Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/7032
DOI URL: http://dx.doi.org/10.21474/IJAR01/7032

RESEARCH ARTICLE

BIOCHEMICALS ASSOCIATED WITH *CALLOSOBRUCHUS CHINENSIS* RESISTANCE IN SOYBEAN.

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Manuscript Info

Manuscript History

Received: 04 March 2018
Final Accepted: 06 April 2018
Published: May 2018

Keywords:-
Antioxidant, flavonoids, median development period, tannins, peroxidase, bruchid

Abstract

Soybean is a very important crop worldwide because of its 40% protein and 20% oil content. However, soybean suffers damage from bruchid (*Callosobruchus chinensis*) during storage. Understanding of factor(s) contributing to bruchid resistance is useful for development of soybean cultivars with genetic resistance to bruchid damage.

Biochemicals associated with resistance to *Callosobruchus chinensis* were investigated on eight soybean genotypes with varying levels of resistance. Significant differences ($P<0.05$) were observed amongst genotypes with regards to antinutritional factors particularly total antioxidants (TAOX), tannins, peroxidase (POD), and flavonoids. There were no significant differences among genotypes with regards to nutritional factors particularly proteins, starch, lipid peroxidation, and reducing sugars. A resistant genotype AVRDC G8527 had the highest concentration of total antioxidants (1.98 AU min^{-1} mg^{-1}) and tannins (1.85mg TAE $100g^{-1}$) followed by Maksoy 3N while the least was in a very susceptible genotype AGS 292 (TAOX=0.39 AU min^{-1} mg^{-1}, Tannin=0.296 mg TAE $100g^{-1}$). AGS 292 had the highest concentration of flavonoids (31.22 mg QE $100g^{-1}$) while AVRDC G8527 had the least (5.13 mg QE $100g^{-1}$). Peroxidase activity was highest in AVRDC G89 (0.27AU min^{-1} mg^{-1}) while AGS 292 had the least (0.07AU min^{-1} mg^{-1}). From the correlation analysis, a significant strong negative relationship between flavonoids and tannin ($r=-0.73^{**}$) and TAOX ($r=-0.71^{**}$) but a positive non significant relationship with phenolic ($r=0.22$) and alkaloids ($r=0.37$) was recorded. Peroxidase activity had a significant relationships with median development period ($r=0.69^{*}$) indicating that increased peroxidase activity resulted into increased seed resistance through prolonged insect development period. There was a strong relationship between tannins and total antioxidants ($r=0.99^{**}$). These results indicate that secondary metabolites; peroxidase, tannin, and TAOX were biochemicals associated with higher resistance to *C. chinensis* in soybean while flavonoids were associated with higher susceptibility.

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Introduction:-

Soybean [*Glycine max* (L.) Merrill] is an annual legume that belongs to the legume family *Fabaceae* and is classed as an oil crop (Singh *et al.,* 2007). Soybean is one of the five oldest cultivated crops and was utilized by the Chinese as a source of food before 2500 BC (Gibson *et al*., 2005). Nigeria is the largest producer of soybean in sub-Saharan Africa (SSA), followed by South Africa (Tefera, 2015). Commercial soybean production on large farms takes place in Zambia, Zimbabwe and South Africa (Tefera, 2015). However, it is mostly cultivated by small-scale farmers in other parts of Africa where it is planted as a minor food crop among sorghum, maize, or cassava. In Africa, Uganda is the second largest consumer of soybean following Nigeria (Murithi *et al.,* 2016). Soybean is among the major industrial, feed and food crops grown in every continent (Dugje *et al.*, 2009). Soybeans contain 35-40% protein on a dry weight basis (O'Bryan *et al*., 2014). Soybean has all of the essential amino acids in adequate quantities except for methionine and tryptophan (Zarkadas *et al*., 2002) which makes soy products almost equivalent in the quality of the protein to animal sources but with far less saturated fat and zero cholesterol (Young, 1991 and Zarkadas *et al*., 2007).

Soybean production is however being threatened by post harvest losses due to bruchids which are the major storage pests in legumes responsible for post harvest losses world wide (Onyido *et al*., 2011). The seeds damaged by bruchids are unfit for propagation, direct human and animal consumption and processing. One of these bruchids is the adzuki bean weevil, *Callosobruchus chinensis* (L.). *C. chinensis* larvae utilize a variety of dried legume seeds as their hosts, primarily *Vigna species* and genera such as *Cajanus* and *Lens*. Spradbery, (2013) reported that this insect often causes considerable damage to stored mungbean (*Vigna radiata* L.), cowpea (*Vigna unguiculata* L.), adzuki bean (*Vigna angularis*), faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), soybean (*Glycine max* L. Merr.) and lotus seed (*Nelumbo nucifere* Gaertn.). Usually, *C. chinensis* causes 32-64% losses varying between crops as well as genotypes within the crop (Swella and Mushobozy, 2009).

Use of pesticides to manage bruchids is effective, however it is expensive, dangerous to humans, the environment and pests easily develop resistance. The most environmentally friendly and cost effective method especially in the developing world to control the bruchids would be the use of resistant varieties. Resistance to seed damage by bruchids is conferred by both physical and chemical factors of the seeds (War *et al.,* 2017). Soybean has been reported to contain and produce biochemicals that make it fairly resistant to insect pests. Soybeans produce a wide range of secondary metabolites, or allelochemicals, to protect themselves from devastation by animals, insects and pathogens (Guo *et al*., 2012). Plant toxic secondary metabolites are important defensive traits involved in plant defense against insect pests (War *et al.,* 2013). They act either directly on insect pests through antibiosis or indirectly through antixenosis by developing the non-preference for insects feeding on the seeds (War *et al.,* 2013). The toxic secondary metabolites when ingested by insects, they decrease larval weight, extends generation time and induce mortality (Guo *et al*., 2012).

