status (Alicai *et al.* 2007). Thus, without concerted efforts to control CBSD, it is likely to continue spreading to all major cassava growing regions.

Symptomatically, CBSD causes leaf chlorosis that appears in a feathery pattern along the minor veins. These

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symptoms can appear as early as three months after planting (Ndunguru *et al.* 2015). In some genotypes, the leaf symptoms are coupled with purple/brown lesions on stems, which in severe cases cause death of nodes, internodes and the axillary buds, resulting into dieback (Hillocks and Thresh 2000). On the roots, the disease causes yellow and/or brown, corky necrosis within the starch bearing tissues along with black streaks rendering roots unusable and thus causing up to 70–100% yield loss in susceptible genotypes (Hillocks and Thresh 2000). Transient symptom expressions have also been observed (Mohammed *et al.* 2012). Starch quality notably quantities of amylose and amylopectin are respectively reduced by 30 and 50 percent by CBSD (Nuwamanya *et al.* 2015). In this paper, we present empirical CBSD field data generated from over 250 clones across four propagation cycles. However, we limit detailed genomic studies to storage root symptoms, as the storage roots are the most economically important part of the cassava (Tumuhimbise *et al.* 2015).

The pioneering formal cassava breeding program in Africa, which also coincided with first attempts to breed for CBSD and cassava mosaic disease (CMD) resistance was initiated at Amani Research Station, Tanzania in the 1930s (Jennings 1957, Nichols 1947). During that time, due to a lack of virus resistance in cultivated cassava, wild relatives that included *Manihot glaziovii* Muell-Arg. (Ceare Rubber), *Manihot dichotoma* Ule (Jaquie Manicoba Rubber), *Manihot catingea* Ule), *Manihot saxicola* Lang and *Manihot melanobasis* Muell-Arg were reported to exhibit high levels of resistance to CBSD (Jennings 1957, 1959). One of the outstanding CBSD resistant inter-specific hybrid generated from these crosses was *Namikonga* (also referred to as clone No. 46106/27 or Kaleso). For CMD resistance, the outstanding clone was No. 58308, which became the main source of CMD resistance used in the breeding program started in the early 1970s at IITA in Nigeria. Results of CBSD were not as successful as those for CMD. *Namikonga* and/or its improved versions were not extensively used as a CBSD resistance progenitor.

Based on the information available, the following is apparent for CBSD and its associated CBSVs. First, the virus has co-evolved with cassava in eastern and southern Africa (outside its center of origin) for at least 90 years. Second, for the last 74 years (between 1930 and 2004), the disease was only limited to the low lands i.e., less than 500 m.a.s.l. It is only after 2005, that the rapid spread of the disease has been observed at altitudes >500 m.a.s.l (Alicai *et al.* 2007, Ndunguru *et al.* 2015), which is likely to continue if not controlled. Third, most studies have focused on CBSD epidemiology and virus diversity, with limited published work on breeding for CSBD resistance except perhaps the excellent work done in 1930s (Jennings 1957) and more recently in Mozambique (Zacarias and Labuschagne 2010), Kenya (Munga 2008) and Tanzania (Kulembeka *et al.* 2012).

This paper, therefore, responds to the information gaps highlighted. Specifically, it provides: 1) insights into CBSD resistance screening efforts to identify sources of resistance and/or tolerance to CBSD; 2) an update on population development comprising field performances of full-sibs, half-sibs and S_1 families; and 3) insights into chromosomal regions and genes potentially involved in CBSD resistance based on genome-wide association analysis. It is hoped that this information will provide direction to ongoing and future efforts in combating CBSD in all breeding programs in the CBSD endemic regions, and help prepare regions currently not affected by the disease.

Materials and Methods

Screening for sources of resistance and tolerance to CBSD

Two major CBSD resistance and/or tolerance screening efforts have been undertaken at a CBSD and CMD hot spot area, Namulonge located in central Uganda (Abaca *et al.* 2012a, 2012b, Legg and Fauquet 2004). This has been undertaken in the last decade (2004 and 2014). Firstly, *Manihot esculenta* germplasm introduced as botanical seeds from Tanzania (referred to as germplasm-1). Secondly, wild relatives and cassava F_1 families sourced from Brazil and introduced as seeds (referred to as germplasm 2).

Germplasm 1: Tanzanian open-pollinated seed

At the onset of the CBSD epidemic in Uganda, we introduced germplasm from Tanzania in the form of open pollinated (OP) seeds (~5,000 OP seeds). These were derived from a polycross that had at least 10 CBSD tolerant cassava clones. In April 2005, a seedling nursery was established at Namulonge for CBSD resistance screening. Both seedlings and subsequent surviving clones were established at spacing of 1 m × 1 m. We employed the independent culling selection method targeting CBSD for seven consecutive years (between 2005 and 2011). Thus, for each annual selection event, only clones with <10% CBSD root incidence and <5% foliar incidence were advanced; the rest were discarded. During each evaluation trial, spreader rows of CBSD susceptible variety TME 204 were established to augment CBSD pressure; single rows of TME 204 were also included in the evaluation trials to act as susceptible checks. CBSD was scored using the 1–5 severity scale as described in previous studies (Kaweesi *et al.* 2014).

Briefly, for this scale 1 = no root necrosis; 2 = mild root necrotic lesions (1–10%); 3 = pronounced root necrotic lesions (11–25%); 4 = severe root necrotic lesions (26–50%) combined with mild root constriction; and 5 = very severe root necrotic lesion (>50%) coupled with severe constriction. This seven-year culling exercise was undertaken at Namulonge using single-row plots of 10 plants/row. In 2012, the surviving 16 clones were established in replicated plots for final CBSD evaluation. In addition, open pollinated seeds were also generated from these surviving 16 clones. The generated progeny were established in the field at Namulonge for CBSD evaluation as described earlier; this was done in 2013. Thus, for the Tanzanian material, both

the introduced germplasm and its progeny (generated by inter-mating the surviving 16 clones) were evaluated.

Germplasm 2: Wild relatives and cassava families from Brazil

For the wild relatives, open pollinated seeds were sourced from Brazil and also screened for CBSD field resistance at Namulonge in 2013. The introduced seeds were sourced from eight wild relatives: *Manihot anomala* Pohl. *Manihot caerulescens* Pohl., *Manihot carthaginensis* ssp. *glaziovii* Müll.Arg., *Manihot dichotoma* Ule., *Manihot esculenta* ssp. *flabellifolia* Pohl., *Manihot glaziovii* Muel. Arg., *Manihot irwinii* D.J. Rogers & Appan., and *Manihot peruviana* Müll. Arg. For the cassava families, F_1 seeds were generated by crossing elite cassava clones in Brazil (BGM1332 × Fécula Branca, BGM1428 × Fécula Branca, BGM1584 × Fécula Branca and BGM1662 × Fécula Branca). The generated F_1 seeds were introduced to Uganda and evaluated for both CMD and CBSD resistance. The germplasm from Brazil was kindly provided by Dr. Eder Jorge de Oliveira, of Empresa Brasileira de Pesquisa Agropecuária (Embrapa).

