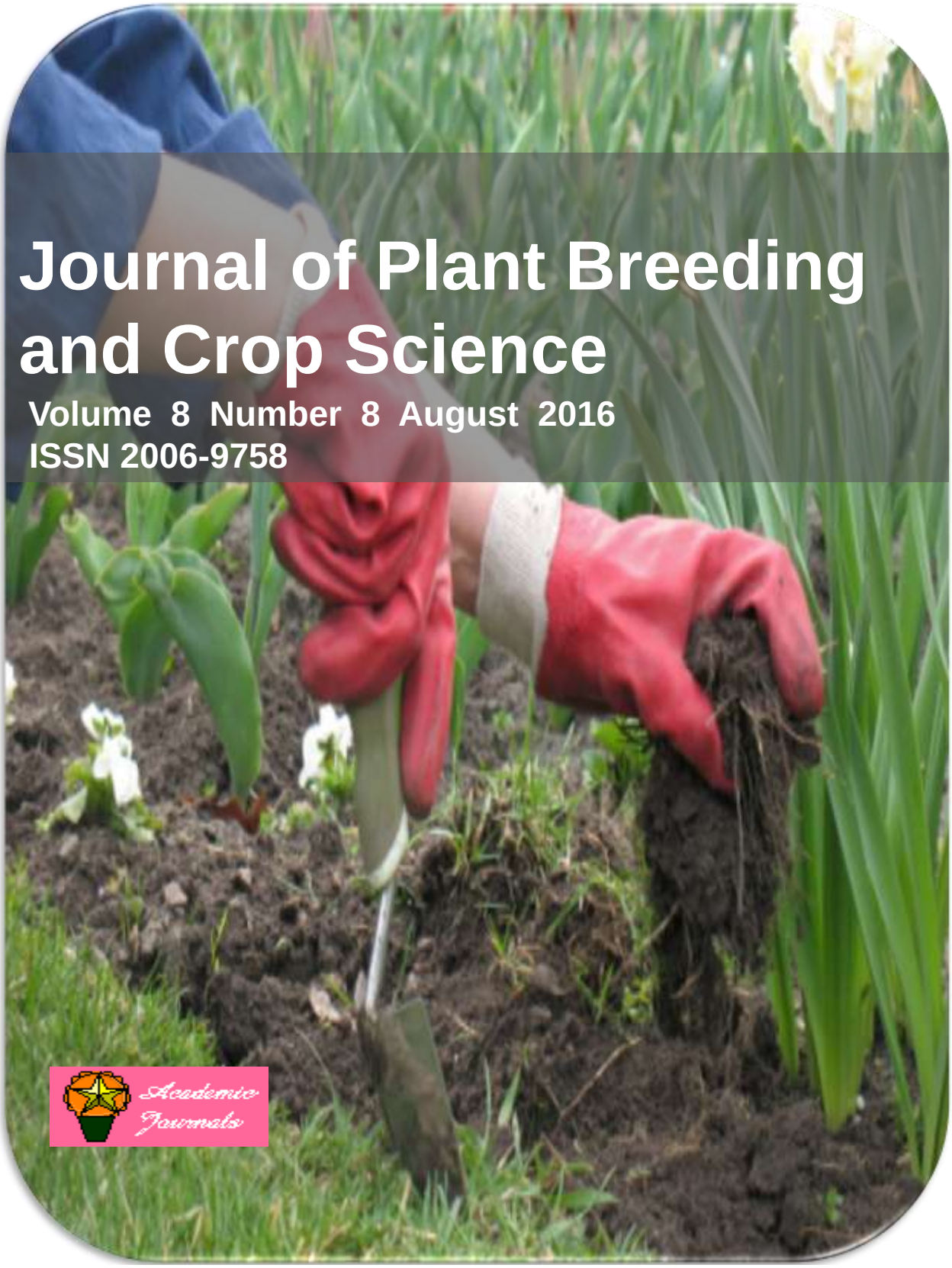

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Full Length Research Paper

Characterization of leaf rust resistance in international barley nurseries

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Barley nurseries comprising 820 lines with 479 unique pedigrees sourced from the International Centre for Agricultural Research in the Dry Areas (ICARDA) were screened for seedling and adult plant resistance (APR) against Australian isolates of barley leaf rust pathogen *Puccinia hordei* Otth. Ninety three percent of the lines were postulated to carry the seedling leaf rust resistance gene *Rph3* based on their susceptibility in the greenhouse and field against *Rph3* virulent pathotype and resistance to *Rph3* avirulent pathotypes. The remaining lines showed either presence of uncharacterised seedling resistance (1%) and uncharacterised APR (1%). Five percent of lines were susceptible at both seedling and adult plant growth stages. Of the six lines identified to carry uncharacterised APR, three likely carried *Rph20* based on the presence of the *Rph20*-linked marker *bPb-0837*. The results suggested that most of the ICARDA germplasm tested is not suitable for leaf rust resistance in Australia due to the presence of virulence for *Rph3*. Lines carrying uncharacterised seedling resistance and APR are potentially new sources of resistance, and are recommended for genetic analysis.

Key words: *Hordeum vulgare*, *Puccinia hordei*, adult plant resistance, *Rph3*.

INTRODUCTION

Cultivated barley (*Hordeum vulgare* L. subsp. *vulgare*) is an important cereal crop (Ullrich, 2011) which ranks fourth in the world's production after wheat, maize and rice (Schulte et al., 2009).

It is grown widely in Australia, where it is an important multi-billion dollar industry. The gross value of barley production in Australia is, however, hampered by many constraints, of which diseases alone account for an estimated average annual loss of $252 million (Murray and Brennan, 2010). Of the diseases that afflict barley, leaf rust (caused by *Puccinia hordei* Otth.) is considered to be most destructive in many parts of the world (Clifford, 1985). Significant losses due to leaf rust epidemics have been reported in Australia, New Zealand, Europe and USA (Murray and Brennan, 2010; Arnst et al., 1979; Cotterill et al., 1992; Griffey et al., 1994; Melville et al., 1976). Many of the known seedling leaf rust resistance genes have been rendered ineffective by the emergence of new pathotypes (pts) of *P. hordei* with matching virulence (Park, 2003).

During the years 1992 to 2001, eight new pts, each virulent for *Rph12*, were detected in Australia (Park, 2008) and recently, following the release of several barley cultivars with *Rph3*, a new pathotype (pt) with

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Table 1. Details of the four International Barley Observation Nurseries introduced from ICARDA and used in this study.

IBON	Year of release	No. of lines	No. of unique pedigrees
31st	2003	205	117
32nd	2004	266	190
33rd	2005	205	160
34th	2006	144	112

IBON: International Barley Observation Nursery

Table 2. Detail of *Puccinia hordei* pathotypes used for greenhouse and field screening of four international barley nurseries sourced from ICARDA.

Pathotype	PBI Culture no.	Virulence for genes
$200P^-$	518	*Rph8*
$253P^-$	490	*Rph1, Rph2, Rph4, Rph6, Rph8*
$5610P^+$	520	*Rph4, Rph8, Rph9, Rph10, Rph12, Rph19*
$5653P^+$ +*Rph13*	542	*Rph1, Rph2, Rph4, Rph6, Rph8, Rph9, Rph10, Rph12, Rph13, Rph19*
$5652P^+$	561	*Rph2, Rph4, Rph6, Rph8, Rph9, Rph10, Rph12, Rph19*
$5457P^+$	612	*Rph1, Rph2, Rph3, Rph4, Rph6, Rph9, Rph10, Rph12, Rph19*

PBI: Plant Breeding Institute.

virulence matching *Rph3* ($5457P^+$) was detected (Park, 2010). Currently, only six seedling resistance genes *Rph7*, *Rph11*, *Rph14*, *Rph15*, *Rph18* and lately mapped *Rph21* (Sandhu et al., 2012) are effective in Australia. In this context, several previous studies (Golegaonkar et al., 2009; Park, 2003, 2008) stressed the need to identify new sources of resistance to leaf rust in barley, including APR. Seedling resistance gene, temporally designated *RphMBR1012*, conferring resistance to the most virulent European leaf rust pathotypes, was mapped to the telomeric region of chromosome 1HS (König et al., 2012).

The first gene conferring APR to leaf rust in barley, *Rph20*, was mapped on chromosome 5HS (Hickey et al., 2011). Two markers linked to *Rph20*, *EBmag0833* and *bPb-0837*, were reported by Liu et al. (2010), who proposed the use of *bPb-0837* in marker assisted selection for APR against *P. hordei*. Recently, Singh et al. (2015) mapped the second APR gene *Rph23* on chromosome 7H in *H*. *vulgare* which provides additive resistance against *P. hordei* under field conditions.

In addition to barley leaf rust, in the presence of heavy inoculum, stem rust caused by either *Puccinia graminis* Pers. f. sp. *tritici* Eriks., E. Henn., *P. graminis* Pers. f. sp. *secalis* Eriks., E. Henn., or the scabrum rust (Park, 2008), can affect barley in Australia. The barley stripe rust pathogen *Puccinia striiformis* f. sp. *hordei* does not occur in Australia (McIntosh et al., 2001; Park, 2008) but *P. striiformis* f. sp. *pseudo-hordei* (barley grass stripe rust; BGYR, Wellings, 2011; Wellings et al., 2000), can also infect some barley genotypes and wild barley grass in Australia.

The rust resistance of entries in several recent International Barley Observation Nurseries (IBONs) developed at ICARDA were examined in an attempt to identify potentially new sources of seedling resistance and APR to leaf rust. Four different IBONs (31st to 34th), released from 2003 to 2006, were examined.

MATERIALS AND METHODS

Plant materials

Eight hundred and twenty lines representing four IBONs were introduced from ICARDA (Table 1), and screened for rust response in the greenhouse and field. The original nurseries were provided by the international nurseries program at ICARDA, Aleppo, Syria. Set of differential lines carrying seedling genes was used as controls for *P. hordei* (Table 4) as described by Park (2003) and Sandhu et al. (2012).

Pathogen material

Different pts of *P. hordei* were sourced from the rust collection maintained in liquid nitrogen at the Plant Breeding Institute (PBI), University of Sydney. For seedling tests, two pts of *P. hordei* ($5457P^+$ and $5652P^+$) were used. In field testing, the predominant *P. hordei* pts were $5652P^+$ (2007 and 2008) and $5457P^+$ (2009). For multipathotype tests, four additional pts ($200P^-$, $253P^-$, $5610P^+$ and $5653P^+$ +*Rph13*) were used. The virulence of the pts against seedling resistance (*Rph*) genes is detailed in Table 2.

Greenhouse screening

For greenhouse tests, all lines along with differential sets were planted in pots filled with the mixture of fine bark and coarse sand and fertilized using “Aquasol®” (100 g per 10 L of water per

Table 3. Classification of ICARDA germplasm with respect to rust resistance based on the field and greenhouse tests with *Puccinia hordei* pathotypes 5652P$^+$ and 5457P$^+$.

Category[1]	IBON 31	IBON 32	IBON 33	IBON 34	Total	Postulation
C1	197	233	180	118	728	*Rph3*
C2	0	2	0	3	5	USR[2]
C3	0	2	0	4	6	UAPR[3]
C4	3	21	4	16	44	Susceptible
C5	5	8	21	3	37	Missing lines

[1]C1 = Lines resistant to pathotype 5652P$^+$ and susceptible to 5457P$^+$ both in greenhouse and field; C2 = Lines resistant to pts 5652P$^+$ and 5457P$^+$ both in the greenhouse and the field; C3 = Lines susceptible in the greenhouse but resistant in the field to pts 5652P$^+$ and 5457P$^+$; C4 = Lines susceptible in the greenhouse and the field to pts 5652P$^+$ and 5457P$^+$; C5 = Missing; [2] USR = Uncharacterised seedling resistance; [3] UAPR = Uncharacterised adult plant resistance.

200 pots) prior to sowing. Seedlings of differentials and barley lines were raised in 9 cm diameter pots by sowing four clumps (test lines) or five clumps (differentials) of each genotype using 8 to10 seeds per clump. Following sowing, pots were kept in a growth room at 20±2°C for germination. Seven-day old seedlings were fertilised with granular urea using "Incitec Pivot" w/w 46% nitrogen (50 g per 10 L of water per 200 pots). Seedlings at the one and a half leaf growth stage (9 to 10 days old) were inoculated and incubated according to the methods described by Sandhu et al. (2012). The seedlings were then transferred to naturally well lit microclimate rooms maintained at 23±2°C and scored 10 to 12 days after inoculation using 0 to 4 scale as described by Park and Karakousis (2002).

Field screening

All lines were tested at the field site Karalee. The lines were hand-sown as hill plots (30 to 40 seeds/plot) using 30 cm spacing during mid to late June in 2007, 2008 and 2009. A row of the susceptible cultivar Gus was sown as a rust spreader after every five hill plots of barley lines to allow the build-up and uniform distribution of inoculum. Four weeks after sowing, plots were fertilised using granular urea "Incitec Pivot" w/w 46% nitrogen @ 100 kg/hectare followed by irrigation. Plots were irrigated once a week or as required, using fixed sprinklers.

Field epidemics of leaf rust were created following the procedures described by McIntosh et al. (1995). Urediniospores (30–40 mg) were suspended in 1.5 L of light mineral oil (Shellsol®, Mobil Oil) and sprayed over buffer/spreader lines with an ultra-low-volume applicator (Microfit®, Micron Sprayer Ltd., UK). Four to five inoculations were performed during late evening on days that had a strong forecast of overnight dew. On the first and second inoculations, hot spots of disease were established by watering and covering small areas of the rust spreader with plastic hoods overnight to ensure adequate dew formation in case natural dew formation did not occur. Leaf rust was scored at the flag leaf growth stage in all three seasons using a modified Cobb's scale (Peterson et al., 1948). Percentage of leaf area infected was followed by different scales; Immune (0), Resistant (R), Resistant to Moderately Resistant (R–MR), Moderately Resistant (MR), Moderately Susceptible (MS) and Susceptible (S).

Molecular analysis

Genomic DNA was extracted from leaf tissue of seedlings of barley lines characterised with APR as per techniques described by Bansal et al. (2010). The molecular marker *bPb-0837* (Liu et al. 2010), closely linked to the APR leaf rust resistance gene *Rph20* was used to genotype the lines selected from different nurseries. Ten micro litres of PCR reaction contained 2.0 µl of genomic DNA (50 ng), 1.0 µl of dNTPs (0.2 mM), 1.0 µl of 10x PCR buffer (Immobuffer, including 15 mM $MgCl_2$), 0.25 µl of each forward and reverse primer (10 µM), 0.04 µl of *Taq* DNA (500 U Immolase DNA polymerase from Bioline) and 5.46 µl of ddH_2O. The PCR amplification profile comprised an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 30 s denaturation at 94°C, 60 s annealing at 55°C, 60 s extension at 72°C and a final extension step of 5 min at 72°C. Reaction was performed in a 96-well DNA theromocycler (Eppendorf Mastercycler, Germany).