Bearing in mind the importance of soybean in nutrition and economic aspects, it was deemed necessary to carry out an investigation on soybean genotypes with the aim of identifying biochemicals that are associated with *C. chinensis* resistance in soybean genotypes. In this study it was hypothesized that (i) soybean contains more than one type of metabolite which are responsible for and associated with bruchid resistance; (ii) soybean genotypes contain varying amounts of resistance metabolites.

Materials and methods:-

Eight soybean genotypes differing in response to bruchid infestation were used in this study, (Table 1) and all reagents used in the study were sourced from BDH Laboratory suppliers–Uganda. This work was done at Nutritional and Bioanalytical laboratory based at the National Crops Resources Research Institute (NacRRI) in Uganda.

Three replicates of 50g seeds from each genotype were used for estimation of peroxidase, flavonoids, phenolics, reducing sugars, carbohydrates, alkaloids, starch, protein, lipid peroxidation, tannins, phytic acid and antioxidants. Seeds were ground into fine powder using a mortar and pestle.

Proteins were determined using a method by Nuwamanya *et al.,* (2014) with modifications. A sample of 0.3 g of was weighed into clean tubes and 2 ml of distilled water added. The samples were heated in a water bath at 80^{o} C for 30 minutes, then left to settle. Samples were filtered and 0.1ml transferred to clean tubes in triplicate. Then 3 ml of

Bradford reagent were added and mixed with the samples, then left to stand for color development. Absorbance was read at 595nm and bovine serum albumin was used to prepare the standard curve to estimate the protein concentration in samples (Sales *et al.* 2000).

Table 1:-Soybean materials used in the study and their susceptibility variables

			Weights			Insect Susceptibility Variables					
Genotype	**Source**	**Resistance status**	**Initial**	**Final**	**% loss**	**Eggs**	**Adult**	**% IE**	**MDP**	**DSI**	**GI**
AGS 292	Taiwan	VS	10678	9616	9.95	171.7	89.67	51.72	29.67	6.459	3
AVRDC G8527	Taiwan	Resistant	4198	4197	0.02	24	2.67	6.31	27	0.704	0.07
Introduction G89	Taiwan	Resistant	6402	6081	4.46	26.3	4.67	23.86	43.33	1.667	0.12
Maksoy 1N	Uganda	Susceptible	6312	5833	7.6	112.7	56	50.83	32.67	5.221	1.71
Maksoy 2N	Uganda	VS	6698	5995	10.5	186.3	90	48.22	31	6.359	3.02
Maksoy 3N	Uganda	Susceptible	8660	8270	4.5	104.3	40.33	36.45	19.67	4.048	1.36
S-Lines 13.2A	Uganda	MR	3977	3842	3.44	140	12	7.18	23.67	2.12	0.36
S-Lines 9.2	Uganda	MR	6411	4926	15.94	52	14	17.58	24.33	2.123	0.38

Source: *Work by Msiska Ulemu in Uganda unpublished.* VS=Very susceptible, MR=Moderate Resistant, %IE=percent Insect emergence, MDP= Median Development Period, GI= Growth Index, DSI= Dobie susceptibility index.

Starch content was estimated by following the method reported by Parthiban *et al.*, (2012) with some modifications. One hundred mg of the sample was homogenized in hot 80 percent ethanol to remove sugars. The residue was retained after centrifugation. The residue was washed with hot 20% ethanol till the washings did not give colour with anthrone reagent. The residue was dried well in a water bath and 5 ml of water and 6.5 ml of 52% perchloric acid were added. Starch was extracted at 0°C for 20 minutes. The extract was retained after centrifugation and extraction repeated with fresh perchloric acid. The extracts were pooled after centrifugation and the volume was made up to 100 ml with 52% perchloric acid. To 0.2 ml of the extract, 0.8 ml of distilled water and 4 ml of anthrone reagent were added and the resultant mixture was heated for 8 minutes in a boiling water bath and cooled rapidly. The colour intensity was read at 630 nm using a spectrophotometer (Parthiban *et al*., 2012).

A modification of the method by Nuwamanya *et al*., (2014) was used to estimate the reducing sugars. A sample of 0.5 ml from the soaked soya beans was placed into a test tube, followed by addition of 0.5 ml of 5% phenol solution. The contents were shaken well and 1 ml of distilled water was added, followed by addition of 1 ml of concentrated sulphuric acid. The samples were gently shaken and then left to cool and develop a blue color in the dark for 10 minutes. Absorbances were read in a spectrophotometer at 490nm (Parthiban *et al*., 2012). A glucose standard curve was used to estimate the reducing sugars in the samples.

Total phenolics were extracted using methanol (50 ml), from 1 g of the sample with continuous swirling for 1 hour at room temperature using an orbital shaker. Extracts were filtered under suction and stored at -20 °C for further use (Wong *et al.,* 2009). Total phenolic content (TPC) was determined using the Folin-Ciocalteu method as reported by (Wong *et al.,* 2009). Soybean extract of 300 µl in triplicate were introduced into test tubes to which 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times with distilled water) and 1.2 ml of sodium carbonate (7.5% w/v) were added respectively. The reaction mixture was shaken, and then allowed to stand for 30 minutes at room temperature and the absorbance of the resulting blue coloured mixture was measured at 765 nm against a blank prepared by dispensing 300 µl of distilled water instead of sample extract. Total phenol content was expressed as gallic acid equivalent (GAE) in mg/g material.

Total flavonoid content was determined using aluminum chloride method as reported by Kale *et al.*, (2010). A methanolic extract of 0.5 ml was dispensed into test tubes, followed by 1.5 ml of methanol, 0.1 ml of aluminum chloride (10%), 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The reaction mixture was shaken, and then allowed to stand at room temperature for 30 minutes before absorbance was read at 514 nm. Total flavonoid content was expressed as quercetin equivalent (QE) in mg/g material.