In both cases (wild relatives and full-sib cassava families), all seedlings were germinated in nurseries and thereafter transplanted to the field for resistance screening following established procedures (IITA 1990). Field evaluations were done during the period May 2013 to November 2014 at Namulonge. For CMD, plants were assigned a severity score according to the standard five point scoring scale (IITA 1990) at six months after planting (MAP). At harvest (12 MAP), surviving seedlings were uprooted and scored for CBSD root necrosis as described earlier.

The generated datasets were separately subjected to statistical analysis to generate family and/or individual clone means using R statistical program (R Development Core Team 2010). For "germplasm 1" datasets that were largely unbalanced owing to varying number of clones or replicates established and/or evaluated, data were subjected to mixed model analysis and Best Linear Unbiased Predictions (BLUPs) computed for each clone as a basis for comparison (Bernardo 2010).

Populations developed and their response to CBSD

Following the initial CBSD resistance screening efforts that were undertaken during 2004–2010, promising clones were selected and consequently used as progenitors for development of populations that could be used for long-term CBSD breeding. Accordingly, three populations were generated and screened for CBSD field resistance/tolerance during the period 2011 to 2014. These included: 1) full-sibs having *Namikonga* as progenitor; 2) S_1 cassava clones from five selected genotypes; and 3) full-sibs and/or half-sib families generated from other introduced and/or local outstanding clones.

Full-sibs from Namikonga

Namikonga which is believed to be a progeny from wild cassava (Hillocks and Jennings 2003) with high levels CBSD resistance (Kaweesi *et al.* 2014, Maruthi *et al.* 2014), was extensively used as a progenitor. Thus four *Namikonga* derived F_1 families were generated and evaluated for CBSD resistance for a period of four years (2011 to 2014). These families included: 1) NASE 14 × *Namikonga* (referred to as CS1 crosses), 2) TME 14 × *Namikonga* (referred to as CS2 crosses), 3) NASE 12 × *Namikonga* (referred to as CS3 crosses) and 4) NASE 13 × *Namikonga* referred to as CS4 crosses.

With exception of *Namikonga*, all other selected clones were highly resistant to CMD. For CBSD, the clones had varying reactions basing on data collected between 2009 and 2013: *Namikonga* with mean root severity of 1.03 and root incidence of 10%, NASE 14 with mean root severity of 3.3 and root incidence of 38.2%, TME 14 with mean root severity of 4.2 and root incidence of 57.9, NASE 12 with mean root severity of 4.3 and root incidence of 97.9% and NASE 13 with mean root severity of 5 and root incidence of 98%. Thus, with exception of CS1 crosses, all other crosses were between a resistant clone *Namikonga*, and a susceptible clone.

The F_1 seedling evaluation trial was established in 2011, while the respective clonal replicated trials (2 and/or 3 replicates) were established in 2012 and 2013. The clonal evaluations undertaken in 2014 was only for clones that consistently had no or few CBSD root symptoms (i.e., root severity score ≤ 2). All these evaluations were undertaken at Namulonge and for each trial, spreader rows of a CBSD-susceptible variety TME 204 were included to augment CBSD pressure. In addition, single rows of TME 204 were always included in the evaluation trials to act as susceptible checks. In all trials, CBSD data was collected as described earlier.

S_1 cassava families

The procedures for generation and agronomic evaluation of S_1 was adopted from previous studies (Kawuki *et al.* 2011). The five selected S_0 parental lines (*Namikonga*, I00142, I30040, 0040, and Tz 130), had mean CBSD root severity of scores of <3 and root incidences of <35%. A variable number of S_1 progeny were generated (44 to 141) and subjected to CBSD resistance screening beginning with the seedling evaluation in 2011. Thereafter, only surviving clones (lower CBSD severities and able to raise reasonable planting materials) were re-evaluated in clonal trials at Namulonge following established procedures for three consecutive years (2012, 2013 and 2014).

Full-and half-sib families from diverse origin

Other full-sibs and/or half-sibs were generated from 49 different progenitors. These progenitors were *per se* characterized by agronomically important traits that included: CBSD and CMD resistance and/or tolerance, fresh root yield

and dry matter content. Approximately 3,500 resulting F_1 seedlings were established in a seedling trial at Namulonge in 2011. Culling for CBSD was done and ~13% of seedlings associated with low CBSD severities (i.e., root severity score ≤2), were cloned for further screening in 2012. The clonal trial (having 455 clones) was established in 2012 in single-row unreplicated plots comprising of 10 plants/plot.

In 2013, the clones (300 to 450) were re-evaluated in replicated plots of single-rows comprising of 10 plants at two CBSD hot spots, Namulonge and Kasese. In all these trials: seedling trials (2011), unreplicated clonal trial at single site (2012) and replicated clonal trials at two sites (2013), entries were evaluated for CBSD root necrosis as described by Kaweesi *et al.* (2014).

The datasets generated from the three populations (full-sibs from *Namikonga*; S_1 cassava families; and full-sibs and half-sib families) were each analyzed separately using R statistical program (R Development Core Team 2010) to enable family and/or clone comparisons. In addition, regressions analyses were undertaken to compare CBSD foliar and root severity data for both seedling and/or clonal trials; this was done for the S_1 and *Namikonga*-derived F_1 families. For the unbalanced dataset (full-sibs and/or half-sibs population), BLUPs were computed to enable clone comparisons (Bernardo 2010).

CBSD genome-wide association studies

We assembled a diverse panel of cassava breeding lines and subjected them to CBSD evaluation at Namulonge for a period of five years (2009–2013). These clones were either at second and/or or third round of recombination. From this dataset, it was possible to select 190 genotypes that would be classified as resistant (with maximum severity score for the root of 1 and 2 and very low root incidence i.e., < 20%), moderately susceptible (with root severity score 3 and incidence ranging between 20–50%) and highly susceptible (root severity score of 4 or 5 and root incidence >50) to constitute the panel. These genotypes were re-evaluated in 2014, which coincided with the fifth year of virus exposure and/or evaluation. Since cassava roots are the most economic part of the plant, CBSD root necrosis data was considered for this study, and thus, data collected on four root-related traits: mean root severity, root necrosis index, maximum root severity and root necrosis incidence, as described by Kaweesi *et al.* (2014).