PCR products were mixed with 3.0 µl of formamide loading buffer (98% formamide, 10 mM EDTA (pH 8.0), 0.05% (wt/vol) Bromophenol blue and 0.05% xylene cyanol). Two percent agarose gels were prepared by adding 2.0 gm agarose (Bioline) per 100 ml of 1x Tris-borate EDTA (TBE) buffer (90 mM Tris-borate + 2 mM EDTA-pH 8.0). For staining, 1.0 µl of ethidium bromide was added per 100 ml of gel solution. The gel solution was poured into moulds and allowed to cool for 40 min at room temperature. Eight to 10.0 µl of PCR product including loading buffer was loaded per well. One kb DNA marker HyperLadder™ IV (Bioline) was used as reference. Electrophoresis was carried out at 110 V for 1.5 h, and the separated bands were visualised under ultra violet light unit fitted with a GelDoc-IT UVP Camera.

RESULTS

89% (728 out of 820) of the lines tested from the four nurseries were resistant to pt 5652P$^+$ in seedling greenhouse tests and in adult plant field tests during 2007 and 2008. All 728 lines were susceptible in the greenhouse and in the field when tested with the *Rph3* virulent pt 5457P$^+$ in 2009, indicating the very probable presence of seedling gene *Rph3* only in all of these lines (Table 3).

Five lines (IBON 32.34, IBON 32.126, IBON 34.88, IBON 34.95 and IBON 34.126) displayed seedling resistance in greenhouse tests to all pts, and were also resistant in the field against the two *P. hordei* pts used. Six lines (IBON 32.183, IBON 32.202, IBON 34.8, IBON 34.41, IBON 34.54 and IBON 34.110) were resistant only in the field (during all three years), indicating the presence of APR. Line numbers IBON 32.202, IBON 34.41, IBON 34.54 and IBON 34.110 showed resistant

Table 4. Seedling responses of selected lines and control differential genotypes against six *Puccinia hordei* pathotypes.

IBON Line	253P-	200P-	5610P+	5653P++*Rph13*	5652P+	5457P+	Postulated Resistance	Field score	Pedigrees of IBON Lines
IBON 32.34	0;=	0;=	0;=	0;=	0;-	0;	USR	10R	Petunia 1/Winchester//Ciru
IBON 32.126	0;=	0;=	0;=	0;=	;C	0;	USR	0	M9878/Cardo//Quina/3/Petunia 1/4/Ciru
IBON 34.88	0;=	0;=	0;=	0;=	0;=	0;=	USR	0	Br2/I.p//Azaf
IBON 34.95	0;=	0;=	0;=	0;=	0;=	0;=	USR	0	Br2/I.p//Azaf
IBON 34.126	0;=	0;=	0;=	0;=	0;=	0;=	USR	50R	Canela/pfc9201//Msel
IBON 32.183	33+	33+	33+	33+	3+	3+	UAPR	40MR	Atah92/Gob//f101.78/3/Arupo/k8755//Mora
IBON 32.202	33+	33+	3+	33+	3+	3+	UAPR	R–MR	Triumph-bar/Tyra//Arupo*2/abn-b/3/Canela/4/Canela/Zhedar#2
IBON 34.8	33+	33+	33+	33+	3+	3+	UAPR	60MR-MS	Cheng du 105/Cabuya//Petunia 1
IBON 34.41	33+	33+	33+	33+	3+	3+	UAPR	R–MR	Scotia1/wa1356.70//wa1245.68/Boyer/ 3/mja/brb2//Quina/4/La Molina 94
IBON 34.54	33+	33+	33+	33+	3+	3+	UAPR	R–MR	Acuario t95/br2//Msel
IBON 34.110	33+	3+	33+	33+	3+	3+	UAPR	10R	Atah92/Gob//f101.78/3/Arupo/k8755//Mora
Differential/*Rph* gene									
Gus/Nil	3+	3+	3+	3+	3+	3+	Nil	-	-
Sudan/*Rph1*	3+	0;	0;-	3+	0;-	3+	*Rh1*	-	-
Berg/*Rph1*	3+	0;C	0;-	3+	0;-	3+	*Rph1*	-	-
Peruvian/*Rph2*	33+C	;1CN	;1CN	33+	3+	3+	*Rph2*	-	-
Reka 1/*Rph2+RphP*	;1=C	;1=C	;C	3+	3+	3+	*Rph2+RphP*	-	-
Ricardo/*Rph2+ Rph21*	2+3-C	;1CN	12+CN	1+2-CN	2++3-C	11+2C	*Rph2+ Rph21*	-	-
Estate/*Rph3*	;-C	0;-	;-C	0;=	;-C	3+	*Rph3*	-	-
Gold/*Rph4*	2++3	;11-	3+	3+	3+	3+	*Rph4*	-	-
Magnif 104/*Rph5*	0;=	0;=	0;-	;	0;=	0;C	*Rph5*	-	-
Quinn/*Rph2+Rph5*	0;=	0;C	0;-	0;-	0;=	0;C	*Rph2+Rph5*	-	-
Bolivia/*Rph2+Rph6*	1++,3+	;C	;CN	3+	3+	3+	*Rph2+Rph6*	-	-
Cebada Capa/*Rph7*	0;-N	0;N	0;CN	;CN+	0;N	;N	*Rph7*	-	-
Egypt 4/*Rph8*	0;-	3+	3+	3+	3+	1++CN+	*Rph8*	-	-
Abyssinian/*Rph9*	;CN	;CN	3+	33+	3+	3+	*Rph9*	-	-
Clipper BC8/*Rph10*	;1=C	;1=C	33+	33+	3+	3+	*Rph10*	-	-
Clipper BC67/*Rph11*	;1-C	;11+C	1++C	;1C	;11++	2++3C	*Rph11*	-	-
Triumph/*Rph12*	0;C	0;CN	3+	33+	3+	3+	*Rph12*	-	-
PI 531849/*Rph13*	0;=	0;=	0;=	3+	0;-C	;CN	*Rph13*	-	-
PI 584760/*Rph14*	11+C	11++2+C	33+	;CN+	;1=C	;1-CN	*Rph14*	-	-
Bowman+*Rph15*/*Rph15*	;CN+	0;C	;CN	;CN+	;CN	;CN+	*Rph15*	-	-
81882/BS1/*Rph17*	;1-C	;C	;11+C	;1-CN	;1=C	;1-C	*Rph17*	-	-
38P18/8/1/10/*Rph18*	0;=	0;=	0;=	0;=	0;=	0;=	*Rph18*	-	-
Prior/*Rph19*	0;-	0;=	3+	3+	3+	3+	*Rph19*	-	-

USR: Uncharacterised seedling resistance, UAPR: Uncharacterised adult plant resistance.

Table 5. Validation of marker *bPb-0837* on ICARDA barley lines carrying adult plant resistance to *Puccinia hordei* including controls.

IBON No.	Field score	*bPb-0837*
32.183	40 MR	+
32.202	R–MR	-
34.8	60 MR-MS	-
34.41	R–MR	-
34.54	R–MR	+
34.110	10 R	+
Controls		
Flagship	10 R	+
Stirling	70 S	-
Pompadour	10 R	+
Baronesse	10 R	+
WI 3407	5 R	+
Ricardo	10 R	-
Gus	90 S	-

+ and - indicates presence and absence of marker respectively.

responses of R–MR to 10R at adult plant growth stages and line numbers IBON 32.183 and IBON 34.8 showed MR to MR–MS responses under field conditions (Table 5). Adult plant responses against *P. hordei* pt 5457P$^+$ under field conditions are shown in Figure 3.

An overall analysis of the total 783 lines tested (excluding 37 missing lines) showed that 93% carried the major seedling resistance gene *Rph3*, 5.6% of the lines were susceptible at both growth stages, 0.65% of the lines were resistant to both *P. hordei* pts at both growth stages, and 0.75% of the lines possessed uncharacterised adult plant resistance (UAPR) as shown in Figure 1.

Multipathotype testing

The five lines in category C2 and six lines in category C3 (described in Table 3) were subjected to multipathotype tests in the greenhouse using six pts of *P. hordei*. Sets of differential lines included in these tests showed the expected infection types (ITs) against all six pts (Table 4).

Barley lines IBON 34.8, IBON 34.41, IBON 34.54, IBON 34.110, IBON 32.183 and IBON 32.202, which were resistant in the field, displayed high ITs against all six pts when tested in the greenhouse, indicating that they lacked detectable seedling resistance genes and confirming the presence of UAPR in all six lines. In contrast, lines IBON 32.34, IBON 32.126, IBON 34.88, IBON 34.95 and IBON 34.126, resistant in the field, also showed resistance in the greenhouse against all six *P. hordei* pts (Table 4).

Lines 88 and 95 from IBON 34 had the same pedigree and are hence sib-lines. Based on the resistance of these lines against the six pts used, they could carry either *Rph5*, *Rph7*, *Rph11*, *Rph14*, *Rph15*, *Rph18*, *Rph21* or another uncharacterised seedling resistance (USR) gene. However, differences in the ITs produced by these lines in comparison with control genotypes carrying these genes suggested that the gene(s) present in each may be different. Differentials produced the expected ITs as shown in Figure 2.

Molecular marker analysis

Barley lines IBON 34.8, IBON 34.41, IBON 34.54, IBON 34.110, IBON 32.183 and IBON 32.202, which were resistant at adult plant stage in the field and displayed high ITs against all six pts at the seedling growth stage (Table 4), were selected for genotyping with the *Rph20* linked marker *bPb-0837* (Liu et al. 2010). Barley lines IBON 32.183, IBON 34.54 and IBON 34.110 amplified a 245 bp band, whereas no amplification occurred in lines IBON 32.202, IBON 34.8 and IBON 34.41 with marker *bPb-0837*. The control cultivars Pompadour, Baronesse, WI 3407 and Flagship amplified 245 bp bands, while no band was produced in tests with Stirling, Gus and Ricardo (Figure 4 and Table 5).

DISCUSSION AND CONCLUSION

Of the designated major seedling genes that confer resistance to *P. hordei* in barley (*Rph1* to *Rph21*), only *Rph7*, *Rph11*, *Rph14*, *Rph15*, *Rph18*, *Rph21* (Park, 2003; Park, 2010; Sandhu et al., 2012) and *Rph20* (Hickey et al., 2011) are effective in Australia. It is well known that major genes can be easily overcome by new pts of *P. hordei*. This situation has occurred in Australia, with the frequency of virulence for *Rph4* increasing following the widespread use of cultivar Grimmett carrying *Rph4* (Cotterill et al., 1995), for *Rph12* following the releases and widespread cultivation of barley cultivars including Franklin, Tallon, Lindwall and Fitzgerald, all of which carry *Rph12* (Park, 2008) and more recently for *Rph3*, following the releases of cultivars Fitzroy, Yarra and Starmalt carrying *Rph3* (Park, 2010). Plant breeders therefore have a limited choice in terms of resistance sources against *P. hordei*. In view of this, the present study sought new sources of resistance to *P. hordei* in four of the IBONs, which are distributed annually by ICARDA.

Tests of leaf rust response indicated that 44 lines were susceptible at both seedling and adult plant growth stages to all of the pts tested. During 2007 and 2008, more than 93% of the entries tested showed high levels of resistance to leaf rust in both seedling greenhouse and adult plant field tests. In early 2009, for the first time in

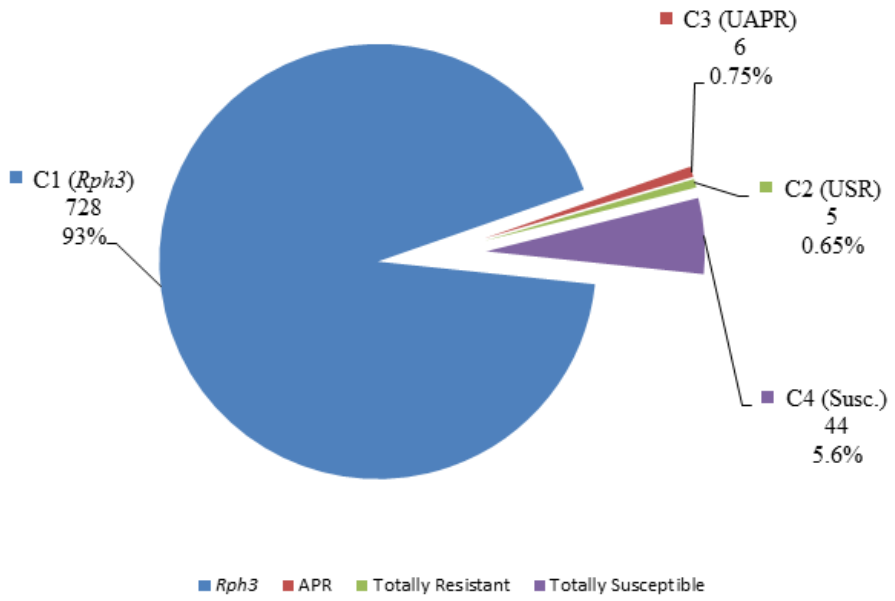

Figure 1. Percentage of leaf rust resistance among 783 barley lines from four international barley observation nurseries distributed by ICARDA. Categories (C1 to C4) of resistance as described in Table 3.