The total potential antioxidant activity of the investigated soybean genotypes was determined using a method by Ahmed *et al.,* (2015) with some modifications. Plant material was extracted using 50% aqueous methanol and then centrifuged to separate residues from supernatant. In a test tube, 0.1 ml of the plant extract was added, followed by 2.5 ml of phosphate buffered saline (PBS). Thereafter, 2.5 ml of 1% potassium ferricyanide was added and the solution incubated for 20 minutes at 50^0 C. After, 2.5 ml of 80% phosphoric acid was added. The sample was centrifuged at 10000 rpm for 10 minutes and 5ml of supernatant was transferred into a fresh tube. Then 5ml of distilled water was added, followed by 1 ml of 0.1% ferric chloride. The sample was mixed well and absorbance read at 700nm.

Alkaloids were determined using the method of Babu *et al*., (2012) modified by Vijay and Rajendra, (2014) where 1 g of sample was ground in 5 ml of 90% ethanol and then refluxed at 85^o C for 40 minutes. The sample was filtered and the filtrate evaporated to dryness at 45^o C in a vacuum concentrator. The resulting residue was dissolved into 3 ml of phosphate buffer (pH = 4.5) and transferred into a separating funnel. The resulting solution was mixed with 3 ml of bromocresol green solution and let to stand for 30 minutes. Then 5 ml of chloroform was added and vortexed for 2 minutes, then let to settle for 10 minutes. The lower layer in the funnel was then separated off. The extraction was continued for 3 more times and the extracts were mixed in a volumetric flask. Using 90% ethanol as blank, extracts were analyzed by using a UV–Vis spectrophotometer at a wavelength of 418 nm (Vijay and Rajendra, 2014).

Lipid peroxidation was determined in terms of content of malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, which is considered a useful index of general lipid peroxidation. The MDA was calculated using the extinction coefficient, ε= 155 $nMol^{-1}$ cm^{-1}), following the method of Heath and Packer (1968) as reported by (Hodges *et al*., 1999):

$$\text{MDA equivalents (nmol.ml}^{-1}) = \left[\frac{(A_{532} - A_{600})}{155000}\right]10^6$$

where A_{532} nm represented the maximum absorbance of the TBA-MDA complex, A_{600} nm the correction for nonspecific turbidity, and 155000 the molar extinction coefficient for MDA.

Ground soybean sample (0.1 g) was homogenized in 5 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged for 5 min (15000 x g, 4.0 ^{o}C) and 1ml aliquot of the supernatant collected was mixed with 4 ml of 0.5% (w/v) thiobarbituric acid (TBA) diluted in 20 % (w/v) TCA. The mixture was incubated in water bath at 95 ^{o}C for 30 minutes and then the reaction was stopped by quickly cooling the sample in an ice bath. The sample was further centrifuged for 10 minutes (10000 x g, 4,0 ^{o}C) (Taulavuori *et al.,* 2001). Absorbance was measured at 532 and 600 nm. The value of non specific absorption at 600nm was then subtracted. MDA content was then expressed as nmol MDA per g.

Peroxidase activity was determined using a method by Shannon *et al*., (1966). A 100mg sample of each genotype was extracted with 2 ml of 0.1 M potassium phosphate buffer (pH=7.5) containing 1% mM EDTA, 1% polyvinylpyrolidone (PVP) and 10mM β- mercaptoethanol using prechilled pestle and mortar. The contents were then transferred into eppendorf tubes and centrifuged (PrismaR, Edison, New Jersey, USA) at 1,000 rpm for 20 minutes. Thereafter, 0.02 ml of the supernatant were placed into a test tube containing 3 ml of 0.1M potassium phosphate buffer (pH=6.5). The reaction mixture, without the H_2O_2, was measured as a blank. The reaction was initiated by adding 0.8M H_2O_2 and the breakdown of the H_2O_2 was monitored for 2.5 minutes at a 30 second interval, at 24 ºC, by recording absorbances at 470 nm in a spectrophotometer (Biowaveii+, Cambridge, England). The activity of the enzyme was calculated and expressed as absorbance units/min/mg (Mizobutsi *et al*., 2010).

The quantitative tannin content in samples was estimated by the method of Price and Butler, (1977) as reported by Mrudula and Prabhu, (2014) with some modifications. Known concentration of methanolic extract were taken and made up to 0.5ml using distilled water. To this reaction mixture, 1 ml 1% K3Fe(CN)6 and 1ml 1% FeCl3 were added, and the volume was made up to 10 ml with distilled water. The reading of the resultant solution was measured spectrophotometrically at 720nm after 5 min using tannic acid as a standard. The tannin content was expressed as mg of tannic acid equivalent/100g of sample.

Phytic acid was determined using a method reported by Yahaya *et al.*, (2013). Four grams of ground sample was soaked in 100 ml of 2% hydrochloric acid (HCl) for 3 hours and then filtered through two layers of filter paper, 25 ml of the filtrate was placed in a 250 ml conical flask and 5 ml of 0.3% ammonium thiocyanate (NH_4SCN) solution was added as an indicator, 53.5 ml of distilled water was then added to reach the proper acidity. This mixture was titrated against ferric chloride ($FeCl_{3)}$ solution, which contains about 0.00195 g of iron per ml of $FeCl_3$ solution until a brownish yellow colour that persisted for 5 minutes was obtained (Ileke, 2014). The result was multiplied by factor 1.95 to obtain phytate P. Phytate P result was multiplied by factor 3.55 to convent to phytate.

Data Analysis:-
All measurements were done in triplicate. One way Analysis of variance (ANOVA) with genotype as a treatment structure of seed metabolites concentrations was done using Genstat 12th edition statistical software package. Correlation analysis was carried out between the metabolites and the morphological susceptible parameters in Genstat 12th edition.