DNA was extracted from leaf samples following established procedures (Dellaporta *et al.* 1983). Genotyping was done at Cornell University, using a genotyping-by-sequencing (GBS) approach as described by Elshire *et al.* (2011) using *Ape*KI enzymes. The bioinformatics procedure in TASSEL-GBS pipeline (Glaubitz *et al.* 2014) was used to process that raw data, and SNP calls were based on cassava genome sequence v6.0. To clean up the raw dataset, indels were removed and imputation done using Beagle software v4.0 as described by Swarts *et al.* (2014). A total of 162,951 SNPs were identified and only filtered SNPs with minor allelic frequency (MAF) of 10% (145,391) were used in the genome-wide association analysis.

Marker-trait association was implemented in Trait by aSSociation, Evolution and Linkage (TASSEL) software version 5.2.3, using a mixed linear model (MLM) where kinship and population structure were included as random and fixed effects respectively. For population structure, the first three principal components were used. Kinship matrix was generated using filtered SNPs using scaled identity by state (IBS) method. As opposed to the pairwise IBS, the scaled IBS method produces a kinship matrix that is scaled to give a reasonable estimate of additive variance. The four traits (mean root severity, root necrosis index, maximum root severity and root necrosis incidence) were separately analyzed in the GWAS analysis. Q-Q plots were used to evaluate the best trait for association analysis. After identifying the significant SNPs through association analysis, the sequence that flanked the significant SNPs was obtained as an open reading frame using Artemis (Rutherford *et al.* 2000). A BLAST search was performed against the entire National Center for Biotechnology Information (NCBI) non-redundant protein database and Phytozome 10.3 (Goodstein *et al.* 2012), to establish whether or not these sequence encoded proteins with known functions. Best hits were used as reference for interpretation of putative biological functions of the sequence from which the SNPs were obtained.

Results

Screening for sources of resistance and tolerance to CBSD

Data on performance of the surviving 16 clones (out of the introduced 5000 seeds from Tanzania) and their respective progeny at seedling stage is presented in **Table 1**. After eight years of CBSV virus exposure, lowest CBSD root incidences were registered on clone Tz-80 with respective BLUP and mean incidences of –32.1 and 12.2% (**Table 1**). Clones Tz-65, Tz-90 and Tz-177, also had BLUP values that ranged between –26.5 to –24.6, with mean root incidences of <20%. However, other clones like Tz-61, Tz-64, and Tz-100 had mean incidences of >70% with root severity scores of ≥3 (**Table 1**). It is also evident that each clone had progeny that were classified as resistant with root severity scores of 1 and/or 2 (**Table 1**). This finding confirm presence of CBSD resistance/tolerance genes in the introduced Tanzanian germplasm.

Data on performance of wild relatives is presented in **Table 2**. A total of 239 seedlings established successfully by one MAP. However these reduced to 210 and 173 respectively at three and six MAP. At six MAP, CMD severity ranged from 1 for *M. caerulescens* Pohl, to 4.1 for *M. peruviana* Müll. Arg. *M. esculenta* individuals had average CMD severity of 3.8, indicating higher levels of CMD susceptibility. At Namulonge, the most prevalent strains of cassava mosaic geminiviruses are East African Cassava Mosaic Virus-Ug (EACMV-Ug) and the African Cassava Mosaic Virus (ACMV), with EACMV-Ug strain being the

Table 1. Best Linear Unbiased Predictions (BLUPs) of CBSD root necrosis of cassava parental lines and their respective progeny evaluated at NaCRRI, Uganda

Parental line	Performance of parents		F_1 seedling evaluation trials					
	CBSDRi[a]	CBSDRs[b]	Score 1 & 2	Score 3	Score 4 & 5	No. F_1s	CBSDRs	CBSDRi
Tz_100	37.5 (81.9)	1.25 (3.06)	6	0	6	12	0.03 (1.71)	0.74 (27.2)
Tz_110	0.19 (44.5)	–0.05 (1.76)	27	1	6	34	–0.02 (1.64)	–1.57 (24.4)
Tz_146	12.4 (56.8)	–0.01 (1.80)	7	1	0	8	–0.03 (1.63)	–1.27 (24.8)
Tz_163	–23.6 (20.7)	–0.54 (1.26)	17	1	2	20	–0.04 (1.62)	–1.22 (24.7)
Tz_175	–1.66 (42.7)	–0.13 (1.67)	16	1	6	23	0.06 (1.74)	2.17 (28.8)
Tz_177	–24.6 (19.7)	–0.58 (1.22)	21	3	3	27	–0.01 (1.66)	–0.97 (25.2)
Tz_61	31.5 (75.9)	0.76 (2.57)	16	3	7	26	0.06 (1.74)	2.49 (29.5)
Tz_62	–14.8 (29.5)	–0.36 (1.45)	20	2	11	33	0.04 (1.71)	1.26 (27.7)
Tz_64	35.7 (80.1)	1.34 (3.16)	21	1	5	27	–0.02 (1.65)	–0.84 (25.2)
Tz_65	–26.5 (17.8)	–0.54 (1.26)	29	2	8	39	–0.01 (1.66)	–1.40 (24.5)
Tz_66	19.2 (63.6)	0.35 (2.17)	38	3	13	54	0.06 (1.74)	2.28 (29.1)
Tz_69	5.5 (49.9)	–0.01 (1.80)	31	3	5	39	–0.05 (1.60)	–2.93 (22.9)
Tz_73	–12.0 (32.3)	–0.38 (1.43)	15	0	1	16	–0.03 (1.63)	0.43 (26.8)
Tz_80	–32.1 (12.2)	–0.67 (1.14)	29	6	6	41	0.02 (1.70)	1.71 (28.2)
Tz_88	8.5 (52.9)	0.12 (1.94)	41	2	6	49	–0.06 (1.60)	–1.03 (25.1)
Tz_90	–25.5 (18.8)	–0.58 (1.22)	20	1	7	28	0.01 (1.69)	0.17 (26.5)
P-values	0.026	0.007					0.51	0.406
H^{2c}	0.60	0.70					0.10	0.14

[a] Cassava brown streak disease root incidence assessed at 12 MAP; [b] Cassava brown streak disease root severity assessed at 12 MAP; [c] Broad-sense heritability. CBSD root necrosis was assessed on a scale of 1 to 5, where 1 = no necrosis, 2 = mild necrotic lesions (1–10%), 3 = pronounced necrotic lesion (11–25%), 4 = severe necrotic lesion (26–50%) combined with mild root constriction and 5 = very severe necrotic lesion (>50%) coupled with severe constriction. Data in parentheses are least-square (LS) means for parents and family progeny means, respectively.