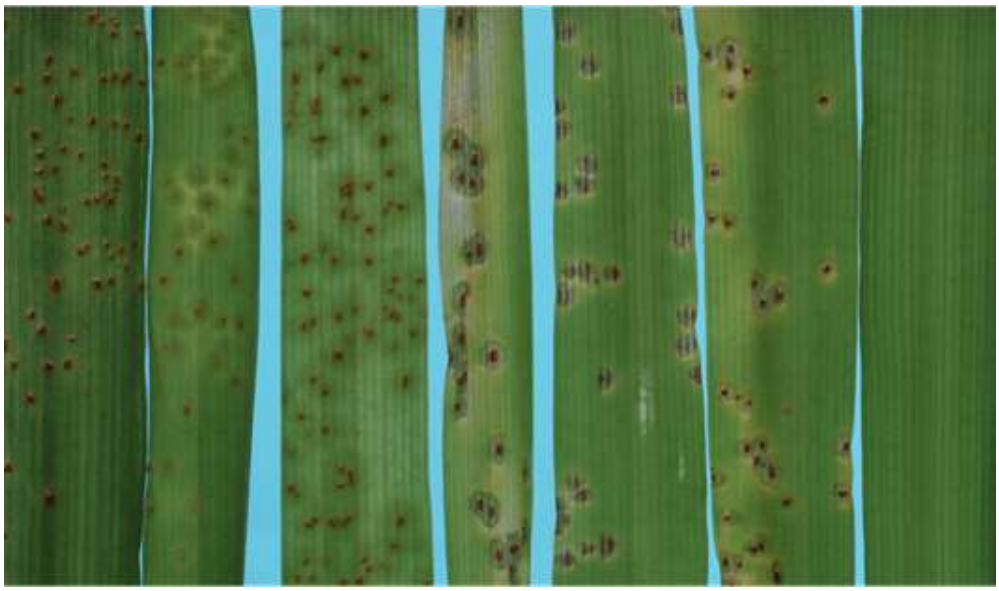

Figure 2. Rust response of control lines against *Puccinia hordei* pathotype 5457P$^+$ L to R: Gus (3+), Ricardo (11+2C), Estate (*Rph3*, 3+), Egypt 4 (*Rph8*, 1++CN+), Cebada Capa (*Rph7*, ;N), 81882/BS1 (*Rph17*, ;1-C) and 38P18/8/1/10 (*Rph18*, 0;=)

Australia, virulence for the seedling resistance gene *Rph3* was detected with the identification of a pt 5457P$^+$ from the northern NSW (Park, 2010). When the nursery entries were tested with this new pt, 93% were susceptible in both the greenhouse and the field, strongly indicating that the resistance detected in previous tests was due to a single major gene, *Rph3* and that no additional resistance was present in these lines. The occurrence of *Rph3* only, in 93% of this germplasm indicated that it is highly vulnerable to leaf rust.

Five entries showed resistance to leaf rust that was effective in the field against pts 5652P$^+$ and 5457P$^+$ as

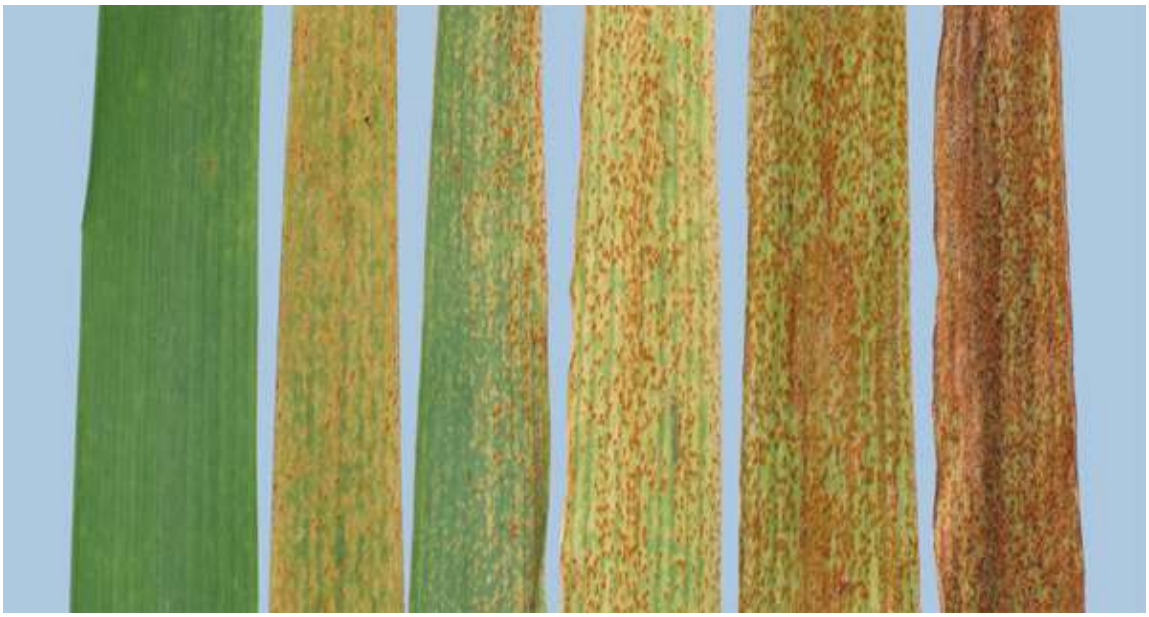

Figure 3. Field responses of barley lines; L to R: 0, R, MR, MS, S and leaf death due to high infection of leaf rust pathotype 5457P^{+}.

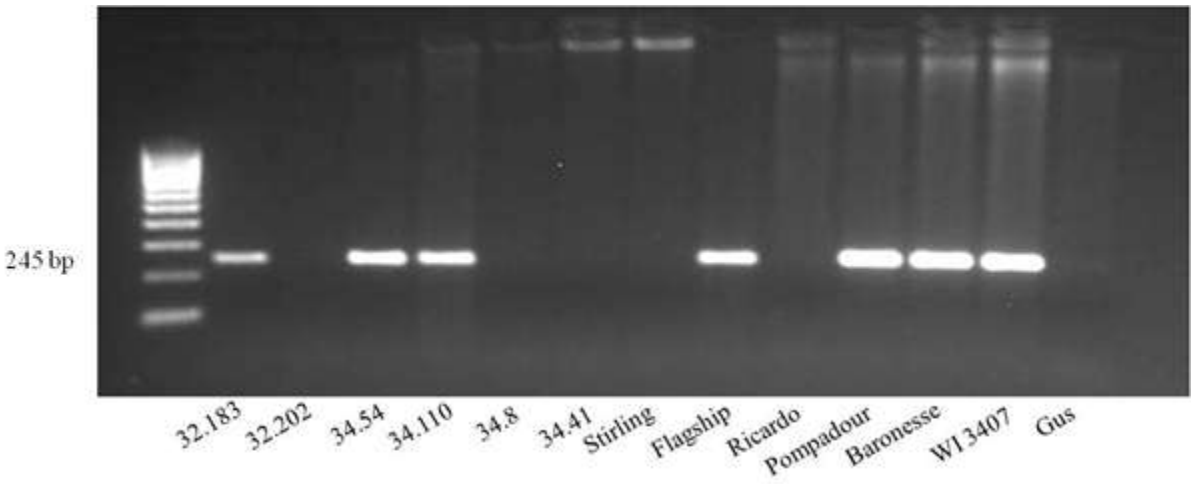

Figure 4. APR gene *Rph20* linked marker *bPb-0837* amplifications at 245 bp; L to R: Lines 183, 202 (IBON 32) and 54, 110, 8, 41 (IBON 34) and controls Stirling, Flagship, Ricardo, Pompadour, Baronesse, WI 3407 and Gus.

well as to a range of pts in the greenhouse (Table 4). Based on the ITs generated against a range of pts, it was postulated that these lines carry one or more unknown seedling resistance genes. While it is possible that these lines may carry one of the resistance genes (*Rph5*, *Rph7*, *Rph11*, *Rph14*, *Rph15*, *Rph18* and or *Rph21*) that is effective against all of the pts used, this was considered unlikely because all five entries showed ITs that differed from those shown by all of the known effective genes except *Rph18*. It was considered unlikely that these nursery entries carried *Rph18* because this resistance gene is derived from *Hordeum bulbosum* (Pickering et al., 2000) and has not yet been deployed in breeding programs. Genetic studies are therefore needed to characterise the seedling resistance identified in these five lines.

On the basis of multipathotype testing in the greenhouse and field testing for three consecutive seasons, six lines that carry APR to leaf rust were identified. It is known that many European cultivars carry APR to *P. hordei* (Golegaonkar et al., 2009; Park, 2008). Positive validation of the molecular marker *bPb-0837* amplified 245 bp bands in lines IBON 32.183, IBON 34.54 and IBON 34.110 indicated the likely presence of the APR gene *Rph20*, reported on chromosome 5H (Hickey et al., 2011) and closely linked to this marker (Liu et al., 2010).

Similar amplification of 245 bp bands from DNA of reference stock; Pompadour, Baronesse, WI 3407 and Flagship by marker *bPb-0837* (Liu et al., 2010) also supported the likely presence of *Rph20* in genotypes IBON 32.183, IBON 34.54and IBON 34.110. The marker *bPb-0837* failed to amplify a product in lines IBON 32.202, IBON 34.8 and IBON 34.41, indicating the likely presence of uncharacterised APR in each. These lines were resistant in the field and susceptible in the greenhouse to a range of *P. hordei* pts. The lines IBON 32.202 and IBON 34.41 showed identical field responses and it is possible that they might have a gene in common. The cultivar Ricardo was resistant in the field but failed to produce a PCR product when genotyped using marker *bPb-0837*, indicating the likely presence of an unknown resistance under field conditions. It will be useful therefore to undertake genetic analysis and allelic studies of unknown APR present in lines IBON 32.202, IBON 34.8 and IBON 34.41, along with Ricardo, to determine the mode of inheritance and their genetic relationship with the only other named APR gene for leaf rust in barley, *Rph20* on chromosome 5H.

Given that virulence for seedling resistance gene *Rph3* is now present in eastern Australia, 93% of the germplasm tested here carries *Rph3* only is of limited value for leaf rust resistance. The diversity of leaf rust resistance among these four ICARDA nurseries is very narrow as only 11 lines (6 with unknown APR and 5 with unknown seedling resistance) were identified in the study. Out of six lines with APR, three likely carry *Rph20*. Eight lines (three with APR and five with seedling resistance) were identified that carry potentially uncharacterised resistance to leaf rust and are therefore potentially valuable as new sources of resistance.

It is recommended to undertake genetic analysis of these eight lines to understand their inheritance and genetic relationship with other known genes for their effective utilisation in breeding programs. The present studies again stress the importance of identifying new sources of leaf rust resistance to diversify the genetic base of resistance and for additional and better choice of resistance in breeding programs. At the same time, deployment of single major known effective genes *(Rph7, Rph11, Rph14, Rph15, Rph18 and Rph21)* should be conducted wisely by avoiding the release of cultivars with single major genes only. Efforts should be made to pyramid the resistance genes available to reduce the chance of matching virulence developing in pathogens. Given that only one gene conferring APR to leaf rust in barley has been characterised to date, the identification of one or more potentially new sources of APR could provide a means of achieving durable resistance via APR genes pyramiding.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Comparing pollination control bag types for sorghum seed harvest

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Efficacy of pollination bags made of new nonwoven fabrics was compared with the traditional paper bags in sorghum during 2015 using three cultivars comprising BR007B (red seeded), SC283 (white seeded) and 1167048 hybrid with tannin (brown seeded). The five pollination bag treatments were: no bagging, traditional paper bag, paper bag plus plastic screen bag for extra bird protection, duraweb® SG2 polypropylene bag and duraweb® SG1 polyester bag. There was no bird damage on tannin hybrid but birds damaged bags to access grains of the other two varieties. Varieties and bag types differed significantly, and also showed significant interactions for panicle weight (at *P*<0.06), seed weight and average seed weight per panicle. The tannin hybrid was consistently a better performer for all traits regardless of bag type. The paper bags were the worst for bird damage. Duraweb® SG1 was the best performer for all traits including bird damage followed by duraweb® SG2. The joint regression analysis showed that BR007B performed consistently under all bag types with average response. On the other hand, SC283 improved its response with the increasing quality of bag type at an above average rate for panicle weight and seed traits. It was concluded that new nonwoven fabric bags could replace paper bags in providing better seed production potential and greater protection against bird damage.

Key words: Sorghum, pollination bags, panicle weight, seed weight, bird control.

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) has great inherent variation with as many as 40,000 germplasm accessions in the US sorghum collection alone, in addition to germplasm collections of many countries of their own (Dahlberg et al., 2011). Maintenance of these germplasm accessions and breeding lines at numerous research stations is facilitated by isolating the genetic accessions and breeding lines from contamination with foreign pollen. This is achieved by the use of pollination control bags. Pollination bags are not only used in artificial hybridization or self-pollination but also for controlling bird damage in the extremely small plots of thousands of germplasms accessions and breeding lines (Ormerod and Watkinson, 2000; Gitz et al., 2013, 2015). Traditionally, plant breeders have been covering the panicles of sorghum with paper bags for pollen control and to protect developing seeds from bird damage and for hybridization of different types of sorghum for genetic

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improvement. Such bags are not very effective against bird damage because the birds over time associate the paper bags, which they can tear off with their beaks, with food of developing seeds underneath. Also paper bags get torn off in the rainy season and heavy winds during the hybridization process. This may lead to high losses of the valuable hybrid seed in the breeding process. Gitz et al. (2013) tested the efficacy of Tyvek® polyethylene bags and found them resistant to bird damage. Gitz et al. (2015) compared the polyethylene and paper bags for pollen transmission and microenvironment within them as this affects seed development. They reported no pollen transmission differences between hard form Tyvek® polyethylene and paper bags but the soft form Tyvek® polyethylene bags allowed 35 to 40% wind borne pollen through the pores. However, heating within the soft and hard polyethylene bags was 25 and 50% that of paper bags, respectively. These studies clearly indicated the need for studies on alternatives to commonly used paper bags in sorghum.

An enormous variety of synthetic fabrics can be made with both woven and nonwoven techniques, and by using knowledge of the polymers, manufacturing processes and fiber properties it is possible to identify fabrics which may produce near-ambient micro-environment within pollination bags for seed development. However, plant breeders have not paid much attention to pollination bags and limited studies have been conducted to compare their efficacy particularly in sorghum (Gitz et al. 2013; Gitz et al. 2015). Gitz et al. (2013) while looking for solutions for maximizing seed yield of breeding/germplasm lines for mechanical sowings by minimizing bird damage could not find off the shelf pollination bags and were unable to identify bags specifically for sorghum. A few studies on rye grass (Griffiths and Pegler, 1963; Foster, 1968; McAdam et al., 1987), switchgrass (Vogel et al., 2014) and trees (McGranahan et al., 1994; del Rio and Caballero, 1999; Neal and Anderson, 2004) highlight the importance of choosing the most efficient pollination bags. PBS International has developed a nonwoven material, duraweb®, specifically for plant breeding purposes, although the researchers believed that this particular material could be developed further for the purposes of this application to increase airflow.

The objectives of this study were to compare the efficacy of two novel materials identified and developed by PBS International for the purpose of experimentation in sorghum against traditional paper bags and to evaluate the effect of different bag types on the performance of different varieties for some seed harvest traits. Such traits include their relative protection against bird damage. The overarching aim was to maximize seed production during segregating generations of crosses, germplasm maintenance and hybridization processes for breeding purposes.

MATERIALS AND METHODS

The present investigation was carried at the Embrapa Milho e Sorgo in Sete Lagoas, MG Brazil research station during 2015. The experiment was conducted during the winter season in a split-plot design with three varieties in the main plots and five bag types in the sub-plots. There were four complete replicate blocks in the experiment. Each sub-plot consisted of one five meter row with 70 cm spacing between rows having 8 to 10 plants per meter.