Results:-

The results on the quantitative estimation of secondary metabolites associated with resistance to *Callosobruchus chinensis* in soybean are presented in Table 2. Significant differences (P<0.05) were observed amongst genotypes for tannins, flavonoids, total anti-oxidants and phenolics. Genotypes did not significantly differ in the concentration of alkaloids. Table 3 presents results on primary metabolites estimation in different soybean genotypes. Genotypes did not show significant differences for lipid peroxidation, phytic acid, proteins and starch but for reducing sugars. Comparison of the concentrations of phenolics, flavonoids, tannins and alkaloids is presented in Figure 1. Tannins were the most abundant secondary metabolites.

Table 2:-Mean concentrations for the secondary metabolites in 8 soybean genotypes

Genotype	Tannins	TAOX	Flavonoids	Alkaloids	Phenolics
	mgTAE /100g	$AUmin^{-1}mg^{-1}$	mgQE/100g	$AUmin^{-1}mg^{-1}$	mg GAE /100g
AGS 292	0.296	0.391	31.22	0.27	554.4
AVRDC G8527	1.845	1.978	5.13	0.17	148.5
AVRDC G89	1.21	1.324	5.88	0.19	1051.1
Maksoy 1N	1.013	1.176	6.11	0.16	139.1
Maksoy 2N	0.394	0.509	22.14	0.14	572.1
Maksoy 3N	1.651	1.778	8.37	0.16	368.6
S-Line 13.2A	0.441	0.565	8.56	0.26	367.6
S-Line 9.2	0.968	1.112	10.91	0.25	249.7
P-Value	0.021	0.023	<.001	0.369	0.043
LSD	*0.937*	*0.957*	*9.209*	*0.143*	*532.985*

TAOX= Total antioxidants, TAE= tannic acid equivalent, QE =Quercetin equivalent, GAE= Gallic Acid equivalents, AU= Absorbance Units

Analysis of variance showed significant differences in tannins among soybean genotypes (P<0.021). Tannin concentration for the studied genotypes ranged from 0.296 to 1.845 mg of tannic acid equivalents per 100g sample. Genotype AGS 292 had the lowest tannin concentration while AVRDC G8527 had the highest. Tannin concentration was highest in resistant genotypes and lowest in susceptible genotypes indicating that tannins may be the basis for resistance to *C. chinensis* in soybean.

Total antioxidants (TAOX) concentration was highest in resistant genotypes and lowest in susceptible genotypes (P<0.023) indicating that TAOX negatively impacted bruchid growth and development. AVRDC G8527 had the highest total antioxidants concentration (1.98 AU $min^{-1}mg^{-1}$) followed by Maksoy 3N (1.78 AU $min^{-1}mg^{-1}$) while susceptible AGS 292 had the least concentration (0.39 AU $min^{-1}mg^{-1}$).

Flavonoids concentration was highest in susceptible genotypes than in the resistant genotypes (P<0.001) indicating positive impact on bruchid development. The most susceptible genotype, AGS 292 had the highest concentration of

flavonoids (31.22 mg QE per 100g) followed by Maksoy 2N (22.14 mg QE/100g) while AVRDC G 8527 had the least content of flavonoids (5.13mg QE/100g).

Alkaloid concentration in soybean seeds ranged from 0.14 - 0.27 mg of AE per g of extract. The highest content was measured in AGS 292 and the lowest in Maksoy 2N. Nevertheless there was no significant differences in alkaloid concentration between the resistant genotypes and susceptible genotypes indicating that alkaloids may not be the basis for soybean resistance to *C. chinensis.*