Table 2. Field response of selected *Manihot* species to CMD and CBSD assessed at NaCRRI, Uganda during 2013–2014

Manihot Species	Number[a]	CMDs[b]	CBSDs[c]
Manihot caerulescens	22	1.0	1.5
Manihot dichotoma	21	1.5	1.1
Manihot esculenta ssp. *flabellifolia*	12	3.3	1.2
Manihot sp.	4	1.5	1.0
Manihot carthaginensis ssp. *glaziovii*	8	1.6	1.1
Manihot irwinii	11	1.0	1.0
M. esculenta F_1	74	3.8	1.4
Manihot glaziovii	2	2.5	1.0
Manihot peruviana	19	4.1	1.0
		CBSDRi[d]	CBSDRs[e]
Manihot peruviana ssp. *glaziovii*	4	0.0	1.0
M. esculenta F_1	23	85.4	3.5
Manihot glaziovii	3	0.0	1.0

[a] Number of genotypes evaluated; [b] Mean severity of cassava mosaic disease assessed at six MAP; [c] Mean severity of cassava brown streak disease assessed on the foliar parts at six MAP; [d] Cassava brown streak disease root incidence assessed at 12 MAP; [e] Cassava brown streak disease root severity assessed at 12 MAP using the 1–5 scale. Data presented are family means.

most virulent (Legg and Fauquet 2004). Thus, all individuals that had scores 1 or 2 can be classified as truly resistant and thus sources of CMD resistance genes.

At harvest, a variable number of roots were obtained. For instance, 143 seedlings had no roots purportedly due to the CMD pressure, 16 seedlings had one root and two seedlings had five roots. Thus, CBSD root necrosis was only assessed on 30 seedlings. Up to 20 individuals all of which are *M. esculenta* F_1 had average CBSD root necrosis severity scores >3 (**Table 2**). Seven individuals from *Manihot carthaginensis* ssp. *glaziovii* and *Manihot glaziovii* had no root necrosis (**Table 2**). Individuals belonging to the *M. esculenta* F_1 families registered the highest CBSD root incidence (85%) and highest CBSD root severity (3.5). In all these evaluations, the susceptible check (TME 204) registered mean CBSD root severities scores >4 and incidences of >80%.

Populations developed and their response to CBSD

Three populations were generated and their respective progeny evaluated for CBSD resistance and/or tolerance: 1) full-sibs having *Namikonga* as progenitor, 2) S_1 cassava families, and 3) full-sibs and/or half-sib families derived from 49 progenitors. Data on the performance of *Namikonga*-derived crosses across four years of screening is presented in **Table 3**. It is evident from these datasets that: 1) progenitor *Namikonga* provided CBSD resistance alleles to the progeny as reflected by the number of individuals (18 to 32) that had no and/or few CBSD root symptoms after the 3rd and 4th year of evaluation; 2) there is a persistent poor correlation between CBSD foliar and root symptoms i.e., R^2 values <22%; and 3) it requires at least three seasons of CBSD evaluation in a hot spot to reliably quantify the CBSD field response, as reflected by the sharp contrast of clones with scores 1 and 2 in 2011 (214 clones) and those observed in 2013 (only 46 clones) or 2014 (24).

Data on the CBSD reaction of the S_1 families is presented in **Table 4**. Though a drastic reduction (~66%) was observed in number of progeny evaluated between seedling (2011 trial) and first clonal trial (2012 trial), each family had

Table 3. Field reaction of *Namikonga*-derived progeny to cassava brown streak disease at NaCRRI, Uganda evaluated across three propagation cycles

Year	Family	CBSDs[a]	CBSDRi[b]	F_1s used for scoring CBSD			Rootless[c]	Total[d]	$R^{2\,e}$
				Score 1 & 2	Score 3	Score 4 & 5			
2011	CS1	1.85	21.6	138	6	31	148	323	0.013
	CS2	2.10	32.6	42	3	13	32	90	0.031
	CS3	1.50	13.5	28	2	2	30	62	0.113
	CS4	2.54	29.3	6	1	4	21	32	0.148
2012	CS1	3.02	33.2	59	24	60	0	143	0.069*
	CS2	3.77	56.1	7	8	21	0	36	0.052
	CS3	2.64	31.1	12	6	7	0	25	0.218*
	CS4	3.77	58.6	2	1	6	0	9	0.398*
2013	CS1	2.79	40.3	32	23	39	8	102	0.016
	CS2	1.59	16.18	7	1	1	5	14	0.045
	CS3	1.20	5.13	7	–	–	2	9	0.208
	CS4								
2014	CS1	1.37	23.5	18	1	–	–	19	0.0001
	CS2	1.10	5.4	4	–	–	–	4	
	CS3	1.2	10.0	2	–	–	–	2	

[a] Cassava brown streak disease root severity; [b] Cassava brown streak disease root incidence; [c] Number of F_1s without roots at evaluation; [d] Total number of F_1s per family. [e] Regression coefficient of CBSD root severity regressed on CBSD foliar severity: no regression was done for data for some families of 2014 owing to the small sample size i.e., <10 observations. CBSD root necrosis was scored on a scale of 1 to 5, where 1 = no necrosis; 2 = mild necrotic lesions (1–10%); 3 = pronounced necrotic lesion (11–25%); 4 = severe necrotic lesion (26–50%) combined with mild root constriction; and 5 = very severe necrotic lesion (>50%) coupled with severe constriction. CS1 = NASE 14 × *Namikonga*; CS2 = TME 14 × *Namikonga*; CS3 = NASE 12 × *Namikonga*; and CS4 = NASE 13 × *Namikonga* referred to as CS4 crosses. Check variety TME 204 had CBSDRs > 4 and CBSDRi > 80%. Data presented are family means.

Table 4. Field response of S_1 cassava partial inbreds to CBSD over three propagation cycles at NaCRRI, Uganda

Year	Family	CBSDs[a]	CBSDRi[b]	F_1s used for scoring CBSD			Rootless[c]	Total[d]	$R^{2\,e}$
				Score 1 & 2	Score 3	Score 4 & 5			
2011	*Namikonga*	1.04	10.6	32	–	2	10	44	0.116*
	I00142	1.63	19.1	93	5	14	29	141	0.0003
	I30040	1.23	16.9	92	3	6	–	101	0.01
	0040	1.20	2.28	79	–	4	17	100	0.13*
	Tz130	1.87	22.6	55	5	12	16	88	0.0000
2012	*Namikonga*	3.57	49.2	4	1	9	0	14	0.38*
	I00142	4.00	46.8	3	2	11	0	16	0.001
	I30040	3.22	36.2	15	5	16	0	36	0.01
	0040	3.88	44.2	6	4	17	0	27	0.03
	Tz130	3.54	36.6	16	7	30	0	53	0.11*
2013	*Namikonga*	1.45	18.4	4	0	0	0	4	–
	I00142	2.21	29.8	1	1	2	0	4	–
	I30040	1.68	17.7	11	0	1	0	12	–
	0040	1.92	19.9	4	1	0	0	5	–
	Tz130	2.59	38.2	9	0	6	0	15	–
2014	*Namikonga*	1.12	6.25	4	0	0	0	4	–
	I30040	1.04	1.54	6	0	0	0	6	–
	0040	1.0	0	2	0	0	0	2	–
	Tz130	1.55	23.9	7	–	1	0	7	–