Three varieties were distinct for the seed coat colour. This was purposely done to see if there is any relationship of seed coat color and bird choice. The varieties were: BR007B with red seeds; SC283 with white seeds, and 1167048 – a brown seeded experimental hybrid with tannin (bird resistant) and referred to as Tannin line hereafter. Panicles were covered by pollination bags before pollination. There were five bag treatments:

1. No bagging (control)
2. Normal Kraft paper pollination bag
3. Normal Kraft paper pollination bag covered by a plastic screen bag for extra protection following pollination and at seed formation.
4. Duraweb® SG2 pollination bag of size 400 mm x 215 mm made from nonwoven polyester with a smooth paper like surface.
5. Duraweb® SG1 pollination bag of size 400 mm x 215 mm made of coarse nonwoven polypropylene with a point-bonded surface

Of the 5 rows of a variety whole-plot in a replication block, one row was allocated to each of the 5 bag treatments. Five panicles were covered by each pollination bags in a row of a variety plot. Observations were made on all 5 panicles in each plot. Data were collected on number of panicles per treatment, panicle weight (g) and average seed weight (g) per panicle. Each panicle was threshed separately in a head thresher and seed weight was recorded in grams. There was slight variation in the panicle number per treatment. Therefore, we performed a covariance analysis using panicle number as the covariate following Snedecor and Cochran (1974) for all traits and using MINITAB 16 package. When the covariance with panicle number was not significant then the analysis of variance was re-performed without the covariate.

The analysis of varieties x pollen control treatment interactions was performed by fitting linear regressions of variety mean values on to the mean values of each bag type following Yates and Cochran (1938), Finlay and Wilkinson (1963), Eberhart and Russell (1966) and Perkins and Jinks (1968). The mean of bag type equates to environmental indices in these studies. A joint regression analysis was used to characterize the sensitivity (inversely instability) of varieties due to bag effects by partitioning the variety x bag type interaction into heterogeneity of regressions and residual interactions. Since regression of panicle weight was significant on panicle number in the covariance analysis adjusted mean values were used for the joint regression analysis for panicle weight.

RESULTS

Bird damage

It was observed that bag treatments 3 (Paper bag+

Figure 1. Tearing of paper bag by the pushing panicle (left) and no tearing effect on duraweb® SG2 bags.

plastic screen bag at grain filling), 4 (duraweb® SG2) and 5 (duraweb® SG1) were similar and more effective in protecting against birds and insects. Bird damage under no bagging treatment 1 (control) and paper bag treatment 2 was high on white and red seeded varieties. However, no bird damage was observed on the brown seeded hybrid with tannin. The astringency from the tannins is what causes the dry and 'pucker' feeling in the mouth following the consumption of unripe seed (McGee, 2004).

Tannin is a polyphenolic biomolecule that binds to proteins and various other organic compounds including amino acids and alkaloids. The tannin compounds are found in many species of plants where they play a role in protection from predation, and perhaps as pesticides, and in plant growth regulation (Katie and Thorington, 2006). This deters birds unless there is no other food source available.

The bird pressure in the 2015 winter season was medium as the above average rainfall provided alternative food sources for the birds. No bird damage was observed on the tannin variety. The birds preferred the white and red varieties which appeared equally appealing. It was estimated that about 50% of the panicles were damaged in the uncovered treatment (treatment 1) and in the kraft paper bag condition (treatment 2) about 20 to 25% bags were damaged.

From images taken in the experimental field, we observed that paper bags suffered damage made by the birds and by the growth of the panicles bursting the end of the bag indicating their weakness in protection. We have experienced that in some years, when the bird pressure is particularly high, as much as 100% of paper bags are torn open and the plastic screen bags can even be removed by birds requiring multiple visits to re-enforce them. In contrast, the experimental treatments 3, 4 and 5 in the year of this research did not suffer any damage due to the strength of the materials (Figures 1, 2 and 3). It shows that new bags 4 and 5 have strength similar to a paper bag plus protective plastic screen.

Covariance analysis

Since panicle number was variable across treatments it was introduced as covariate in the analysis of variance for panicle weight, seed weight and average seed weight per panicle. The covariance of panicle number was significant for panicle weight (g) but was non-significant for seed weight and average seed weight per panicle (Table 1). Therefore, analysis for panicle weight reported here is adjusted for the significant regression of panicle weight on variation in panicle number. The covariance takes 1 *df* from the error *df* for panicle weight which are 1 less than that for the other two traits (Table 1).

Analysis of variance and mean performance

There were significant differences between varieties and bag types for all traits. The varieties showed significant

Figure 2. Bird damage holes on paper bag (left) and no damage on duraweb® SG1 bags.

Figure 3. Extent of bird attack with small black birds sitting on panicles of their preferred varieties of white and red seed coats. No such bird attack occurred on brown seeded hybrid with tannin in the seed coat.

interaction with bag types for seed weight and average seed weight per panicle at $P < 0.01$ (Table 1). There was also a near significant interaction between main effects for panicle weight at $P = 0.06$.

Variety mean yield showed that the hybrid with tannin was superior for all three traits. Varieties SC283 and BR2007B were statistical similar for all traits although they changed ranks for seed weight and average seed weight compared to panicle weight. SC283 with higher mean panicle weight showed lower mean values for seed weight and average seed weight than BR007B (Table 2).

Mean performance of bag types showed that bag type 5 (duraweb® SG1) was superior to all other bags (Table 2, Figure 4) for panicle weight and average seed weight. It was followed by bag 4 (duraweb® SG2) which was superior to all for seed weight. Bag 3 (paper with screen)

Table 1. Mean squares from analysis of variance for panicle weight (g), seed weight (g) and average seed weight per panicle (g).

Source	*df*	Panicle weight (g)	Seed weight (g)	Av. seed weight per panicle
Panicle number	1	5431**	NS	NS
Rep	3	1282	606	19.79
Variety	2	131796**	103808**	4731.58**
Error (a)	6	754	442	26.63
Bag treatments	4	5137**	4987**	219.69**
Variety *Treatments	8	1353+	1438**	59.02**
Error (b)	35 (36)	646	424	14.92
Total	59	-	-	-

**P<0.01; +P=0.06; NS =Not-significant. Error (b) *df* in bracket are without covariate analysis for panicle number for seed weight and average seed weight where covariance with panicle number was non-significant.

Table 2. Mean performance of bag types over three varieties and varieties over five bag types for different traits. Grouping was carried out using Tukey Method at 95% confidence for all traits.

Bag type	Panicle wt (g)	Seed wt (g)	Av seed wt (g)	Variety	Panicle wt (g)	Seed wt (g)	Av seed wt (g)
5	162.10^{A}	106.26^{A}	23.34^{A}	Tannin	234.78^{A}	175.41^{A}	37.11^{A}
4	153.52^{AB}	108.62^{A}	22.37^{A}	SC283	98.27^{B}	48.56^{B}	9.92^{B}
3	149.69^{AB}	103.26^{AB}	20.86^{AB}	BR007B	90.26^{B}	52.80^{B}	11.06^{B}
2	129.38^{BC}	82.11^{BC}	17.38^{BC}	LSD 5%	16.32	13.22	2.48
1	110.82^{C}	61.04^{C}	12.87^{C}	LSD 1%	21.89	17.73	3.33
LSD 5%	21.06	17.06	3.20	-	-	-	-
LSD 1%	28.26	22.90	4.30	-	-	-	-

Mean values followed by the same letter do not differ significantly. Bag types; 1= no bagging, 2= paper bag, 3= paper bag + plastic bag, 4= duraweb® SG2, 5= duraweb® SG1. LSD = least significant difference.

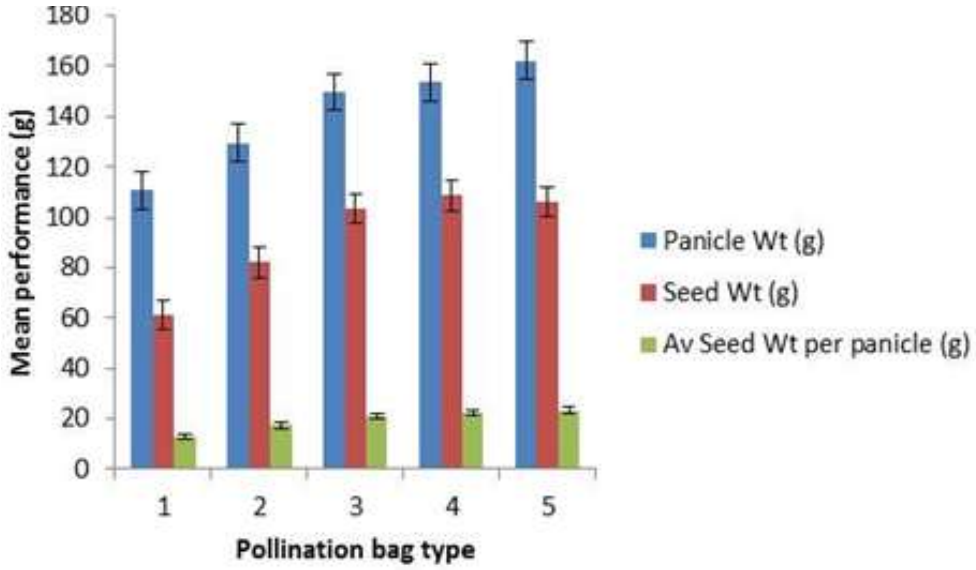

Figure 4. Mean performance of bag types over three varieties for different traits.

Table 3. Variety x pollination bag types interactions for panicle weight (g) after allowing for its covariance with number of panicles, seed weight (g) and average seed weight per panicle (g).

	Bag type[ξ]						
Variety	**1**	**2**	**3**	**4**	**5**	**Var Mean**	**LSD 5% Var Mean**
Panicle weight							
BR007B	32.57	94.56	113.34	100.70	110.12	90.26	16.32
SC283	64.14	79.76	110.20	112.77	124.47	98.27	-
Tannin	235.75	213.82	225.54	247.08	251.69	234.78	-
Bag Mean	110.82	129.38	149.69	153.52	162.10	141.10	-
LSD 5% Bag mean	21.06	-	-	-	-	-	-
LSD 5% Interactions	36.48	-	-	-	-	-	-
Seed weight (g)							
BR007B	0.53	60.06	74.44	71.09	57.88	52.80	13.22
SC283	5.35	27.6	63.15	69.01	77.71	48.56	-
Tannin	177.25	158.66	172.19	185.78	183.18	175.41	-
Bag Mean	61.04	82.11	103.26	108.62	106.26	92.26	-
LSD 5% Bag mean	17.06	-	-	-	-	-	-
LSD 5% Interactions	29.56	-	-	-	-	-	-
Average seed weight per panicle							
BR007B	0.11	12.01	15.51	14.22	13.45	11.06	2.48
SC283	1.14	6.47	12.63	13.80	15.54	9.92	-
Tannin	37.35	33.66	34.44	39.09	41.02	37.11	-
Bag Mean	12.87	17.38	20.86	22.37	23.34	19.36	-
LSD 5% Bag mean	3.20	-	-	-	-	-	-
LSD 5% Interactions	5.44	-	-	-	-	-	-

[ξ]1= no bagging, 2= paper bag, 3= paper bag + plastic bag, 4= durawed ® SG2, 5= duraweb® SG1.

was inferior to 4, and 5 for seed weight and average seed weight but was similar to bag 2 (paper bag). Statistically, Bags 4, 5 and 3 fall in the same group for all traits. Treatments 1 (no bag) and 2 (paper bag) were similar and inferior for all traits.

Variety x bag type interaction

Variety x pollination bag type interaction was significant for all traits (Tables 1 and 3, Figures 5, 6 and 7). The hybrid with tannin showed consistently higher mean values with all bag types though the magnitude varied over bag types. Thus the hybrid with tannin was least interactive with bag type and performed well with any type of bag. The other two varieties showed a change of ranking resulting in cross-over interactions (Tables 1 and 3, Figure 5, 6 and 7). For instance, for panicle weight Tannin and SC283 varieties showed highest mean values with bag type 5 but BR007B variety with bag type 3. The lowest panicle weight for Tannin was with bag 2 but with bag 1 for the other two varieties. Similarly, rank changes are noticeable for seed weight and average seed weight.

Correlations of bag type with mean values for all traits were positive and significant (Table 4) showing that as the mean performance of the bag type improves from bag type 1 to 5 so does the mean performance for all traits. Mean values of all three agronomic traits were highly correlated over the five bag types (Table 4). How the three varieties performed under different bag types was indicated from their separate correlations for the five bag types.

Variety SC283 consistently showed highly significant correlation with bag type for all three traits showing that its mean performance was associated with improvement in bag type and that it produced better performance under better bag type. This variety is most sensitive to bag change and hence bags for SC283 need to be carefully chosen. For the other two varieties all correlations were non-significant showing that varietal performance for any of the traits was independent of bag type and that any bag type will be equally effective. However, for BR007B the trend for average seed weight was close to significance level and perhaps could be significant if there were more than five bag types providing more degrees of freedom. Thus, variety

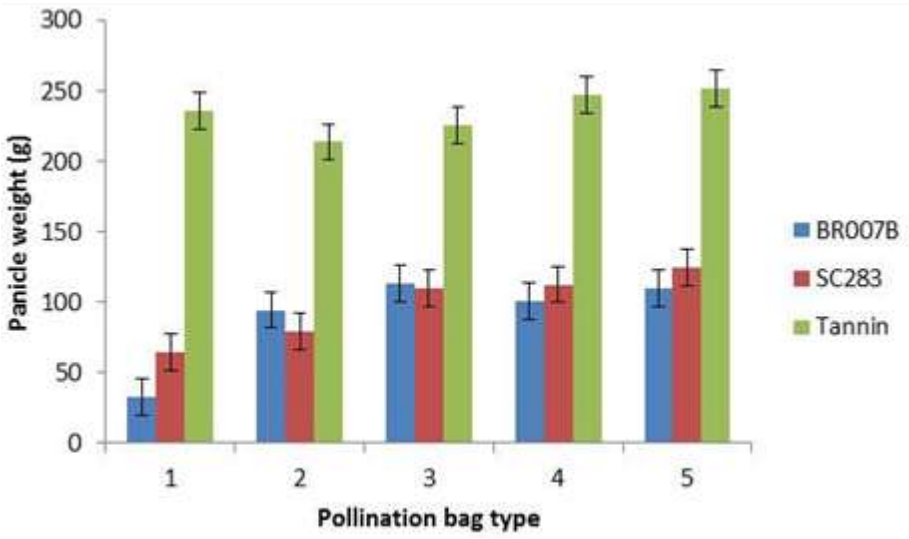

Figure 5. Mean performance of each variety for panicle weight (g) against different pollination bag types.