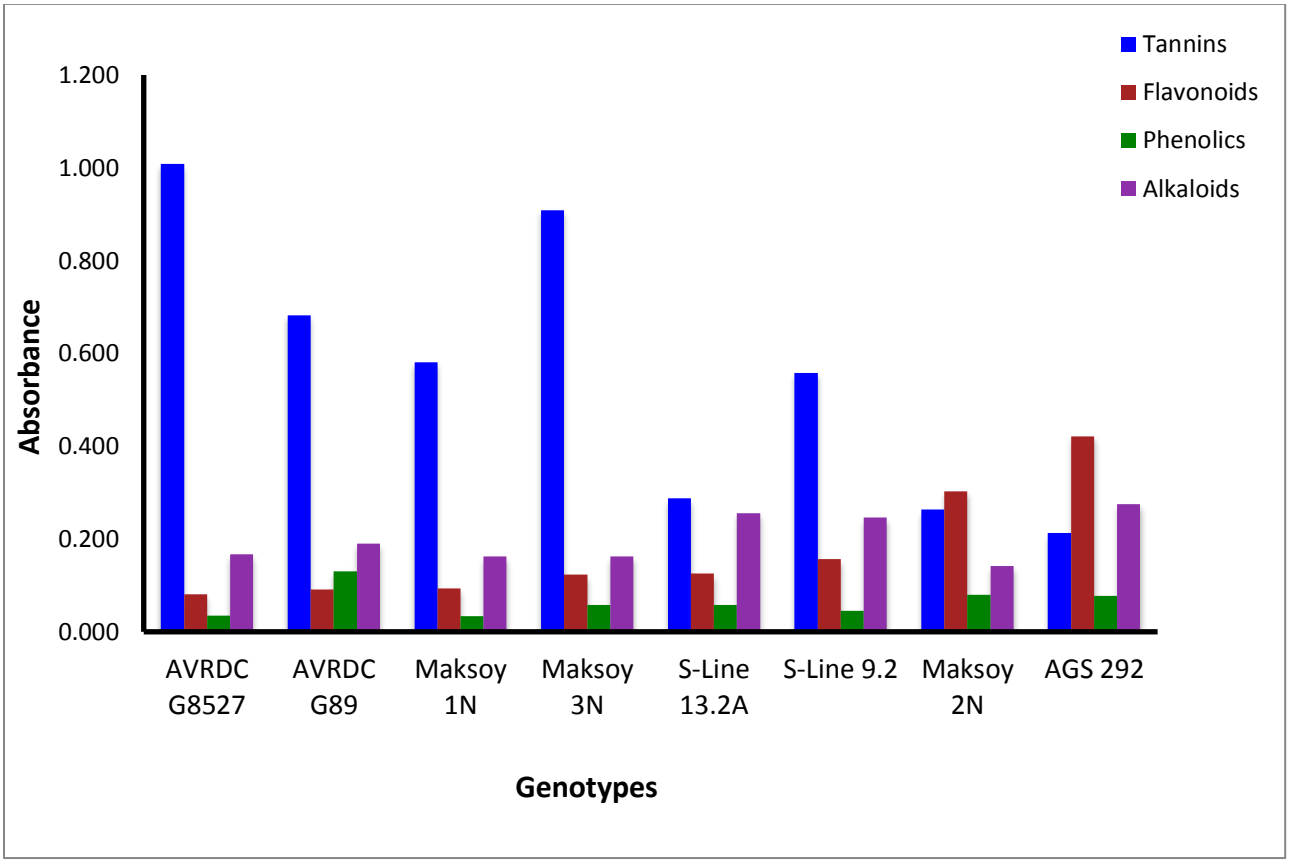

Figure 1:-Comparisons of secondary metabolites Absorbance concentrations

The highest phenol concentration was recorded in a resistant genotype AVRDC G89 (1051 mg of GAE per 100g) followed by AGS 292 (554.4 mg GAE per 100g) while the least concentration was recorded in Maksoy 1N (139.1 mg GAE per 100g). However though genotypes showed significant difference, there were no defined differences in phenol concentrations between the susceptible and resistant genotypes suggesting that it may not be a good trait for determining resistance or susceptibility to bruchids in soybeans.

The results of Phytic acid content in different soybean genotypes showed that there were no significant differences among the soybean genotypes. The highest phytic acid was in S-Line 13.2A (11.19mg/100g) and lowest was in AGS 292 with 8.88mg per 100g (Table 3).

There were no significant differences in lipid peroxidation amongst soybean genotypes indicating no difference in extent to which genotypes caused *C. chinensis* organ damage. The highest lipid peroxidation activity was observed for AVRDC G89 (1.16 nmol $MDA^{-1}g$) followed by Maksoy 3N (0.51 nmol $MDA^{-1}g$) and lowest in AVRDC G8527 and Maksoy 1N (0.01 nmol $MDA^{-1}g$).

The results for starch concentration in seed coats and cotyledons are shown in Table 3. There were no significant differences in starch content among the different genotypes indicating that starch had no effect on seed resistance to

C. chinensis. Highest concentration was observed in S-Line 13.2A (64.13 mg^{-1}100g) and lowest was in AGS 292 (18.69 mg^{-1}100g)

Protein concentration did not differ significantly amongst the studied soybean genotypes. Highest protein concentration was observed in Maksoy 3N (48367.49 mg^{-1} 100g) and the lowest in S-Line 13.2A (28900.27 mg^{-1}100g). The results indicate that proteins were not responsible for resistance to *C. chinensis* in soybean.

Genotypes showed significant differences in concentration of reducing sugars (P<0.035). The highest reducing sugar concentration was on AGS 292 and Maksoy 3N (0.007mg^{-1}100g) while the lowest reducing sugar concentration was recorded in Maksoy 2N (0.005 mg^{-1}100g). However there was no trend observed between the resistant and susceptible genotypes signifying that reducing sugars may not be the basis for resistance to *C. chinensis* in soybean.

Table 3:-Mean concentrations for the primary metabolites in 8 soybean genotypes

Genotype	LP (nmol MDA/g)	Phytic A (mg/100g)	Protein (mg/100g)	RS (mg/100g)	Starch (mg/100g)
AGS 292	0.02	8.88	35913.02	0.007	18.69
AVRDC G8527	0.01	10.15	33180.78	0.006	48.89
AVRDC G89	1.16	9.23	47069.67	0.006	50.13
Maksoy 1N	0.01	10.04	39943.08	0.006	38.84
Maksoy 2N	0.02	9.58	43221.77	0.005	38.56
Maksoy 3N	0.51	10.61	48367.49	0.007	27.58
S-Line 13.2A	0.06	11.19	28900.27	0.006	64.13
S-Line 9.2	0.15	10.96	40785.52	0.006	49.01
P-Value	0.462	0.175	0.952	0.035	0.065
LSD	*0.583*	*1.878*	*38590.56*	*0.00117*	*27.165*
LP= Lipid peroxidation, PA= Phytic acid, RS= Reducing sugars, MDA= Malondialdehyde					

Peroxidase Activity:-

The results on peroxidase activity for the studied genotypes over a period of 150 seconds are presented in Figure 2. Significant variations were observed amongst genotypes for peroxidase activity (P<0.001). Peroxidase activity was not statistically affected by time (P=0.998) and the interaction between time and genotypes (P=1.00). Resistant genotype G89 had the highest peroxidase activity (0.27 AU min^{-1} mg^{-1}) followed by genotype S-Line 13.2A (0.18 AU min^{-1} mg^{-1}) while the least activity was observed on a susceptible genotype AGS 292 (0.07 AU min^{-1} mg^{-1}). As indicated by non-significant interaction between time and genotypes, The reaction of genotypes did not vary with time, genotypes showed consistency in their response; for example genotype G89 had highest activity at all observational times.