[a] Cassava brown streak disease root severity; [b] Cassava brown streak disease root incidence; [c] Number of F_1s without roots at evaluation; [d] Total number of F_1s per family. [e] Regression coefficient of CBSD root severity regressed on CBSD foliar severity: no regression was done for data for some families owing to the small sample size i.e., <16 observations. CBSD root necrosis scored on a scale of 1 to 5, where 1 = no necrosis; 2 = mild necrotic lesions (1–10%); 3 = pronounced necrotic lesion (11–25%); 4 = severe necrotic lesion (26–50%) combined with mild root constriction; and 5 = very severe necrotic lesion (>50%) coupled with severe constriction. Check variety TME 204 had CBSDRs > 4 and CBSDRi > 80%. Data presented are family means.

individuals (3 to 16) that showed no and/or limited CBSD root symptoms (**Table 4**). It was also evident that correlations between CBSD foliar and root severities were negligible (i.e., most r values were <20%) for both seedling and clonal trials (**Table 4**). By the fourth year (2014 trial), the number of S_1 clones that remained symptomless were: seven for Tz 130, six for I30040, four for *Namikonga* and two for 0040. These S_1 clones had mean harvest index of 0.18 (ranging from 0.03 to 0.47) and mean root dry matter content of 28.6% (ranging from 16.9%–38.8%). It is these

Table 5. Best Linear Unbiased Prediction (BLUP) of CBSD root necrosis of five-best and five-worst progeny from selected parental lines evaluated at NaCRRI, Uganda

Clone	Female	Male	BLUPs[a]	CBSDRs[b]	Clone	Female	Male	BLUPs[a]	CBSDRs[b]
Top TMS 30572 Progeny					Worst TMS 30572 Progeny				
Ug120001	TMS30572	NASE 12	–46.79	1.49	Ug120013	NASE 11	TMS30572	44.01	3.06
Ug120104	TMS30572	TMS30572	–46.70	1.67	Ug120042	NASE 11	TMS30572	46.34	3.72
Ug120058	TMS30572	MM96/0686	–39.97	1.83	Ug120111	TMS30572	MH04/236	54.83	4.02
Ug130126	TMS30572		–39.63	1.57	Ug120011	NASE 11	TMS30572	55.29	3.37
Ug130087	TMS30572		–30.90	1.68	Ug120061	TMS30572	NASE 4	57.76	3.52
Top TMS 60142 Progeny					Worst TMS 60142 Progeny				
Ug120002	NASE 11	TMS 60142	–30.64	1.66	Ug120249	SE95/00036	TMS60142	21.83	3.18
Ug120303	TMS 60142	NASE 14	–34.65	1.66	Ug120005	SE95/00036	TMS 60142	21.87	3.22
Ug120251	TMS 60142	NASE 9	–22.70	1.74	Ug120009	NASE 11	TMS 60142	48.33	3.34
Ug120267	TMS 60142	TME 14	–28.69	1.80	Ug120250	SE95/00036	TMS60142	36.84	3.58
Ug120289	TMS 60142	NASE 11	–13.51	1.91	Ug120010	NASE 11	TMS 60142	17.39	3.70
Top NASE 14 Progeny					Worst NASE 14 Progeny				
Ug120124	NASE 14	MH04/2767	–38.71	1.95	Ug120125	NASE 14	MH04/236	27.23	3.14
Ug120123	NASE 14	MH04/2767	–31.76	1.97	Ug120200	SE95/00036	NASE 14	34.76	3.33
Ug120135	NASE 14	MH04/2575	–30.36	1.80	Ug120121	NASE 14	MH02/0441	46.24	3.07
Ug120116	NASE 14		–20.87	1.94	Ug120202	SE95/00036	NASE 14	46.24	3.31
Ug120105	I92/0067	NASE 14	–19.49	2.53	Ug120201	SE95/00036	NASE 14	46.34	3.72
Top TME 14 Progeny					Worst TME 14 Progeny				
Ug120095	TME 14	TME 14	–33.84	1.62	Ug120134	TME 14	26B/27	24.20	3.47
Ug120274	TME 14		–33.57	1.92	Ug120233	TME 14	Nyaraboke	24.20	3.47
Ug130005	TME 14	26B-27	–32.84	1.76	Ug120212	NASE 12	TME 14	39.20	3.50
Ug130009	TME 14	11B-91	–32.03	1.65	Ug120295	TME 14		40.55	3.55
Ug130110	TME 14	Nyaraboke	–31.69	1.73	Ug120292	TME 14		47.35	3.21

[a] Best linear unbiased predictions, based on CBSD root necrosis data collected from trials established during 2012 (unreplicated trials at NaCRRI) and 2013 (replicated trials at both NaCRRI and Kasese); [b] Cassava brown streak root severity scored using the 1–5 severity scale. This dataset is based on evaluation of 300 to 450 clones that were established in single row plots of 10 plants/row. Parental lines SE95/00036, NASE 12, NASE 4, and NASE 11 are highly susceptible to CBSD, while parental lines TMS 30572, TMS 60142 and NASE 14 are classified as tolerant to CBSD. The best clone was Ug120198 (with BLUP value of –48.2) and the worst performing clone was Ug120278 (BLUP value of 65.8).

few outstanding individuals (19 out of the original 474 S_1 clones) that are of interest for CBSD breeding, as they demonstrate the benefit of inbreeding in cassava, particularly when combined with stringent selection.

Data on the performance of the best-five and worst-five progeny generated from four of the 49 parental lines is presented in **Table 5**. The selected parental clones, all of IITA pedigree included: NASE 1 (TMS 60142), NASE 3 (TMS 30572), NASE 14 (MM96/4271) and TME 14. The parental lines NASE 1, NASE 3 and NASE 14, have all been associated with low CBSD symptoms. Progeny of TME 14 were added for comparison purposes. It is evident from the data that each of the parental lines had outstanding progeny; among the top-best, the most CBSD resistant progeny (Ug120001 and Ug120104) were all derived from TMS 30572, as they had BLUP values of –46.7 (**Table 5**). The best (Ug120002) and worst (Ug120009) progeny of parental clone TMS 60142, were all derived from the same cross combination (**Table 5**), an illustration of the heterozygosity challenge in cassava breeding.