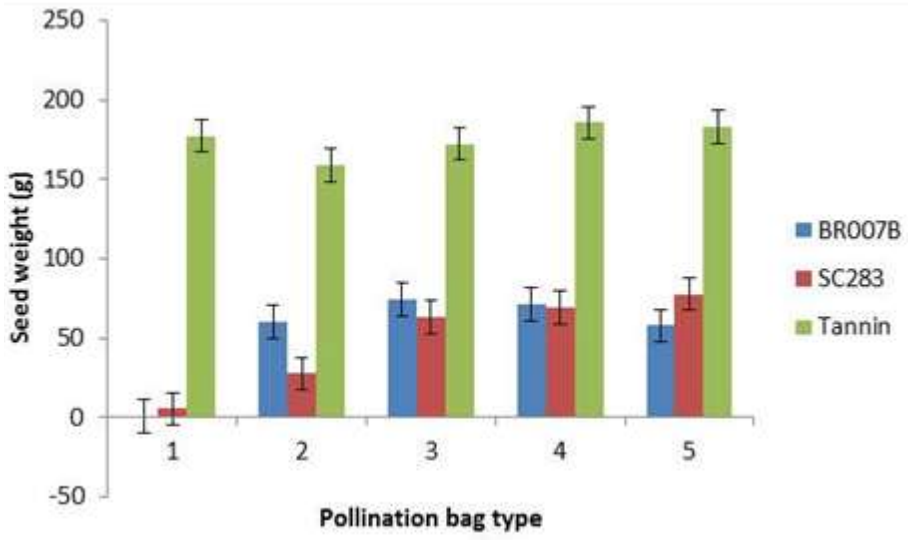

Figure 6. Mean performance of each variety for seed weight (g) against different pollination bag types.

BR007B also has the tendency to show inter-relationship with bag type.

Joint regression analysis

A joint regression analysis of individual variety means on to the all variety means for each bag type was performed for all traits (Table 5). For panicle weight mean values over four replications adjusted for covariance with panicle number were used. The significant heterogeneity among regressions for all traits showed that linear interactions were important (Table 5). However, for average seed weight the significant remainder mean squares indicated the importance of both linear and non-linear interactions (Tables 5).

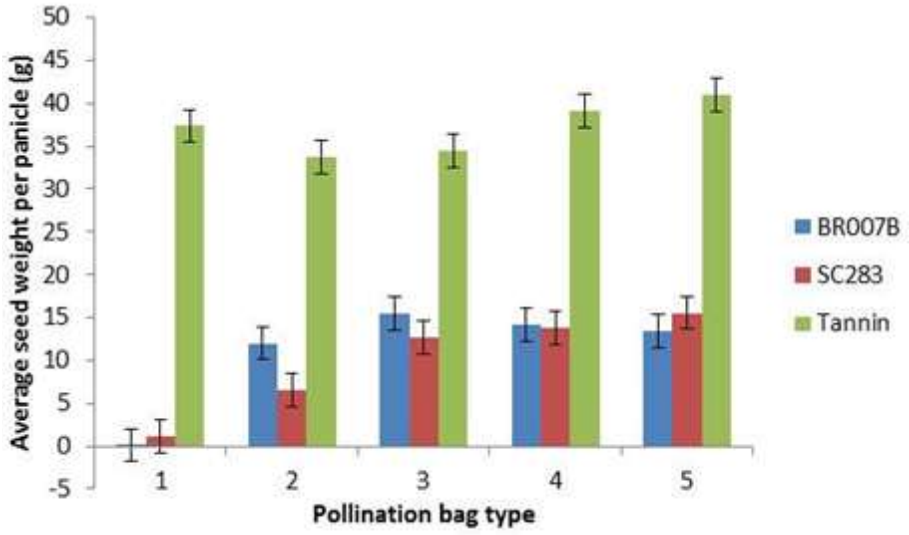

Figure 7. Mean performance of each variety for average seed weight per panicle (g) against different pollination bag types.

Table 4. Correlations between mean performance of agronomic traits over bag types and with five bag types.

Variable	Bag type	Panicle Wt (g)	Seed Wt (g)	Variety	Panicle wt Vs bag type	Seed Wt Vs bag type	Av seed Wt Vs bag type
Panicle Wt (g)	0.965**	-	-	BR007B	0.770	0.661	0.839
Seed Wt (g)	0.907*	0.978**	-	SC283	0.963**	0.956**	0.971**
Av Seed Wt (g)	0.958*	0.996**	0.987**	Tannin	0.664	0.573	0.305

*P<0.05; **P<0.001. Table value of r at 3 df = 0.878 at 5% and 0.959 at 1% levels.

Table 5. Joint regression analysis (mean squares-MS) of varieties on to the mean of all varieties for a bag type for panicle weight (g) on means adjusted for the covariance with number of panicles, seed weight and average seed weight per panicle (the analysis was based on means over four replications).

Source	*df*	MS for panicle weight (g)	MS for seed weight (g)	MS for Av seed weight (g)
Variety	2	32987**	25952**	1183**
Bag types	4	1292**	1247**	55**
Variety x Bag types	8	340+	360**	15**
Heterogeneity of regressions	2	671*	845**	31**
Remainder	6	230	198	9*
Error	35 (36)	161	106	4

*P<0.05; **P<0.01; +P = 0.05-0.10. Error df in bracket are for seed weight and average seed weight without adjustment for covariance for panicle number.

The Tannin cultivar clearly showed higher productivity with highest panicle weight, seed weight and average seed weight per panicle with all type of bags; this variety did not show any dependency on bag types and all types of bags were equally suitable for this variety. Apparently, Tannin hybrid did not have a significant regression on bag types for any trait (Table 6 and Figure 8).

Trends for other two varieties were similar for all traits

Table 6. Estimates of regression parameters for varieties on to mean of all varieties under different pollination bag types for panicle weight (g), seed weight (g) and average seed weight per panicle (g).

Variety	Panicle wt (g)	Seed wt (g)	Av seed wt per panicle (g)
BR007B	-109.67+1.42±0.42*,ns; R^2=79%	-67.44+1.30±0.40**,ns; R^2=78%	-14.05+1.30±0.39**,ns; R^2=79%
SC283	-72.31+1.21±0.07**,**; R^2=99%	-88.24+1.48±0.16**,**; R^2=97%	-17.04+1.39±0.07**,**; R^2=99%
Tannin	181.98+0.37±0.37ns; R^2=25%	155.68+0.21±0.28ns; R^2=17%	31.09+0.31±0.38ns; R^2=19%

ns= non-significant; *P<0.05; **P<0.01. The first significance for regression coefficients is from zero and the second is from 1.0.

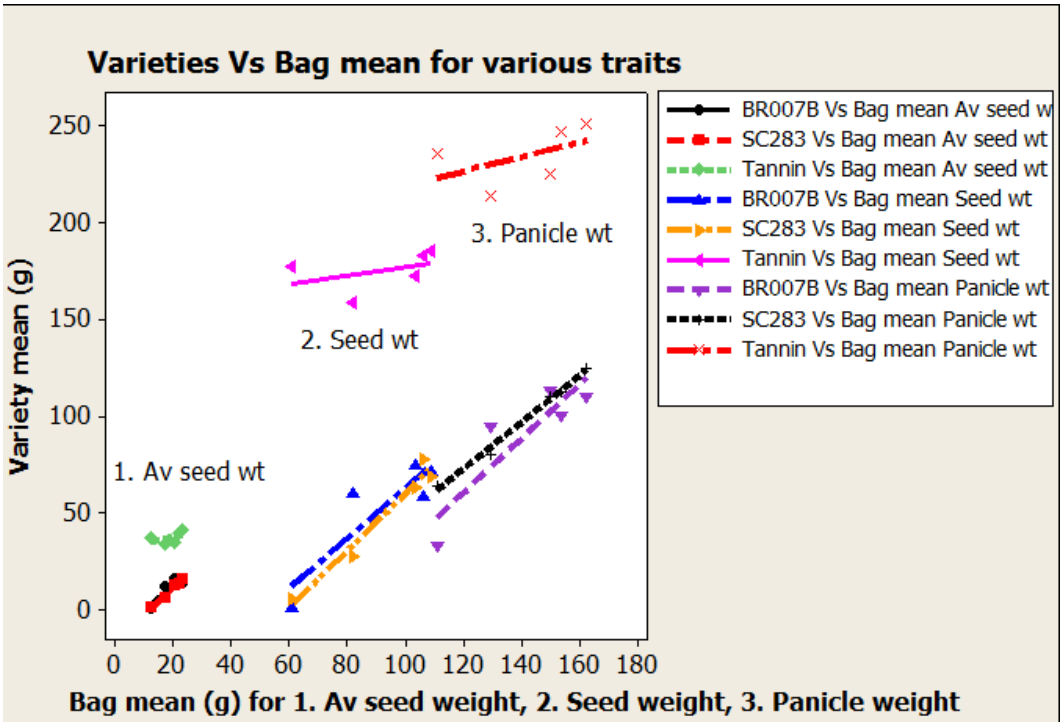

Figure 8. Scatter plots for regression of mean performance of varieties for 1. Average seed weight (g), 2. Seed weight (g), and 3. Panicle weight (g) on to mean of all varieties for each pollination bag type. For estimates of regression parameters see Table 6.

(Table 6 and Figure 8). Variety BR007B showed an average regression of unity for all traits. Hence this variety responds generally well to all bag types showing an average increase in performance with the improvement of bag type.

However, variety SC283 shows an above average response of greater than unity for all traits (Table 6 and Figure 8) which means its performance increases at above average level in response to the improvement of bag type's performance as was also shown by significant correlation coefficients in Table 4. Consequently, it is specifically suitable for bags with higher performance, that is, duraweb® SG2 and duraweb® SG1 (bags 4 and 5). It may be noted that as the bag type improves the difference in performance of BR007B and SC283 gets reduced due to higher rate of response of SC283.

DISCUSSION

The primary function of pollination bags is to facilitate the process of pollen control and hybridization between potential parents. Bagging of plants creates a fabric barrier between reproductive parts and the environment, and is practised to control pollen transmission by insects, wind or other agents and also to collect pollen for artificial cross pollination. Another use of bagging is to facilitate self-pollination of plants and to protect against bird

damage to developing grains particularly in very valuable materials in the breeding nursery including inbred lines and germplasms.

A plant breeder always aims at maximising the seed production under controlled breeding for experimentation while minimising the seed loss from bird attack, insects or damage from environmental vagaries by protecting with pollination bags. While paper bags are commonly used in sorghum other types of materials made from muslin, micromesh, polyethylene, cellulose acetate, micropore acetate bread bags have been in vogue in other plant breeding researches on various types of plants (Pickering, 1982; Ball et al.,1992; Wyatt et al., 1992; McGranahan et al., 1994; del Rio and Caballero, 1999; Neal and Anderson, 2004; Gitz et al., 2013, 2015).

New synthetic materials have been developed which have greater strength for bird owitchgrassr wind resistance, more air permeability, less moisture absorption and resistance against pollen contamination (PBS International, 2013). Polyester bags have been successfully used to control pollination in tree species such as *Elaeis guineensis*, *Melaleuca alternifolia*, *Grevillea robusta* and *Phillyrea angustifolia* (PBS International, 2016). Although materials used for bagging plants have specific merits and demerits the sorghum breeders have not changed over to any other materials than brown paper bags perhaps because of their low cost, availability or adherence to age-old practice.

It is important that studies on performance of paper bags and new fabrics are conducted to build the confidence of sorghum breeders to try new options of nonwoven materials for bagging plants in the breeding processes. Studies have shown that different materials vary in permeability and pollen proofing (McAdam et al. 1987; Adhikari et al., 2014; Vogel et al., 2014). Vogel et al. (2014) obtained four to tenfold increase in seed produced per cross in micro-mesh fabric pollination bags in switchgrass that allowed larger progeny for evaluation in replicated trials. Adhikari et al. (2014) reported that polyester bags were more reliable than traditionally used bags in controlling contamination by foreign pollen using simple sequence repeats (SSR) markers to identify extent of contamination by outcrossing in the bagged panicles of switchgrass for selfing of progenies.

The micro-environment within pollination bags can vary greatly depending upon the type of fabric. Therefore, identifying fabrics that create appropriate environmental conditions within the bag is crucial (Foster, 1968). Gitz et al. (2015) compared the microenvironments within novel spun-bond polyethylene and brown paper bags in sorghum. A considerable increase in temperature was measured within brown bags throughout the season as compared to ambient temperatures.

However, temperatures within polyethylene bags were lower than paper bags because of air permeability. Humidity was lower in soft polyethylene bags than hard polyethylene and paper bags that resulted in moulds especially in the recently irrigated plants. Hayes and Virk (2016) found in *Miscanthus* that duraweb® bags exhibited a narrower range of temperature and humidity than those shown by the Orchard and Glassine bags which could impact the success of crossing and seed set rate. The duraweb® bags made from nonwoven polyester seem to allow air-permeability and moisture absorption for micro-environmental adjustments conducive for better seed set and development.

Bird attack is a major problem in sorghum breeding and germplasm maintenance. Paper pollination bags are damaged by rains and provide minimal deterrent to birds (Gitz et al., 2013). The study results show that covering of panicles with synthetic nonwoven bags provides protection against birds and the damage and seed loss by birds was nearly eliminated under the novel bags. This observation is specifically relevant to areas where bird damage on sorghum breeding stocks is serious. This also is relevant to areas with unpredictable climatic conditions.

Plant breeding experiments often have differential plant stand especially in dry and rainfed conditions due to uneven seedling survival. Trabanino et al. (1989) reported that sorghum seedling stands in Central Honduras are influenced by the infestation by ants, white grubs and armyworms. In the event of variable plant stand resulting from any causes an analysis of covariance that combines the features of analysis of variance and regression is highly useful in computing adjusted means (Snedecor and Cochran, 1974). The study found that the total panicle weight was influenced by the variation in panicle number but seed weight and average seed weight were not affected by the variation in panicle number. Thus adjustments by covariate analysis were justified.

The study showed that paper bags were consistently inferior in performance whether for resistance against bird damage or for panicle and seed traits irrespective of the variety. The new bags, on the other hand, produced more panicle weight, seed weight and average seed weight perhaps due to better micro-environments within them as reported by Hayes and Virk (2016) in *Miscanthus* and Gitz et al. (2015) in sorghum. There were significant interactions of varieties with bag types for seed weight and average seed weight. Variety Tannin did not show significant interaction with bag types and hence its performance for various traits did not depend on bag types. This variety was the highest performer for all traits and showed no bird preference.

While SC283 showed greater than unity regression with above average response to bag types compared with BR007B that showed an average response to changes in bag types showing that its performance improves at the rate of improvement in bag type performance.

Table 7. Factors for comparing pollination bags for economic analysis.