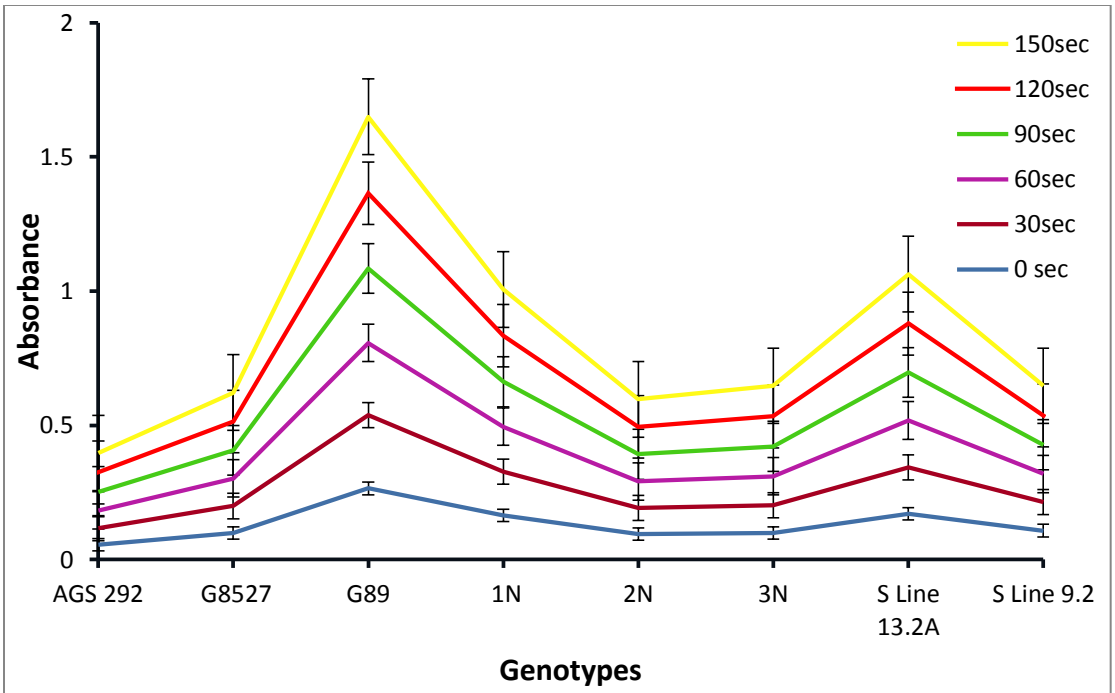

Figure 2. Peroxidase absorbances of soybean genotypes over 150 seconds

Correlation Analysis:-

Correlations coefficients for biochemical and susceptibility variables are presented in Table 4. Peroxidase had a significant positive correlation with median development period (r=0.69*). Peroxidase activity had negative relationships with percent seed weight loss (r=-0.32), adult emergence (r= -0.50), DSI (r= -0.42), eggs (r= -0.41), insect growth index (r=-0.51) although not significant. These results indicate that peroxidase activity had a significant association with *C. chinensis* resistance through median development period.

Total antioxidants correlated negatively with resistance parameters such as percent seed weight loss (r=-0.15), number of eggs (r=-0.38), number of adult insect emergence (r=-0.17), dobie susceptibility index (r=-0.27), insect growth index (r=-0.18) and positively with median development period (r=0.38) even though not significantly. Tannins had a significant relationship with total antioxidants (r=0.99**) and flavonoids (r=-0.71*). Further more tannins negatively correlated with percent seed weight loss, adult emergence, DSI, and insect growth index. Flavonoids had a significant strong relationships initial seed weight (r=0.70**). Results further showed a strong negative relationship between flavonoids and TAOX (r=-0.73**), a moderate positive relationship with adult emergence (r= 0.54) and DSI (r=0.53). Flavonoids had a negative association with MDP (r=-0.34). Phenols showed a negative relationship with adult emergence, weight loss and DSI. Phenols positively correlated with MDP (r=0.54) and peroxidase (r=0.55).

Table 4:-Correlations for biochemical and susceptibility parameters of the different soybean genotypes

	Pro	**% wtL**	**AE**	**Alk**	**DSI**	**Eggs**	**Flav**	**GI**	**I wt**	**MDP**	**Prx**	**Phe**	**RS**	**TOX**
Prot	-													
%wtL	0.36	-												
AE	0.22	0.43	-											
Alk	-0.5	0.37	0	-										
DSI	0.26	0.45	0.98***	-0.02	-									
Eggs	-0.07	0.3	0.85**	0.21	0.85	-								
Flav	-0.1	0.26	0.54	0.37	0.53	0.46	-							
GI	0.23	0.42	0.99***	0	0.98**	0.85**	0.55	-						
I wt	0.39	0.58	0.85**	0.2	0.83**	0.59	0.70**	0.86**	-					

MDP	0.44	-0.06	0.01	-0.08	0.02	-0.21	-0.34	0.01	0.2	-				
Prx	0.24	-0.32	-0.5	-0.11	-0.42	-0.41	-0.58	-0.51	-0.4	0.69*	-			
Phe	0.47	-0.13	-0.08	0.05	-0.05	-0.13	0.22	-0.06	0.27	0.54	0.55	-		
RS	0.27	0.4	0.62	0.26	0.51	0.39	0.22	0.63	0.75*	0.47	-0.22	0.16		
TOX	0.29	-0.15	-0.17	-0.51	-0.27	-0.38	-0.73**	-0.18	-0.26	0.37	0.13	-0.25	0.27	-
Tan	0.29	-0.16	-0.17	-0.51	-0.27	-0.38	-0.71**	-0.17	-0.24	0.37	0.12	-0.23	0.29	0.99**

Key for Table 4:-

Prot= Protein, %wtL= Percent seed weight loss, AE=Adult Emergence, Alk= Alkaloids, DSI=Dobie susceptibility Index, Flav=Flavonoids, GI=Growth Index, Iwt=Initial seed weight, MDP=Median development period, Prx= Peroxidase, Phe=Phenolics, RS=Reducing sugars, TOX=Total antioxidants, Tan=Tannins

Discussion:-

Callosobruchus chinensis has been considered to be one of the most important storage pest on soybean in Uganda Tukamuhabwa (2015 Soybean breeder, Makerere University Agricultural Research Institute personal communication). Host plant resistance is an important component of an integrated pest management strategy for the control of *C. chinensis* (Nahdy, 1995). Currently some genotypes were identified as sources of resistance however little information has been available on levels and basis of resistance already present within the soybean germplasm in Uganda.