However, when all progeny from different combinations were analyzed together (including the introductions from Tanzania; data not shown), we observed that the top five clones were: Ug120198 (introduction from Tanzania, with BLUP value of –48.2), Ug120024 (F_1 of NASE 14 × *Namikonga*, with BLUP value of –48.1), Ug120190 (introduction from Tanzania, with BLUP value of –48.1), Ug120022 (F_1 of NASE 14 × *Namikonga*, with BLUP value of –47.7) and Ug120001 ((F_1 of NASE 3 × NASE 12, with BLUP value of –46.7). The worst clone Ug120278 (an F_1 of NASE 10 × NASE 9) had BLUP value of 65.8 with respective root mean severity of 3.64.

CBSD genome-wide association studies

Five SNPs all located on chromosome 11 had significant signals (**Table 6**, **Fig. 1**). However, these SNPs did not reach genome-wide Bonferroni significance threshold (of $P = 3.44 \times 10^{-7}$). Four of these SNPs were identified with mean root severity. Two SNPs (S11-22909579 and S11-19872319) were identified with both mean root severity and disease index data. SNP S11-23228224 was the only significant signal that was obtained using maximum root severity. These SNPs are physically located between 19872319 to 23751929 bp, a segment which most likely harbors the gene locus conditioning resistance and/or tolerance to CBSD root necrosis. On average the identified SNPs explained 14.6% of CBSD root necrosis phenotypic variation.

Based on the BLASTx plant protein search, four different

Table 6. SNP markers significantly associated with CBSD root necrosis resistance and their respective *P*-values

Trait	CHR[a]	Marker	Position (Mb)	*P*-value	$R^{2\,b}$	Candidate gene	
						Gene[c]	BLASTx plant protein
Mean Sev[d]	11	S11-19872319	19872319	4.27×10^{-6}	0.14	*Cassava4.1_019379m*	*lysM* domain containing protein
	11	S11-23751929	23751929	4.42×10^{-6}	0.14	*Cassava4.1_028097m*	*glycine-rich* protein
	11	S11-22909579	22909579	5.78×10^{-6}	0.14	–	*Ankyrin-3-like* protein
	11	S11-22909532	22909532	7.65×10^{-6}	0.16	–	–
Index[e]	11	S11-22909579	22909579	5.43×10^{-6}	0.13	–	*Ankyrin-3-like* protein
	11	S11-19872319	19872319	6.11×10^{-6}	0.13	*Cassava4.1_019379m*	*lysM* domain containing protein
Max Sev[f]	11	S11-23228224	23228224	3.15×10^{-6}	0.17	*Cassava4.1_00037m*	*3.5.2.9-5-oxoprolinase* enzyme

[a] Chromosome; [b] Proportion of genetic trait variation explained by SNPs; [c] Obtained through BLAST search against Phytozome 10.3; [d] Mean root severity; [e] Disease index as described by Kaweesi *et al.* (2014); [f] Maximum root severity.

proteins were identified from this study. These included l*ysM domain* containing protein, *glycine-rich* protein, *ankyrin-3-like* protein and *3.5.2.9-5-oxoprolinase* enzyme (**Table 6**). The Q-Q plots which were used to evaluate the best trait for CBSD association tests are displayed in **Fig. 2**. It's evident that 99% of the SNPs had *P*-values greater than 0.001 for mean severity and disease index showing that the bulk of distribution behaved the way it should, based on the no association hypothesis. However, for root incidence and maximum severity, there was an early deviation from the perfect diagonal (which corresponds to the null hypothesis), which means that statistical distribution is not appropriate for association tests.

Discussion

Since the first reports of CBSD breeding in the early 1930's (Jennings 1957, Nichols 1947), limited genetics and/or breeding information has been generated. This is evident in the few published work between the 1930s and 2015, as cited herein. Thus, this paper presents and discusses empirical CBSD data generated in the last 11 years. From these datasets, important information relating to CBSD and/or CBSV evaluation, scoring methodologies and breeding strategies have been gained. This information will specifically be relevant for on-going CBSD breeding efforts and consequently, inform the future breeding interventions aimed at combating CBSD.

It is evidently clear that CBSD and/or CBSV evaluations be conducted in a truly CBSD hotspot for a minimum of three years to reliably classify clone responses. The sharp contrast in number of clones with CBSD root severity scores of 1 and/or 2 (classified as resistant) in 2011, and in 2014 for both F_1 and S_1 families (**Tables 3**, **4**) is testimony for this. This also provides an opportunity to assess degeneration purported to arise from increased viral load observed during the clonal propagation cycles. It is preferable to measure virus load using real-time PCR during CBSD evaluations.

However in situations where large-scale virus monitoring is not possible for each individual plot and/or clone, then representative plots and/or clones could be sampled and monitored to get insights into virus species dynamics (Ndunguru *et al.* 2015) and/or virus load during the evaluation periods. For example, if evaluations are conducted for three seasons/years, virus monitoring should be done for each season; 10–20 plots/clones with severe disease symptoms and 10–20 plots/clones with transient and/or no symptoms should be assayed. This is particularly relevant in early selection stages i.e., clonal trials which often involve evaluation of >150 clones. Fortunately, optimal sampling schedules (with details of plant growth stage and plant part) for CBSVs have been described (Kaweesi *et al.* 2014, Ogwok *et al.* 2015). Thus, as cheaper and reliable methods to quantify CBSV viral loads become available, each individual plot and/or clone can be monitored and its respective viral load compared with the disease severity.

It is also evident from the generated field datasets that entailed evaluation of >250 clones for minimum of three years, that no consistent relationship exists between CBSD foliar and root symptoms i.e., most R^2 values were <10% (**Tables 3**, **4**). This could suggest that these are different traits under different genetic and/or biochemical mechanisms. Thus, in terms of measurement, both traits can be measured and final categorizations of CBSD response based on both as proposed by Kaweesi *et al.* (2014). In fact, in absence of immunity, foliar CBSD assessments will continue to be critically important in early selection stages (for purposes of culling) and for cassava seed certification.

In practice, methodologies for measuring root severity and/or root incidence can be variable. From experience, we observe that different genotypes exhibit varying frequencies of total harvested roots and/or root severity scores per plant, a situation that complicates genotype categorization and/or comparison. It is commonplace for breeding programmes to use a maximum score, on the scale of 1–5, for the entire plot, others may use a maximum score per plant and then compute plot averages; clearly, the two do not equate. It's also evident that a root that is assigned a score of 1 is economically very different from a root that is assigned a score of either 3, 4 or 5. Thus, discussions are on-going to find solutions to this by way of accounting for the variable number of roots sampled per genotype through the development of a CBSD root necrosis index.