Treatment[ξ]	Bird damage observed	Relative bag cost	Other cost implications	Risk of catastrophic loss	Reusability
2	20-25%, up to 100% in high pressure seasons	$	Extra bags, labour to check / replace bags; Over-planting to compensate for loss	Yes, under high pressure	Not reusable
3	0% in current study. 20% observed in high pressure years	$$	Extra labour cost to attach screens	Some risk under high pressure; damage and lower seed yield	Screen bag reusable
4	0% (not tested under high pressure)	$$	No extra labour in normal year	Little (not tested under high pressure)	Highly probable† (not tested here)
5	0% (not tested under high pressure)	$$	No extra labour in normal year	Little (not tested under high pressure)	Highly probable† (not tested here)

[ξ] 2=paper bag, 3= paper bag + plastic screen bag, 4 =duraweb® SG2, 5= duraweb® SG1. † Hayes and Virk (2016) found duraweb® bags reusable in *Miscanthus*.

It means that better performing bags will be comparatively more useful for all varieties that are more prone to bird attack and that higher specification bags may be required for some varieties such as SC283. Clearly, more research needs to be conducted before generalisations are made about different bag types but what is clear is that the novel bags performed better than the traditional practice of paper bags in all circumstances within our experimentation.

Economic analysis

While these studies do not support a proper economic analysis to compare various bag types we can examine essential factors that determine their comparative advantages as a preliminary attempt. We have set out a scenario in Table 7. It should be emphasised that pollination bags have more relevance in the breeding processes than in commercial seed production.

During the filial generations, seed produced is always in small quantities from individual lines or plants and if such progeny are lost due to bird damage then the whole year is virtually wasted at the loss of labour and effort used in the season. The necessity of protection against bird damage becomes more severe when there is high bird pressure especially in the off-seasons where alternative sources of food are scarce. We have noticed that under the medium pressure as, in the present study, the mean seed weight of variety BR007B under no bagging was only 1% of the overall mean performance under all treatments tendering a loss of 99% (Table 3).

Similarly, variety SC283 showed only 11% performance of mean registering a loss of 89%. However, there was no loss in the Tannin variety. Thus on average, 90% seed weight is lost in bird susceptible varieties which can be avoided by putting bags 3, 4 or 5. On average, new bag types 4 and 5 produced 32 and 29% more seed weight than the paper bags on the basis of mean over all varieties (Table 3). This is a significant economic benefit from the novel bags even under medium bird pressure in the present experiment.

To allow for light or moderate bird pressure, excess resources such as labour, seed, land and consumables have to be used to ensure that the target seed yield is achieved. For example, if 25% seed loss is typical, 33% more seeds should be sown to allow for bird related reduction. In addition, extra labour is required to patrol the fields and replace damaged bags as and when required.

However, in some years the bird pressure is severe and up to 100% seed loss results when paper bags are used. Under these circumstances the entire direct cost of the programme (which may be as high as several hundred thousand dollars) is wasted, and an entire breeding cycle is lost, delaying the progress of the work. Although the new materials have not been tested under these circumstances, it is thought that the protection against bird damage may reduce the risk of catastrophic loss of this type. In addition to this the researchers felt anecdotally that the seeds produced under the paper bags were of lower quality, a topic for future research.

Finally, the nonwoven bags are likely to be re-useable, thus reducing the cost-per-cross of the bags, although

this was not tested in this experiment. These preliminary results need confirmation with more robust experiments to explore the economic implications more fully, and to establish whether micro-environmental differences in the bags explain differences in their seed harvest outcome.

Conclusion

The use of carefully selected nonwoven bags instead of commonly used paper bags for germplasm maintenance and crossing purposes is recommended, since these bags provide better protection against bird damage as well as higher panicle weight, seed weight and average seed weight per panicle across all three types of varieties of sorghum.

Conflict of Interests

The authors have not declared any conflict of interests.

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Inbreeding enhances field resistance to cassava brown streak viruses

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Cassava brown streak disease (CBSD) is currently the major disease affecting cassava production in Eastern and Southern Africa. Breeding for resistance has been hampered by a lack of sources of resistance and the complexity of CBSD. This study was initiated to assess the possibility of exploiting inbreeding, as a strategy for generating new sources of resistance to CBSD. This was based on the premise that inbreeding increases the additive variance upon which selection for desirable phenotypes can be made. Eight cassava progenitors (S_0): Namikonga, 182/006661, Kigoma Red, Tz/130, Tz/140, 130040, 0040 and 100142 were selfed for one generation to produce the first inbred generation (S_1). The S_1 progenies generated were evaluated for two seasons (seedling and clonal evaluation trial) in a high CBSD pressure area. Promising clones were re-evaluated to confirm their CBSD reaction status. Results obtained showed that within each family, a few S_1 inbreds (1-15) had higher levels of resistance compared to the S_0 progenitors with the highest number observed in Tz/130. It is possible therefore to get transgressive progenies through inbreeding.

Key words: Cassava brown streak disease, inbreeding, cassava partial inbreds, new sources of resistance, inbreeding depression, resistance breeding.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) is one of the most important root crops grown widely in tropical countries notably in sub-Saharan Africa, South America and Asia. In recent years, cassava production has been greatly hindered by a myriad of biotic stresses. Of these, cassava brown streak disease (CBSD) is the major disease affecting cassava production in Eastern and Southern Africa (Pennisi, 2010). The disease is caused by two virus species, cassava brown streak virus (CBSV) and Uganda cassava brown streak virus (UCBSV), both are *Ipomoviruses* of family Potyviridae characterized by an elongate flexuous filament 650 to 690 nm long (Monger et al., 2001; Mbanzibwa et al., 2011). The presence of two distinct species of virus that causes

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CBSD and a lack of natural resistance has posed a great challenge to breeding efforts tailored towards increasing cassava productivity in CBSD affected areas. These viruses are distributed in Tanzania (Ndunguru et al., 2015), Kenya (Munga, 2008), Uganda (Alicai et al., 2007), Democratic Republic of Congo (Mulimbi et al., 2012), Rwanda (Tomlinson et al., 2013), Burundi (Bigirimana et al., 2011), Malawi (Mbewe et al., 2015) and Mozambique (Zacarias and Labuschagne, 2010). Undocumented reports of CBSD outbreaks in Zambia have also been made.

Breeding for CBSD resistance is the most efficient way to combat the disease. The pioneering breeding program for cassava mosaic disease (CMD) and CBSD was started more than 70 years ago at Amani Research station in Tanzania. The program initially focused on searching for sources of resistance among different cassava genotypes. According to Jennings (1957), limited progress was made which led to the use of cassava wild relatives in the program. Several crosses were made between *M. esculenta* and wild *Manihot* species (*Manihot glaziovii, Manihot melanobasis, Manihot cathartica, Manihot dichotoma* and *Manihot saxicola*) in order to introgress CMD and CBSD resistance genes into preferred cassava genotypes (Hillocks and Jennings, 2003). Through interspecific hybridization and backcrossing several hybrids with reasonable levels of CBSD resistance, such as Namikonga (also known as Kaleso in Kenya) were developed and incorporated in the farming system. IITA have actively been breeding for CBSD resistance in Tanzania since 2004, incorporating germplasm derived from the Amani program.

Diallel studies of the inheritance of CBSD resistance/tolerance conducted in Kenya (Munga, 2008), Uganda (Tumuhimbise et al., 2014) and Tanzania (Kulembeka et al., 2012) have demonstrated the relative importance of additive genetic effects as opposed to non-additive effects). Zacarias and Labuschagne (2010) showed the importance of non-additive genetic effects in germplasm from Mozambique. Additivity presents the possibility for enhancing levels of resistance through the inbreeding of tolerant genotypes. According to Walsh (2005), inbreeding allows “concentration” of desirable genes originally present in the elite clone. By forcing an average of half of the loci to become homozygous, the additive value of a selfed individual or progeny is increased, and through selection, any resultant homozygous deleterious alleles can be purged (Barrett and Charlesworth, 1991). Inbreeding results in progeny at both fitness extremes, that is, extremely high fitness with many homozygous advantageous alleles with few deleterious mutations and extremely low fitness with many homozygous deleterious mutations. Indeed, a recent study on the segregation of selected agronomic traits in cassava inbreds (Kawuki et al., 2011) showed an increase in performance in agronomic traits (harvest index and root dry matter content) in some inbreds compared to their respective non-inbred parents. Here, it was hypothesized that S_1 partial inbreds will not only be better progenitors but will also possess higher levels of resistance to CBSD than their respective non inbred parents. This study was initiated to generate and evaluate cassava partial inbred for resistance and/or tolerance to CBSD in Uganda.

MATERIALS AND METHODS

Generation of S_1 families from S_0 parents

Ten cassava progenitors (S_0): Namikonga, 182/006661, Kigoma Red, Tz/130, Tz/140, 130040, 0040, Kiroba, Nachinyaya and I00142 from Tanzania were selected after CBSD tolerance had been confirmed using quantitative real-time PCR diagnostics (Kaweesi et al., 2014) and established in isolated plots at National Crops Resources Research Institute (NaCRRI), Central Uganda. With the exception of Tz/130 and Tz/140, which were selected in Uganda from open pollinated seeds introduced from Tanzania in 2005, all other progenitors were introduced from Tanzania as stem cuttings in 2009. Each parental line was represented by 20 plants which were established in two-row plots of 10 plants. At flowering, selfing was done by hand according to standard procedures to generate partial inbred lines (S_1). Within a cassava field, it is possible to get mature pollen and mature female flowers of the same clone (from different branches or plants) and thus selfing is possible. After selfing, flowers were bagged for at least 2 to 3 days to avoid contamination, labeled appropriately and the number of flowers selfed and the number of selfed fruits per plant were recorded. Any open pollinated flowers were removed to avoid mixtures. After three months, the mature fruits were harvested and numbers of seeds recorded. The harvested S_1 seeds were established in a nursery at NaCRRI after a two month period to break dormancy. After two months in the nursery, the S_1 seedlings were transplanted in a well-prepared field for CBSD evaluation.

S_1 seedling evaluation trial

Eight S_1 families were evaluated. All seedlings belonging to a single family were established in the same block. Spreaders using a CBSD – susceptible variety (TME 204) were planted after every four rows of test genotypes to augment the CBSD pressure. This trial was planted during the first rains (March - June) of 2011. Data for CBSD were collected on individual seedlings at two-month intervals after the third month after planting (MAP). Cassava raised from seed usually produces a few storage roots (1-10) (Tumuhimbise et al., 2014) which also provide an opportunity for CBSD root necrosis evaluation. However, subsequent evaluations were done on cloned genotypes, thus, after nine months, each plant in the seedling evaluation trial (SET) was individually harvested and data were taken for foliage yield, root yield, CBSD root severity and CBSD root incidence. Thereafter, 8 to 12 cuttings were taken from each parent (S_0) and self (S_1) to generate clones for further evaluation.

Evaluation of S_1 clones for CBSD resistance

S1 clones were evaluated in clonal trials during 2012/13 and 2013/14. Clonal evaluation trials (CET) were established at NaCRRI using single rows of six plants per genotype. Both S_1 progeny and the non-inbred parent (S_0) were established in the CET. The first clonal trial was planted during the first rains (April) of 2012. Each

row represented a single clone and the spacing was 1 m within and between the rows. To control variability in the field, clones from a given family were separated into three groups of roughly equal size and each group of a family was randomly allocated to one of the blocks along with respective parental genotypes for comparison. No selection was done; all seedlings were cloned and evaluated. Spreader rows of TME 204 were established between rows to augment CBSD pressure. The genotypes were grown for 12 months under rain fed conditions with no fertilizer or herbicide applied.

Above-ground CBSD symptoms (on leaves and stem) were assessed visually on every plant in each plot. Both incidence (proportion of cassava plants in a plot expressing CBSD symptoms) and severity (degree of infection of CBSD on the individual plant) were used to quantify the disease. Five data sets at three, five, seven, nine and eleven months after planting (MAP) were collected. A severity scale of 1 to 5 (Gondwe et al., 2003) was adopted for above ground symptoms where 1- no symptom, 2- mild symptom (1-10%), 3- pronounced foliar chlorotic mottle and mild stem lesion (11-25%), 4- severe chlorotic mottle and stem lesion (26-50%) and 5- very severe symptoms (>50%). Severity scores for root necrosis were also taken on all roots harvested per plot at 12 MAP. Severity scores for root necrosis were based on a 1-5 scale where 1- no necrosis, 2- mild necrotic lesions (1-10%), 3-pronounced necrotic lesions (11-25%), 4- severe necrotic lesions (26-50%) and 5- very severe necrotic lesion (>50%).

Clones that maintained a root severity of 1 or 2 were selected and re-evaluated at NaCRRI during the CET-2 established in 2013/2014 season to further confirm their resistance/tolerance levels. Thus, the S_1 inbreds with scores of 1 or 2 were evaluated for three seasons.

Data analysis

Root severity scores were converted into disease severity mean (DSM) using the following formula:

$$DSM = \frac{\sum(\text{severity scores for all affected roots on the infected plant})}{\text{Total number of infected roots on the infected plant}}$$

Disease incidence (DI) of CBSD in harvested roots was quantified as a ratio of the number of roots showing roots symptoms to the total number of roots harvested per plant per genotype. Disease index of every clone was derived as a product of DI and DSM.

Data on disease index was subjected to one-way analysis of variance using Genstat (ver. 14) at a significance level of 5% to compare families. The field reaction of each generated partial inbred to CBSD was compared to that of the respective progenitor (S_0) by subjecting disease index data for the family (S_0 progenitor and its S_1 inbreds) to the analysis of variance using Genstat (ver. 14) (Payne et al., 2011). To determine the effect of inbreeding, the disease index of each partial inbred was compared to the disease index of their respective parent to determine the total number of positively transgressive progenies per family. Each S_1 partial inbred that had a lower disease index compared to its respective progenitor was considered a positively transgressive progeny. The percentage of positively transgressive progenies per family was compared to determine the best progenitors.

To measure the heritability of resistance or tolerance to CBSD, a parent-offspring regression was made using mean values of disease index of parents and offspring based on root necrosis data collected in one environment, NaCRRI. The offspring were regressed on that of their parent using standard linear regression model $y_1 = b_0+b_1x_1 +e$, where y_1 is the mean of offspring of the i^{th} family, b_0 is the intercept, b_1 is the regression coefficient and x_1 is the parent of the i^{th} family and e is the random error. The expression $h^2=2b_1$ was used since partial inbred families are regressed on a single parent. Parent-offspring regression analysis was performed using Genstat (ver. 14).

RESULTS

S_1 seed germination and survival

Ten cassava genotypes (S_0) were evaluated and selfed to produce partial inbreds (S_1). Of the ten S_O progenitors, only eight were able to produce seeds in variable proportions while two genotypes (Kiroba and Nachinyaya) did not produce seed due to male sterility. Due to high heterozygosity of cassava as a crop, it had been hypothesized that a low rate of germination would be obtained due to inbreeding depression. Results show variable germination rates among the families, ranging from 47.8 to 73.2%. Under ideal conditions, the germination rate of non-inbred cassava population is expected to be 90 to 100%. Therefore, these results show moderate effects of inbreeding on germination.