In this study biochemical analysis was done on resistant, moderate resistant and susceptible genotypes to determine the basis for resistance to *C. chinensis.* Results from the present study indicated that resistant genotypes had higher tannin and total antioxidants concentration, peroxidase activity and lower flavonoids concentration compared to susceptible genotypes. Resistant and susceptible genotypes did not differ in concentration of nutritional factors such as starch and protein. Correlation analysis revealed that antinutritional factors such as tannins, total antioxidants and peroxidase were correlated negatively to DSI, adult emergence, seed weight loss, insect growth index and percent insect emergence but positively to MDP.

Variations observed in the anti-nutritional factors particularly, tannins, total antioxidants, flavonoids, and peroxidases amongst soybean genotypes explains the differences observed in resistance levels of the genotypes and therefore be crowned as the basis for soybean resistance to *C. chinensis.* The results indicate that there is an array of compounds found in seeds that act either additively or synergistically against bruchids. They act either directly on bruchid through antibiosis or develop the non-preference for insects feeding on the seeds (War *et al.,* 2017). The secondary metabolites serve to reduce or destroy the palatability of the plant in which they are produced. The results in this study are in agreement with Sharma and Thakur, (2014) who reported that anti nutritional factors were responsible for bruchid resistance in legumes and not nutritional factors.

Results of comparison of soybean antinutritional compounds (Figure 2) indicate that phenols, tannins, alkaloids and flavonoids were all present in all soybean genotypes studied. This finding is in agreement with the findings of Pereira *et al.,* (2009). The findings further highlight that there is a bigger potential in utilizing soybean antioxidants as a basis for resistance in addition to physical factors. Chung, (2009) reported that tannins, flavonoids and isoflavones are the large constituents of the soybean antioxidants. Chung, (2009) further reported that cultivars of soybean have varying concentration of antioxidants. Presence of antioxidants indicate the capacity of a genotype to cause vital damage to organs in insects which is a firm defense mechanism related to bruchids (Kolawole and Kolawole, 2014).

Findings in this study showed that flavonoids were higher in susceptible genotypes than resistant genotypes. This finding is agreement with the report by Lattanzio *et al.,* (2006) who reported that most plants contain an array of flavonoids which phytophagous insects usually differentiate. Lattanzio *et al.,* (2006) went further to explain that some flavonoids are feeding and oviposition stimulants to insects implying that genotypes with high concentrations of such flavonoids will be susceptible to insect pest attack. Further more, in comparison to many other secondary metabolites, flavonoids are apparently not very toxic to and have a low physiological activity in most insects (Harbone, 1980).

Significant differences observed in phenols among genotypes did not show any defined pattern between the susceptible and resistant genotypes suggesting that it may not be responsible for susceptibility of genotypes to bruchids. Similar observations were reported by Mahatma *et al*., (2011). Mahatma *et al.,* (2011) observed that

genotypes reported increased content of phenolics but without conferring any resistance. This could be attributed to the fact reported by Wu *et al*., (2015) that plant phenolics were not toxic to insects unless prophenoloxidase genes are lost or the levels of phenolics exceed the catalytic activity of the gut prophenoloxidases. Prophenoloxidases which are produced in the foreguts detoxify phenols in the midgut of insects.

Tannins are the most abundant secondary metabolites made by plants. The significant differences in tannin concentration amongst resistant and susceptible genotypes implied that tannin played a role in soybean resistance to *Callosobruchus chinensis*. Tannins are generally considered to be deleterious to phytophagous insects. Tannins may affect the growth of insects in three main ways: they have an astringent taste, which affects palatability, and decreases feed consumption, they form complexes of reduced digestibility with proteins and they act as enzyme inactivators (Winkel *et al.* 1998). In cowpea, condensed tannins (proanthocyanidins) contributed to resistance to infestation by *Callosobruchus maculatus* (Belay *et al*., 2017). Barbehenn and Constabel, (2011) reported tannin toxicity in insects is thought to result from the production of high levels of reactive oxygen species which react with high pH guts, forming semiquinone radicals and quinones. When developing larvae fed on the tanniferous soybean, tannins permeated the peritrophic envelopes thereby producing fatal lesions in insects midgut which subsequently led to reduced growth index of the insects in the resistant soybean genotypes (Barbehenn and Constabel, 2011).

Significant differences were observed in peroxidase activity between resistant and susceptible soybean genotypes. Resistant genotypes showed higher levels of peroxidase activity than susceptible ones indicating that peroxidase negatively impacts *C. chinensis'* growth and development in soybean. Similar results were reported in field beans by Babu *et al*., (2012) suggesting that peroxidase enzymes play a defensive role in plants against bruchids. Peroxidases deter the feeding of insects and produce toxins that reduce the plant digestibility, which in turn leads to nutrient deficiency in insects with drastic effects on their growth and development (War *et al.*, 2012). Furthermore peroxidases impair nutrition through forming electrophiles which oxidize mono-or dihydroxyphenols thereby directly causing toxicity in the guts of the insects (Zhu-Salzman *et al*., 2008). Peroxidase in the presence of hydrogen peroxide catalyzes autoxidation of tannin compounds. Oxidized tannins do react with proteins and decrease their nutritional quality (Barbehenn and Constabel, 2011).