Regarding CBSD resistance breeding it is encouraging to note that outstanding CBSD resistant and/or tolerant clones

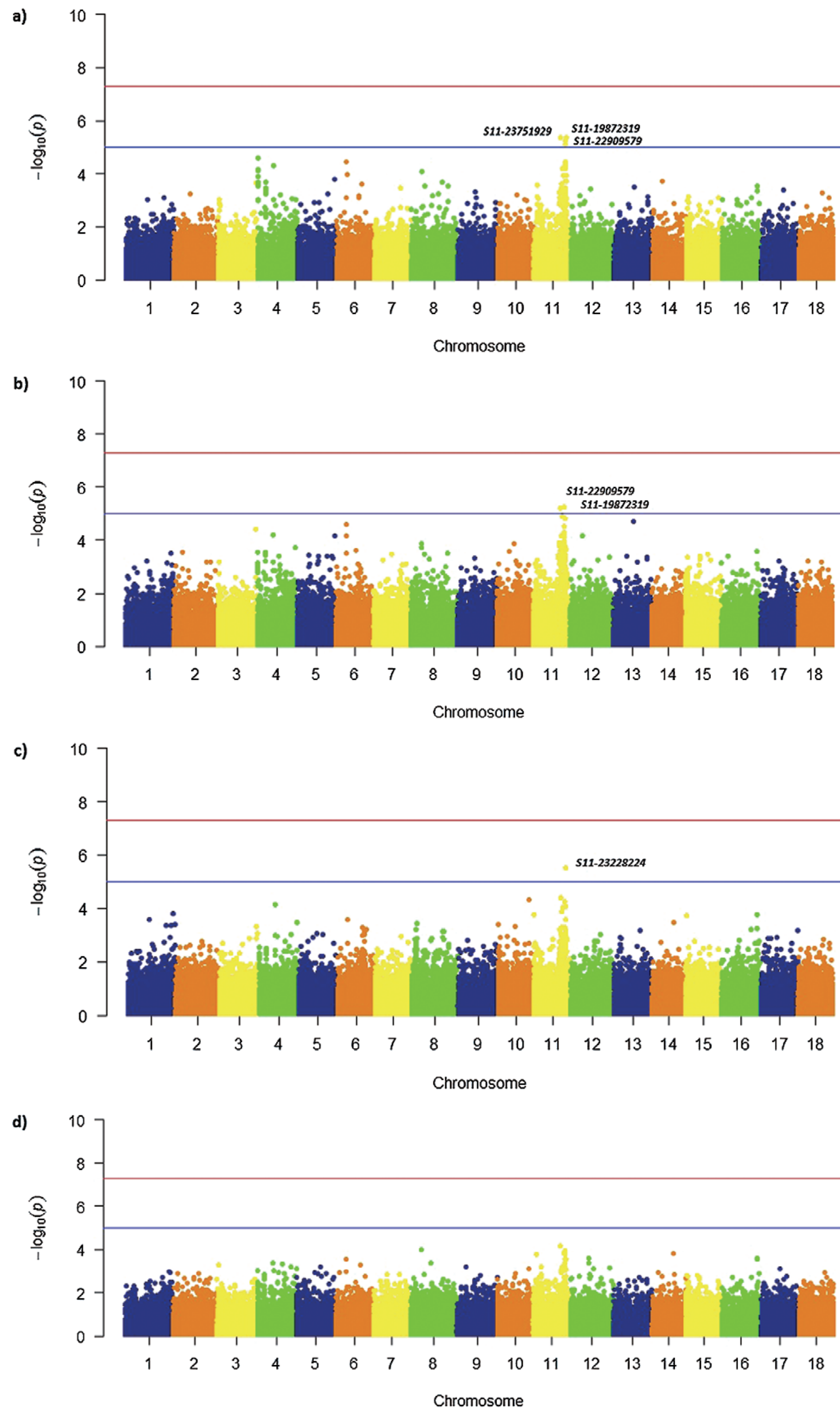

Fig. 1. Manhattan plots for genome wide association analysis for CBSD root necrosis resistance based on mixed linear models. Mean severity of root necrosis (a); disease index of root necrosis (b); maximum root severity (c); and incidence of root necrosis (d). The redline represents the Bonferroni correction threshold that determines SNPs with genome wide significance signal.

have been identified. Based on evaluations undertaken in the past decade, we have identified some clones with reasonable resistance and/or tolerance to CBSD. These clones come from half-sibs, full-sibs and/or S_1 cassava families generated from CBSD tolerant and/or resistant genotypes. Notable of these are progeny derived from *Namikonga*, NASE 1 (TMS 60142), NASE 3 (TMS 30572), NASE 14 and *M. esculenta* selections introduced from Tanzania. *Namikonga* derived F_1s (particularly those involving NASE 14), and the S_1 partial inbreds were truly outstanding (**Tables 3**, **4**).