A general reduction was observed in the survival rate in all the families between the SET and CET. Two S_0 progenitors (0040 and 100142) that produced the highest number of seeds had the lowest survival rate at CET of 13.5 and 6.1%, respectively, as compared to TZ/130 and TZ/140 with a survival rate of 66.7 and 41.2% (Table 1). Though, this study intended to explore the benefits of inbreeding in search of resistance to CBSD, effects of inbreeding on fitness traits were noted. A large proportion of seedlings generated was characterized by a loss of vigour, height and reduction in growth, and therefore, did not survive to be advanced to the clonal evaluation trial. The low survival rate could be partly attributed to inbreeding depression and virus challenges (both CMD and CBSD). Inbreeding depression for sprouting, vigor, height, flowering, harvest index and dry matter content was low or absent in some families of the clones that were advanced to the CET (Supplementary Tables 1 and 2).

Response of partial inbreds to cassava brown streak disease

Both foliar and root symptoms were used to determine the response of the generated partial inbreds to CBSD. Variation in susceptibility to CBSD among different clones of different cassava families was striking. It ranged from 0 to 100% foliar incidence with a severity score of 1 to 4.5. All the parents showed foliar symptoms while a variable number of partial inbreds in each family remained symptomless (Table 2). A similar pattern was observed also for root symptom. Different phenotypic classes (partial inbreds with max root necrosis score 1, 2, 3, 4 and 5) for CBSD were observed differentially in the different families. At SET, most of the partial inbreds had

Table 1. Number of S1 seeds generated, seedling established and clones generated.

S_0 Progenitor	No. of seeds generated	Seedling germination		Clones generated[2]	Clone established[3]	Survival[4] (%)
		No of seedling	Percentage			
0040	418	200	47.8	100	27	13.5
100142	396	280	70.7	160	17	6.1
130040	353	200	56.7	104	40	20
Namikonga	123	60	48.8	46	15	25
TZ/130	123	90	73.2	79	60	66.7
182/00661	79	40	50.6	24	6	15
Kigoma Red	60	40	66.7	20	7	17.5
TZ/140	25	17	68.0	11	7	41.2
Kiroba	0	0	0	0	0	0
Nachinyaya	0	0	0	0	0	0

[2]Number of genotypes cloned per family at 11 MAP in 2011; [3]Number of clones that established. On which clonal evaluation data was taken during 2012; [4] % survival to the end of clonal evaluation trial computed as a ratio of clones established to seedlings generated. Differences in seedling germination could indicate inbreeding depression, while differences observed at survival are a combination of inbreeding depression and virus challenges.

Table 2. Field reaction of partial inbreds (S_1) and their respective parents (S_0) against CBSD in Uganda based on foliar symptoms for CET-1 (2012-2013).

Family	Partial inbreds (S_1)				Parent (S_0)			
	No. of clones	Incidence (%)	severity	Symptomless S_1 clones	Incidence (%)	Severity	Min	Max
TZ/130	60	56.7**	1.99±0.1	26	30.9**	1.59±0.1	1	3
Namikonga	15	53.3**	2.16±0.1	7	3.6**	1.04±0.1	1	2
130040	37	72.9	2.41±0.1	10	76.4	2.64±0.2	1	4
TZ/140	7	57.1	1.79±0.2	3	100	3.67±0.2	2	3
Kigoma Red	6	66.7	2.60±0.2	2	100	3.33±0.2	3	3
182/00661	6	83.3	4.05±0.2	1	100	3.92±0.2	3	4
0040	27	74.0	2.01±0.1	7	-	-	-	-
100142	15	66.0	2.48±0.1	5	-	-	-	-
$LSD_{0.05}$	-	35.4	0.44	-	3.33	0.48	-	-

[1]Severity –Mean disease severity at family basis; **significant difference at (5%). Incid: Incidence; Sev: severity; min: minimum value; Max: maximum value.

a maximum score of 1 for root necrosis while family Kigoma Red and 182/00661 showed even distribution across classes (Figure 1). This distribution changed from the SET and CET-1 with increasing frequency of genotypes with maximum root severity scores 4 and 5 as compared to scores 1 and 2. On the other hand, inbreds from Namikonga exhibited two extremes, that is, 42% had score 1 (resistant), while 42% had score 5 (susceptible).

In comparison to S_0 progenitors, there was no significant difference between the mean of all the generated partial inbreds in a given family compared to their respective S_0 progenitors. However, there were partial inbreds that performed better than their respective parent based on both foliar and root symptoms as hypothesized. These were considered positively transgressive progenies. A small proportion (1-15) of partial inbreds generated from all families (except 182/00661) did not show both foliar and root symptoms after SET and CET. The highest percentage of partial inbreds that remained symptomless was obtained in family Namikonga and TZ/130 (Table 3). The TZ/130 and Kigoma Red families contributed a large percentage of positively transgressive progenies (Table 4). Contrastingly, some families like 182/00661 and TZ/140 did not perform as expected, with a higher number of inbreds with higher disease index than their respective progenitors. These parents produced higher percentages of negatively transgressive progenies (Table 5).

Re-evaluation of selected S_1 partial inbreds in CET-2 (2013/2014)

When S_1 partial inbreds with root severity, scores of 1 and 2 were re-evaluated in 2013/2014, some with a

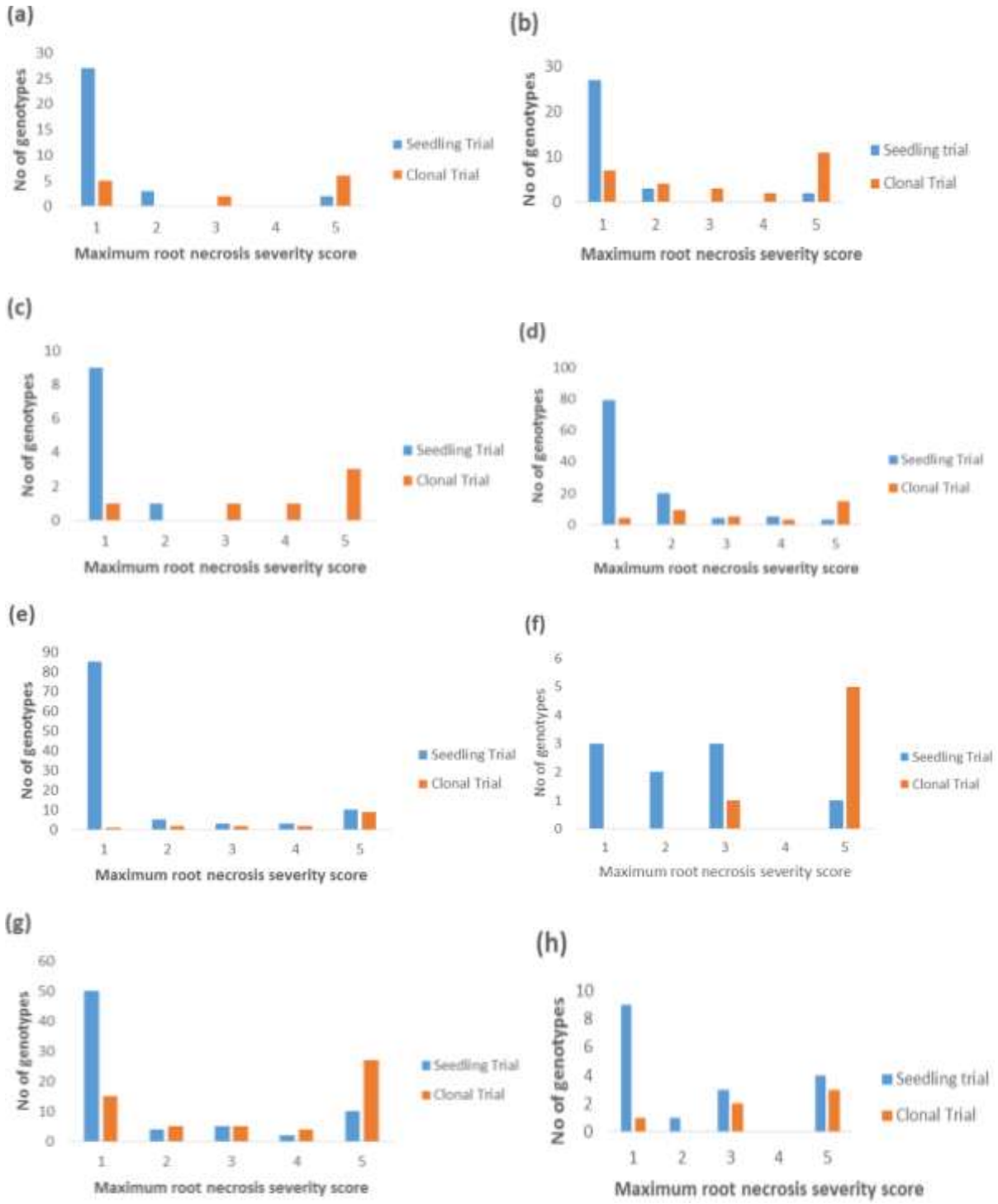

Figure 1. Frequency distribution showing variation in CBSD root necrosis based on maximum root necrosis severity for S_1 inbreds at seedling and clonal stage (CET-1, planted 2012-2013) for (a) Namikonga, (b) 0040, (c) Tz/140, (d) 130040 (e) 100142, (f) 182/00661, (g) Tz/130 and (h) Kigoma Red. In the seedling evaluation trial, some genotypes did not develop tuberous roots that could be scored for CBSD.

maximum score of 1 for root necrosis remained symptomless, while some maintained a very low incidence and maximum severity of 2. Of the 34 S_1 partial inbreds that remained symptomless in 2012/2013, nine S_1 inbreds remained symptomless for CBSD root necrosis, while six S_1 inbred maintained a low incidence

Table 3. Field reaction of partial inbreds (S_1) and their respective parents (S_0) against CBSD in Uganda based on root symptoms during CET – 1 (2012-2013).

Family	Partial inbreds S_1						Parents S_0				
	Clones	Incidence	DSM	D. Index	DSM (range)	symptomless	Incidence	DSM	D. index	Min.	Max.
TZ/130	60	0.46±0.1	3.48±0.2	1.76±0.22	2.00-5.00	15	0.34±0.3	3.16±0.3	1.21±0.5	1	5
Namikonga	15	0.63±0.1	3.26±0.3	2.19±0.5**	2.00 -4.90	5	0.10±0.3	2.00±0.9	0.20±1.4	1	2
130040	37	0.45±0.1	3.02±0.2	1.52±0.3	2.00-5.00	4	0.18±0.8	2.88±0.5	0.49±0.5	1	5
TZ/140	7	0.72±0.1	3.36±0.4	2.56±0.6**	2.30-5.00	1	0.09±0.2	2.00±0.7	0.18±1.0	1	2
Kigoma Red	6	0.84±0.1	3.23±0.4	2.75±0.5	2.10-5.00	1	0.92±0.2	2.62±0.5	2.44±0.7	1	5
182/00661	6	0.74±0.2	2.85±0.5	2.12±0.8	2.30-3.80	0	0.47±0.2	3.39±0.5	1.70±0.7	2	5
0040	27	0.48±0.1	2.98±0.2	1.48±0.3	2.00-4.60	7	-	-	-	-	-
100142	15	0.51±0.1	3.64±0.2	2.06±0.4	2.00-5.00	1	-	-	-	-	-
$LSD_{0.05}$		0.31	0.89	1.35	-	-	-	0.35	1.45	1.69	-

DSM: Disease severity means; D. Index: disease index; min: minimum value; Max: maximum value; LSD: Least significant difference; Data on S_0 progenitors 0040 and 100142, not collected

Table 4. Number of positively transgressive progenies generated from each family after clonal evaluation trial (2012/13) based on CBSD root necrosis.

Family	Disease Index for Parent S_0	No of S_1 clones evaluated in CET	No of S_1 with lower disease index compared to S_0	Percentage of positively transgressive progenies at CET
TZ/130	1.21	60	38	63
Namikonga	0.20	15	6	40
130040	0.49	37	15	40.5
TZ/140	0.18	7	1	14.3
Kigoma Red	2.44	6	4	66.7
182/00661	1.70	6	1	16.7

Data on S_0 progenitors 0040 and 100142, not collected.

(1.25 to 7.96%) and a maximum severity of 2 (Table 6). The absence of root symptoms and/or limited symptom expression after three years of exposure to CBSD at Namulonge suggests the presence of elevated tolerance or resistance levels in S_1 progenies compared to the parents which all showed some symptoms. These results show that inbreeding can not only enhance field resistance to CBSD, but can also be used as a strategy to generate new genetic stocks with high resistance level for CBSD resistance breeding. Offspring-parent regression analysis provided a linear model (y=0.216x + 1.752), with a slope of 0.216, thus providing an estimate of heritability across all the families of 0.43.

DISCUSSION

The objective of this study was to develop and screen S_1 partial inbreds derived from some of the most tolerant genotypes to CBSV and UCBSV in Uganda. Most of these parental lines were sourced from Tanzania, where CBSD has been prevalent for over 50 years, and where an inter-specific breeding program for CMD and CBSD was conducted from the 1930s. Evaluations were done at the seedling and clonal stages in a CBSD hotspot at Namulonge, relative to their parents. Promising S_1 partial inbred clones with a score of

Table 5. Number of partial inbreds with higher disease index compared to their respective progenitors (S_0) based on CBSD root necrosis generated from clonal evaluation trial (2012/2013).

Family	Disease index for Parent S_0	No of S_1 clones evaluated in CET	No of S_1 with higher disease index compared to S_0	Percentage of clones with the higher disease index than S_0	S_1 clones with the highest disease index (most susceptible)	Respective disease index* for these S_1 clones
TZ/130	1.21	60	19	31.7	TZ/130/40 TZ/130/23	4.80 4.80
Namikonga	0.20	15	9	60	Nam 33 Nam 15	4.63 4.54
130040	0.49	37	19	51.4	130040/176 130040/20	5.00 4.50
TZ/140	0.18	7	5	71.4	TZ/140/131 TZ/140/5	5.00 3.31
Kigoma Red	2.44	6	2	33.3	Kigoma 11 Kigoma 30	5.00 3.31
182/00661	1.70	6	4	66.7	182/00661/26 182/00661/27	2.74 2.11

*Maximum disease index for the most susceptible genotype is 5.

1 and 2 were further evaluated in a replicated clonal trial for the third year (2013/2014).