Soybean genotypes did not show variations in protein concentration indicating that proteins were not responsible for resistance. Sales *et al.*, (2000) reported that proteins such as protease, amylase inhibitors, lectins and chitinases are ineffective against host specific bruchids such as *C. chinensis, C. maculatus* and *Zabrotes*.

The correlation analysis results between the susceptibility and biochemical parameters indicated that antinutritional factors such as tannins, total antioxidants and peroxidase were associated with seed resistance in soybean. From the correlation results it can therefore be deduced that soybean genotypes with high tannin and total antioxidants would have lower number of adult insect emergence, longer median development periods, smaller DSI values, lower seed weight percent loss, delayed growth index and consequently would be resistant to the *C. chinensis* attacks.

Findings in this study revealed that high flavonoid concentration would favour high numbers of adult emergence, seed weight loss, shorter development periods and consequently higher DSI which means more susceptibility to *C. chinensis.* These findings are in contrast with what Calatayud *et al.*, (1992) found in cassava mealybug resistance. The results from the current study indicated that different insect species prefer different biochemicals in plants. There is another theory by Sales *et al.,* (2000) which states that legume genotypes containing high flavonoids have become susceptible to bruchids. Sales *et al.,* (2000) reports that bruchids have specialized in detoxification of some antinutritional factors inclusive flavonoids and may even be stimulated to feed by their presence. Alkaline midgut pH, surfactants, and the peritrophic membrane all may help these species tolerate flavonoid concentrations in the diet.

The study established that there were no significant differences in proteins, lipid peroxidation and starch amongst genotypes. This signified that nutritional factors were not associated with resistance to *C. chinensis* in soybean. Even in reducing sugars where genotypes showed significant differences there was no trend observed between the resistant and susceptible genotypes. This finding is of significant importance in soybean breeding programs for resistance to *C. chinensis* because it implies that nutritional factors of soybean will not be affected with breeding towards more bruchid resistance. These findings are in agreement with Sharma and Thakur, (2014) who reported that nutritional factors were not responsible for resistance to bruchids in chickpea, cowpea and soybean.

All genotypes contained phytic acids (PA) but with no significant differences suggesting that the resistance to *C. chinensis* in soybean was not due to PA. This finding was in contradiction with earlier studies in mungbean (Somta *et al.*, 2007) and cowpea (Fawki *et al.*, 2012) which indicated that PA was associated with bruchid resistance. However, Dhole and Reddy, (2016) working on mungbean argued that higher concentrations of PA were required for the resistant reaction. Dhole and Reddy, (2016) further indicated that even though the resistant gene may be present in a plant, reduced concentration of PA decreases tolerance to biotic stress. Under this reasoning therefore it was speculated that PA concentration in soybean was not high enough to affect bruchid's metabolic activities, growth and development.

The negative relationship between peroxidase and DSI, adult emergence, weight loss and insect growth index indicate that peroxidase contributed positively to seed resistance to bruchids. This finding is in agreement with Khan *et al.* (2003) who reported that resistance-related enzymes such as chitinase, β-1,3-glucanase, and peroxidase are also involved in the bruchid resistance. Lattanzio *et al.* (2006) reported that the effectiveness of phenolics as resistance factors to insect feeding is enhanced by oxidation to polymers, which reduce digestibility, palatability and nutritional value. Thus high levels of polyphenol oxidases and peroxidases, the major phenolic oxidising enzymes of plants, can be correlated with plant resistance mechanisms against insects. The non-significant relationship between peroxidase and seed weight parameters indicate that resistance to *C. chinensis* in soybean is independent of seed size. This finding is in agreement with Sharma and Thakur, (2014b) who reported that seed size had no influence in susceptibility parameters of chickpea, soybean and cowpea.

Significant positive correlation between peroxidase activity and median development period implied that peroxidase activity contributed to slow development of *C. chinensis*. Slow development means less number of generations per year. As such, the result consequently means genotypes with higher peroxidase activity would be more resistant to *C. chinensis.* Therefore peroxidase is amongst the biochemicals associated with *C. chinensis* resistance in soybeans. Current studies results are in agreement with (Sharma and Thakur, 2014a), that peroxidase affected insect metabolic activities and inhibited the growth of the larvae in soybean hence the longer development periods. From the study it can be said that peroxidases confer antibiotic resistance to *C. chinensis* through prolonged insect development periods. Peroxidase activity may therefore be used as a biochemical marker for bruchid resistance in soybean. This finding has a practical application in that soybean varieties with higher peroxidase activity can be bred through genetic engineering as it is done for fungal diseases (Dzhavakhiya and Shcherbakola, 2007).

The results indicate that several compounds in soybeans contribute to its antifeedant and/or antibiotic effects against *C. chinensis*. However it was clear from the study that nutritional factors were not responsible for variations in observed resistance. This is a significant finding since it implies that plant breeding for *C. chinensis* in soybean will not conflict with nutritional components for human consumption. However there is further need to isolate these biochemicals and feed them to bruchids to determine if bruchids are directly affected by the biochemicals.

Conclusions:-

In the present research work evaluated soybean genotypes contained varying concentration of biochemical factors. High tannins, total antioxidants, peroxidases and low flavonoids were the biochemical parameters associated with resistance in soybean. Breeding resistance against *C. chinensis* in soybean should therefore consider these biochemical besides physical parameters.

Conflict of Interest:-

The authors have not declared any conflict of interest.

Acknowledgements:-

Authors are grateful to Intra ACP- Mobility Scheme (CSAA) for funding the first author through the PhD programme. We thank the Carnegie Cooperation (New York), Next Generation Competitive Grant through Ruforum for funding the research.

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