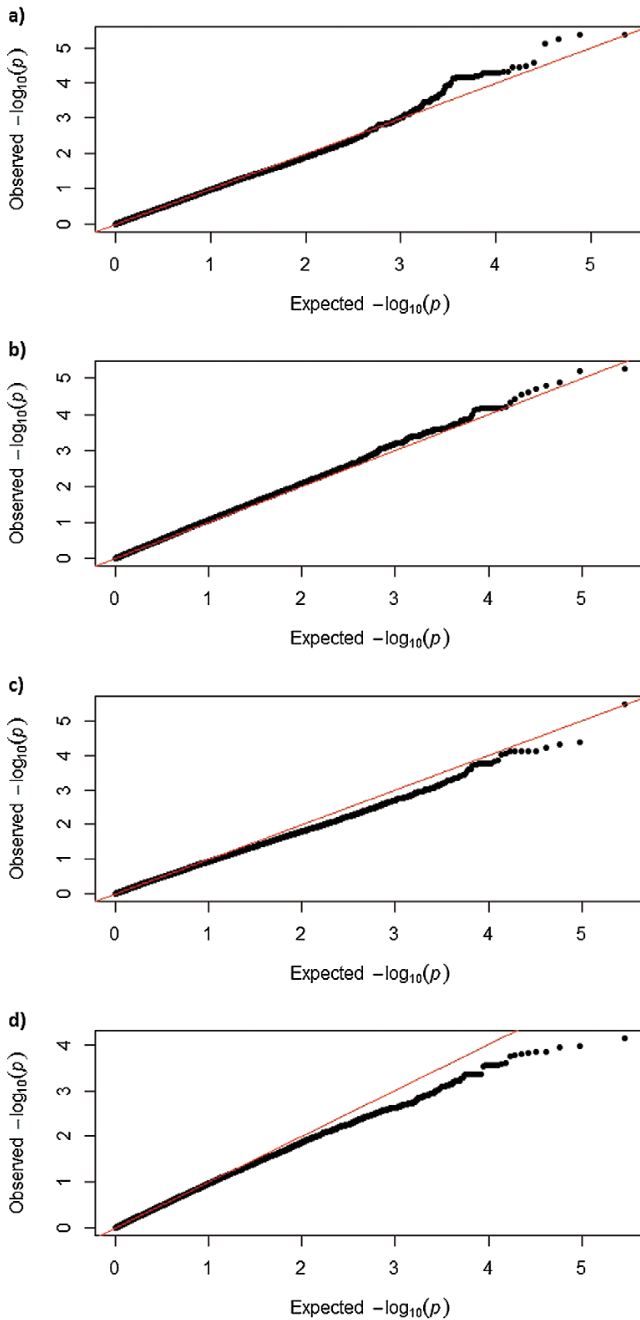

Fig. 2. Q-Q plots of SNPs at marker level (*P*-values). Mean root severity (a); disease index of root necrosis (b); maximum root severity (c); and incidence of root necrosis (d). Deviation from the identity line at different significance levels showed the amount of false positive tests resulted from the analysis of the data; most deviations were observed for maximum severity and root incidence.

These identified clones have remained symptomless or have shown mild CBSD symptoms (maximum severity score 2) with low foliar and/or root incidences (<15%) after five years of evaluation.

Equally striking was the identification of some wild relatives, notably from *M. carthaginensis* ssp. *glaziovii* and *M. glaziovii* that showed no CBSD symptoms. Because evaluations were based on seedlings, it will be necessary to re-evaluate the promising wild seedlings in clonal trials where a larger number of roots can be assessed per clone. In addition, the identified CBSD resistance/tolerance genes particularly those identified within *M. esculenta* germplasm sourced from Tanzania, can be exploited.

On the other hand, CMD datasets (generated from the wild relatives), demonstrated their genetic value. Some individuals from *M. caerulescens*, *M. dichotoma*, *M. irwinii* and *M. carthaginensis* ssp. *glaziovii*, registered CMD severity scores of 1 and/or 2 (**Table 2**), and thus qualifying them as potentially useful sources of resistance to cassava germiniviruses. These CMD resistance sources from the wild will certainly compliment the widely deployed resistance that was exploited from *M. glaziovii* in the 1930s (Jennings 1957). It also suffices to note that during this period cassava varieties NASE 14, NARO-CASS 1 (synonym Tz 130) and NARO-CASS 2 have been officially released for commercial production; NASE 14 and NARO-CASS 1, have also been shared with four other countries (Kenya, Tanzania, Malawi and Mozambique) that are equally challenged by CBSD.

Within limits, the CBSD association genetic study identified five significant SNPs, all physically located between 19872319–23751929 bp on chromosome 11. The consistent presence of more than one SNP in this region suggests that this chromosome region is one of the quantitative trait loci (QTL) for CBSD root necrosis resistance. In this study, Q-Q plots were used to evaluate the best phenotype to use for CBSD genetic association tests. It was evident that 99% of the SNPs had *P*-values greater than 0.001 for mean severity and disease index showing that the bulk of distribution behaved the way it should, based on the no association hypothesis. However, for CBSD incidence and maximum severity, there was an early deviation from the perfect diagonal (which corresponds to the null hypothesis), which limits the utility of the two traits (incidence and maximum severity) for CBSD association studies.

Plants possess pattern recognition receptors (PRRs) for their defense against pathogens (Nicaise *et al.* 2009). Indeed, a number of membrane-bound or soluble PRRs with lectin domain, have been identified as frontiers for plant defense (Lannoo and Van Damme 2014, Van Damme *et al.* 2008). Based on the BLASTx plant protein search, four different proteins were identified: l*ysM domain* containing protein, *glycine-rich* protein, *ankyrin-3-like* protein and *3.5.2.9-5-oxoprolinase* enzyme (**Table 6**). LysM-domain lectins is one of the four lectin receptor kinases (*LecRK*) that have been reported to act both upon biotic and abiotic stresses (Vaid *et al.* 2013).

It can therefore be hypothesized that the *LysM* domain containing protein observed from this study, may have a role in CBSD root necrosis. Lozano *et al.* (2015), observed that cassava chromosome 11 has three TOLL/interleukin-1 receptor (TIR-NBS-LRR) and one coiled- coil N-terminal domain (CC-NBS-LRR). None of these known resistance gene orthologs were identified in this study. This concurs

with the finding of Maruthi *et al.* (2014) where none of the known resistance gene orthologs were uniquely over expressed in CBSD-resistant genotype (*Namikonga*). Thus, more studies are therefore needed to further explore this region and/or other genomic regions to get further insights into genes that contribute to resistance and/or tolerance to CBSD root necrosis. Currently, efforts are underway to undertake detailed CBSD genome wide analysis studies that aim to get further insights into genes and/or chromosomal regions controlling CBSD resistance.

So far, all CBSD genetic studies conducted confirm the preponderance of additive genetic effects (Kulembeka 2010, Kulembeka *et al.* 2012, Munga 2008, Zacarias and Labuschagne 2010) and significant genotype by environment interactions (Pariyo *et al.* 2015, Tumuhimbise *et al.* 2014b). A number of factors are likely to amplify the genotype × environmental interaction for CBSD including genotype susceptibility levels, predominant virus species in locality and/or season, and climatic factors that either influence the abundance of whitefly vectors and/or the growth rate of the crop (Katono *et al.* 2015). The discovery of four distinct virus species (Ndunguru *et al.* 2015), is likely to further complicate the extent of genotype by environment interaction, as CBSD symptom expression (phenotypes) associated with virus species are likely to differ between environments. Therefore, future CBSD breeding strategies have to be designed mindful of these factors.

Accordingly, if selection for hybridization is to be based on phenotypes, then cycle time can be reduced by having field nurseries that serve both as evaluation and hybridization plots. For instance, final CBSD phenotypes (foliar and root) can be scored (during 3rd year of evaluation) at seven MAP and then clones with low severities (combined with desired agronomic traits) crossed to constitute next cycle for selection. This simplistic 3–4 year CBSD breeding cycle can be explored to attain higher levels of resistance. On the other hand, if selection is to be based on genotypic data as implemented for genomic selection (Oliviera *et al.* 2012), then training populations will initially be needed to develop prediction models. It is through this approach that SNP markers associated with CBSD resistance genes can be given more weights in the estimation of genomic estimated breeding values that are used in parental selection.

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