A varying number of the generated S_1 clones remained symptomless for both UCBSV and CBSV (on roots) for the two seasons evaluated in a “hotspot” zone (Tables 3 and 6). Within each family, a few S_1 inbreds (1-15) showed higher levels of resistance than the S_0 progenitors and are therefore considered positively transgressive progenies. These clones are potential sources of resistance to CBSD. Certainly, the absence of root symptoms and/or limited symptom expression after three years of exposure to CBSD at Namulonge suggests the presence of elevated tolerance or resistance levels in S_1 progenies compared to the parents which all showed some symptoms.

Inbreeding increases homozygosity, thereby changing the distribution of genetic variation of a trait (in our case, resistance or tolerance to CBSD). This change increases the visibility of genetic variation to selection and also exposes the phenotypic effects of previously hidden recessives (both beneficial and deleterious) (Charlesworth, 1992). According to Kelly (1999a and 1999b), the extent of these effects depends on the pattern of dominance, linkage disequilibrium and allele frequency in the parent. Our study showed strong parental genotypic differences in inbreeding effects on CBSD resistance/tolerance which is likely to reflect the number of advantageous recessive CBSD resistance alleles in the heterozygous state in a parent, and dominance. For example, family T_Z/130 had a comparatively higher percentage of positively transgressive progenies compared to other families. It is likely this parent possesses more loci influencing CBSD resistance in the heterozygous state. Therefore, selection of S_0 progenitors is important for the success of an inbreeding program. Once outperforming S_0 progenitors are identified based on partial inbred performance, a higher number of partial inbreds from the best performing S_0 progenitors could be generated in addition to further selfing generations. This will increase the chances of generating more positive transgressive progenies with elevated levels of resistance to CBSD.

Previous studies conducted on the genetics of CBSD resistance (Munga, 2008; Kulembeka, 2010; Tumuhimbise et al., 2014) indicate that CBSD resistance is largely under the control of additive genetic effects. Additive effects can easily be exploited with inbreeding. From quantitative genetics theory, additive and non-additive genetic effects in S_1 can be partitioned between and within families in the following proportions; between families (σ^2_A=1, σ^2_D=1/4) and within families (σ^2_A=1/2, σ^2_D=1/2). Consequently, total additive effects will be σ^2_A= 3/2, while total non-additive effects will be σ^2_D=3/4 (Hallauer and Miranda, 1981). This explains to some extent the higher levels of CBSD resistance observed in the few S_1 clones. The positive transgressive segregation observed in some cassava families may also be due to the unmasking of advantageous recessive alleles that are

Table 6. Performance of selected S_1 inbreds in the third year of evaluation (2013/2014) based on CBSD root necrosis.

S_1 Partial inbred	No. of plants	Max. severity at the end 2012/2013	Incidence	DSM	Disease index	Min.	Max.
TZ/130/45	6	1	0.00	1.00	0.00	1	1
TZ/130/22	3	1	0.00	1.00	0.00	1	1
TZ/130/111	7	1	0.00	1.00	0.00	1	1
Nam 5	7	1	0.00	1.00	0.00	1	1
Nam 45	6	1	0.00	1.00	0.00	1	1
130040/80	4	1	0.00	1.00	0.00	1	1
130040/160	7	1	0.00	1.00	0.00	1	1
130040/107	8	1	0.00	1.00	0.00	1	1
0040/92	9	1	0.00	1.00	0.00	1	1
130040/118	10	1	0.01	1.01	0.01	1	2
TZ/130/26	11	1	0.02	1.03	0.03	1	2
130040/2	8	2	0.02	1.03	0.03	1	2
0040/63	6	1	0.03	1.04	0.04	1	2
TZ/130/75	2	1	0.08	1.10	0.10	1	2
0040/119	10	1	0.07	1.15	0.10	1	2
Nam 22	7	1	0.05	1.32	0.11	1	5
Nam 31	8	1	0.08	1.15	0.15	1	2
130040/128	10	2	0.11	1.19	0.16	1	4
130040/97	2	2	0.13	1.17	0.17	1	2
130040/31	7	2	0.09	1.67	0.17	1	3
130040/174	4	2	0.13	1.21	0.17	1	2
100142/233	10	1	0.09	1.46	0.19	1	5
TZ/130/91	9	1	0.07	1.39	0.20	1	3
100142/25	7	2	0.14	1.33	0.23	1	3
130040/45	9	2	0.12	1.33	0.24	1	4
TZ/130/43	4	1	0.05	2.00	0.25	1	5
TZ/130/37	3	1	0.07	1.83	0.25	1	5
0040/49	10	1	0.08	1.79	0.26	1	5
TZ/130/33	3	1	0.12	2.09	0.35	1	5
0040/34	10	2	0.16	2.32	0.47	1	5
TZ/130/76	10	2	0.15	2.63	0.49	1	5
Nam 21	9	2	0.25	1.81	0.52	1	5
TZ/130/18	6	2	0.21	2.59	0.60	1	3
TZ/130/49	3	2	0.13	3.67	0.67	1	5
100142/64	5	2	0.19	3.38	0.72	1	5
100142/12	2	2	0.20	3.75	0.75	1	5
TZ/130/112	6	2	0.19	3.83	0.81	1	5
TZ/130/115	9	2	0.22	4.09	0.89	1	5
130040/115	8	2	0.22	4.09	0.89	1	5
TZ/130/107	5	2	0.23	4.28	0.95	1	5
$LSD_{0.05}$	-	-	0.13	1.04	0.33	-	-

heterozygous in parental lines and the additive action of these unmasked alleles. According to Kawuki et al. (2011), there was an increase in mean performance in amylose content among six cassava S_1 families generated at NaCRRI. That study also observed S_1 individuals with higher dry matter content and harvest index compared to S_0. Desirable phenotypes have also been previously reported in S_1 cassava (Ceballos et al., 2004, 2007). Inbreeding, therefore, presents opportunities to improve traits in cassava especially those that are quantitative in nature. Inbreeding has also been used in other crops to improve plant defense system against biotic stresses. One example is a study by Hall-Sanders and Eubanks (2005), in which inbreeding increased the resistance of

Ipomea hederacea var *intergniuscula* to both specialist and opportunistic generalist herbivore among inbreds compared to the outcrossed.

The heritability estimate of 0.43 obtained in this study implies that only 43% of the observed phenotypic variance in response to CBSD among inbreds is due to additive genetic effects. This is a modest estimate which implies that the response of generated partial inbreds to CBSD can be predicted by severity or disease index of parental genotypes. Moderate estimates obtained in this study also suggest that substantial genetic gain would be obtained when selecting for resistance in partially inbred cassava families though selection would be more effective in later generations (S_3 or S_4).

The CBSD phenotypic class frequency distribution (Figure 1) observed in different families in this study showed that there was continuous variability in all families except for Namikonga which had two distinct classes (susceptible and resistant). Segregation implies that some resistance genes are in the heterozygous state; however, in Namikonga, it is likely that some dominance effects are also operational. Breeding will be easier if we know that resistance genes are fixed in the source genotype, however as soon as these are crossed, they would return to the heterozygous state, and would have to be backcrossed, selfed or intercrossed, to recover the homozygous state.

Therefore, if molecular markers were available that were associated with the quantitative trait loci (QTL) that confer the resistance, breeders could determine whether associated genes were in the homozygous or heterozygous states which would help in the accuracy of breeding. Quantitative trait loci (QTL) mapping experiments are underway to better track contributing alleles and their homozygous/heterozygous states during breeding.

Furthermore, there was variation in the symptom expression within a genotype in the SET and the CET (Figure 1). This variability could be due to virus multiplication and the accumulation over the first season, and carry over, through stakes to the second field season. This study shows the importance of screening cassava genotypes for more than one cycle in a hotspot to properly determine their response to CBSD, if starting with uninfected stakes or seedlings.

Low germination percentage could be attributed to inbreeding depression. In addition, low survival rates at the clonal stage could have resulted from a combination of inbreeding depression and cassava virus accumulation notably CMD. With selfing, some recessive deleterious alleles, once masked by dominance effects in the heterozygous form become homozygous and express these effects on the components of fitness.

According to Charlesworth and Charlesworth (1987), there are two, not necessarily mutually exclusive, hypotheses that describe the decline in fitness with inbreeding; partial dominance and over-dominance. On the over-dominance hypothesis inbreeding depression results from the loss of advantage from the heterozygote state. This hypothesis assumes superiority of the heterozygous state, relative to the homozygous state. On the partial dominance hypothesis inbreeding decline results from the fixation in the homozygous state of recessive or partially recessive deleterious alleles.

It is possible that both of these mechanisms are in operation in cassava, with low germination rates and survival being indicative of the dominance hypothesis. The data generated in this study also indicated a general increase in mean performance for sprouting, vigour, height, flowering, dry matter content and harvest index among some partial inbred families at the CET which cannot be explained by over-dominance. When the surviving clones were evaluated for inbreeding depression (supplementary Tables 1 and 2), it was found that some families did not exhibit inbreeding depression for the evaluated fitness traits while others showed a low inbreeding depression, however it is likely that those individuals showing inbreeding depression did not survive, contributing to low survival rates. The low rate of survival also suggests that inbreeding depression is caused by genes of major effect or dominance (Ritland, 1996).

To our knowledge, this is the first study that has explored inbreeding for purposes of getting new resistance and/or higher resistance levels to CBSD. These findings are encouraging and thus justify the use of inbreeding in cassava, a highly heterozygous crop. Flowering which is critical in advancing generations of selfing appears not to be restrained by inbreeding in the clones used. This provides further motivation to explore inbreeding in cassava.

Conclusion

This study was initiated with a premise that inbreeding would significantly improve resistance to CBSD among partial inbreds as compared to their respective non-inbred progenitors. Results indicate that, within each family, a few S_1 inbreds (1-15) showed higher levels of resistance than the S_0 progenitors. It is, therefore, possible to get higher levels of resistance upon selfing. If field resistance is controlled by several heterozygous loci, it can be envisaged that more cycles of inbreeding for those clones that remained symptomless will lead to the generation of more new sources of resistance and/ or increase in levels of resistance to CBSD once all contributing loci are homozygous for the positive allele.

Alternatively, the generated S_1 can be crossed in a different combination (between families) to exploit both additive and non-additive genetic effects of CBSD. Having molecular markers associated with these QTL would aid in the selection process.

Conflict of interest

The authors have not declared any conflict of interest.

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Supplementary Table 1. Effect of inbreeding on sprouting, growth vigor and height of cassava in selected genotypes.

Parameter	Parent (S_0)		Partial Inbreds				ID*
	Means	No. of plants	Means	No. of genotypes	Min.	Max.	
Sprouting %[1]							
Namikonga	80.0	21	63.3	15	16.7	100	20.8
Tz/130	73	43	74.34	60	16.7	100	-1.0
130040	61.9	25	64.1	40	16.7	100	-3.6
Tz/140	100	6	57.1**	7	16.7	100	42.9
Kigoma Red	45.8	8	63.3	7	16.7	100	-38.2
182/00661	100	18	63.9**	6	16.7	100	36.1
0040	-	-	62.1	27	16.7	100	-
100142	-	-	63.2	17	16.7	100	-
-	LSD = 34.9	Cv% = 30.1	LSD = 29.3	CV% = 43	-	-	-
Vigor[2]							
Namikonga	4.2	21	4.8	15	3	7	-14.2
Tz/130	5.5	43	5.5	60	3	7	0
130040	5.3	25	5.5	40	3	7	-3.8
Tz/140	7.0	6	6.1	7	3	7	12.9
Kigoma Red	6.5	8	5.8	7	3	7	10.8
182/00661	5.0	18	5.0	6	3	7	0
0040	-	-	5.6	27	3	7	-
100142	-	-	5.1	17	3	7	-
-	LSD = 1.93	CV%=23.7	LSD = 1.13	CV%= 28.3	-	-	-
Height[3]							
Namikonga	142	21	163.9	15	54	242	-15.4
Tz/130	198.5	43	153.9**	60	35	321	22.5
130040	142.6	25	174.7	40	62	319	-22.5
Tz/140	172.7	6	198.9	7	64	301	-15.2
Kigoma Red	165.5	8	145.4	7	58	224	12.1
182/00661	133.6	18	150.4	6	86	251	-12.6
0040	-	-	154.9	27	69	311	-
100142	-	-	145.4	17	54	311	-
-	LSD = 33.8	CV% = 20.4	LSD = 49.6	CV%=35.9	-	-	-

ID*- Inbreeding depression. **Significant difference at 5% level. [1]sprouting was assessed as proportions of plants that sprouted/plot at 1MAP; [2]Plant vigour scored on a scale of 3, 5 and 7 with 7 = most vigorous, 3 poor vigour, and 5 = intermediate vigour; [3]Height measurements taken at 12 MAP as length from ground to plant apex on plant basis.

Supplementary Table 2. Effect of inbreeding on flowering and yield of cassava in selected genotypes.

Parameter	Parent (S_0)		Partial Inbreds				ID*
	Means	No. of plants	Means	No. of genotypes	Min.	Max.	
Inflorescence							
Namikonga	34.0	21	49.3	15	0	195	-45
Tz/130	51.0	43	50.8	60	0	288	0.39
130040	56.2	6	60.9	40	0	252	-8.4
Tz/140	10.8	8	36.3**	7	0	130	-236.1
Kigoma Red	38.1	18	38.2	7	3	123	-0.3
182/00661	32.9	25	30.9	6	0	150	6.1
0040	-	-	63.6	27	0	270	-
100142	-	-	51.6	17	0	210	-
-	LSD = 23.6	CV% =71.8	LSD = 24.4	CV% = 99	-	-	-
Harvest Index							
Namikonga	0.16	5	0.25**	13	0	0.14	-56.3
Tz/130	0.34	10	0.32	45	0.14	0.42	5.9
130040	0.28	6	0.29	35	0.04	0.40	-3.6
Tz/140	0.35	8	0.28**	6	0.17	0.40	20
Kigoma Red	0.29	10	0.33	7	0.26	0.38	-13.8
182/00661	0.39	15	0.40	3	0.36	0.43	-10.3
0040	-	-	0.33	20	0.22	0.37	-
100142	-	-	0.32	16	0.22	0.40	-
-	LSD = 0.08	CV%=17.4	LSD = 0.06	CV%= 21.4	-	-	-
Dry matter content							
Namikonga	40.8	5	39.3	13	21.7	44.4	2.5
Tz/130	33.1	10	33.4	45	16.1	51.4	-0.9
130040	36.9	6	33.6	35	22.4	41.0	8.9
Tz/140	30.1	8	31.7	6	19.5	41.5	-5.3
Kigoma Red	29.1	10	32.3	7	25.9	41.8	-10.9
182/00661	35.9	15	36.8	3	33.45	40.22	-2.5
0040	-	-	31.2	20	18.5	43.11	-
100142	-	-	30.5	16	20.4	37.1	-
-	LSD = 5.81	CV% = 10.3	LSD = 5.93	CV% = 18	-	-	-

ID*: Inbreeding depression; **Significant difference at 5% .

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