A TECHNIQUE FOR IDENTIFICATION OF INTRINSIC RESISTANCE OF MAIZE VARIETIES TO *SITOPHILUS ZEAMAIS* (MOTSCH.) (COLEOPTERA: CURCULIONIDAE) INFESTATIONS

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ABSTRACT

A new technique used to identify resistant maize varieties to the maize weevil, Sitophilus zeamais *(Motsch.) infestations is presented. It applies Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS - PAGE) of the insect fat body to determine the levels of fat body vitellogenin (FVg) in the vitellogenic* S. zeamais *females which were reared on different maize varieties. Results on levels of FVg varied and ranged from 83.33% to 43.33% in insects raised in different varieties ANOVA of FVg, maize weight loss and F_1 numbers showed significant differences among the varieties at $P<0.05$. Maize weight loss and F_1 numbers were positively correlated with FVg, $r = 0.74$ and 0.98, $P< 0.05$. The revealed amounts of FVg in* S. zeamais *obtained from the varieties indicated varying intrinsic resistance of the varieties to the insect pest. The varieties exhibited resistance in descending order: ZM 621>H 622> H 511> Situka 1> Situka M1 > ZM 523. In conclusion SDS – PAGE of female* S. zeamais *fat body is hence a useful and robust technique for identification of the intrinsic resistance of maize varieties to S. zeamais infestations.*

Key words: Technique, Fat body, Vitellogenin, Maize resistance, *S. zeamais*

INTRODUCTION

Maize, *Zea mays* L. originated in America in the sixteenth century and is now one of the principle cereal food crops in the tropics and sub-tropics (Makate 2010). The crop is also among the major green fodder and vegetables in Europe and North America. In Tanzania it grows under a wide range of ecological conditions depending on a variety (ASARECA – TUUSI 2009). Basically, breeding programmes for high yielding as well as, field pests and disease resistant varieties have resulted in many hybrids and composite varieties available to farmers. In most cases, however, farmers do not gain the maximum possible benefits from maize production due to various constraints including pest infestations at different levels of the production cycle and particularly at the storage phase.

Great losses of stored maize caused by insect pest infestations in Tanzania for instance, hinder agricultural development as stipulated in the URT (2005) National Strategy for Growth and Reduction of Poverty. Stored crop insect pest management technologies amongst rural communities include applications of chemical pesticides that are unreliable in terms of timely availability, high cost, authenticity and inadequate handling practices (Rugumamu 2011). It is noted that the industrial chemicals do in turn become hazardous to the ecosystem coupled with increased demand on the country's meager foreign earnings.

Stored maize is at high damage risk if conditions in storage ecosystems are conducive to the insect reproduction (Dobie *et al*. 1984, Kramer *et al*. 2000). Maize in

storage is infested mainly by the cosmopolitan phytophagous Maize weevil, *Sitophilus zeamais,* which is a predominant pest in the tropics causing enormous losses and deterioration to the food grain due to its attack and damage (Akob *et al*. 2009). According to Wikipedia, this species attacks both standing crops and stored cereal products, including wheat, rice, sorghum, oats, barley, rye, peas and cotton seeds. The maize weevil is also reported to infest other types of stored, processed cereal products, cassava and fruits while in storage.

It is advanced by Murata *et al*. (2008) among others that, *S. zeamais* exhibit holometabolous type of postembryonic development of 36 days period; a small, single oval white egg is laid and go through cleavage and differentiation inside a tiny hole bored on the grain by the female parent and covered by a waxy secretion that creates a plug. That a single female may lay 300 to 400 eggs during her lifetime and adults can live for five to eight months. Eggs hatch into larvae which voraciously feed within the grain, growing and molting before pupation, larvae are responsible for most of the damage on maize as they eat it away from the inside out until mature (Dobie *et al*. 1984 and Abebe *et al*. 2009). Adults then eat their way out of the grain kernel, females move to the high surface and release sex pheromone which attracts males Hill 1987, Chapman 1998).

The adults immediately start aggressive feeding, reproducing, releasing more crop damaging larvae and hence increased destruction of the grains (Dobie 1974, Dobie *et al*. 1984 and Abebe *et al*. 2009). Given that both larvae and adults feed on grains, they create much dust unfit for human consumption and consequently, great maize weight losses causing economic damage to maize, especially in traditional storage systems where control strategies are limited.

Considerable research effort currently goes into increasing grain production in farm fields, yet farmers may not realize all of the productivity gains if grain is lost to storage pests or if storage reduces its nutritional quality (FAO 1991). This problem is particularly acute for subsistence farmers, who produce and store their harvested maize grains locally, often under conditions favourable for insect colonization (GASGA 1978, Dobie *et al*. and 1984 Abebe *et al*. 2009). Conservative estimates of maize post-harvest losses due to insect infestations in developing countries range from 10% to 35% during a storage period of up to six months (FAO 1991 and Tedele *et al*. 2011). It is against this background that in this research, a new technique using insect fat body to identify resistant maize varieties to *S. zeamais* infestations was conceived and investigated.

The objective of this paper therefore is to present the tested intrinsic resistance of maize varieties to *S. zeamais* as reflected by fat body Vg levels. This innovated technique employs Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS - PAGE) to determine the levels of fat body vitellogenin (FVg) in the vitellogenic *S. zeamais* females reared on different maize varieties. Waddill *et al*. (1972) and Chapman (1998) note, for example, that the fat body of insects is a mass of mesodermal cells floating more or less freely throughout the body mainly in the abdominal haemocoel surrounding the gut where it can quickly take up absorbed nutrients.

It is advanced that some activities in the fat body are a function of the nutritional status of *S. zeamais*. Fat body cells have diverse metabolic functions including, synthesis and storage of proteins, lipids and carbohydrates that circulate in the insect haemolymph (Chapman 1998). As reported by Keeley (1971), Keeley (1974), Hilliard *et al*. (1984) on the effects of dietary nitrogen on the reproductive development in the female boll

weevil, *Anthonomus grandis*; Dorchin *et al.* (2006) on gall inducing wasps and by Chapman (1998) nutrition of an insect determines its reproductive performance. Brent *et al*. (2002) as well report that insect ovarian development and oogenesis processes are often regulated by nutrition. Influence of nutrition on performance of different insects was reported by, among others, Wigglesworth (1974), Hans-Jorg (1993), Kostal (1993), Raikhel *et al*. (1998), Dorchin *et al.* (2006). Further, Chapman (1998) and Aluja *et al.* (2001), note that adequate nutrition is of general importance to insects and that in the absence of sufficient food or a lack of protein many insects fail to produce mature oocytes during vitellogenesis. This study is based on these physiological events unique to insects that might be sensitive to disruption for purpose of reducing insect populations and hence effective pest management.

The synthesis of Vg, a female specific lipoglycoprotein is initiated after the corpora allata (CA) Juvenile hormone (JH) stimulates the vitellogenin gene expression in the fat body of most insects (Amdam *et al.* 2002). Since the physiology of insects is characterized by dynamic processes that involve movements and other actions by molecules, Vg is transported in the haemolymph for processing to maturing oocytes (Xavier 1994, Nelson *et al*. 2007). Further, JH stimulate water loss and shrinkage by the ovarian follicle cells that surround the oocyte and then the shrinkage exposes the oocyte membrane to the hemolymph which carries the Vg. The Vg receptor can then bind the Vg for transport into the developing oocyte and yolk formation (Amdam *et al*. 2002). Hence, as a principal yolk protein, Vg amount in the insect's fat body is a sign of its fecundity after its feeding on a particular type of food. The types of food in this study are maize varieties and therefore fat body Vg levels in insects reared on different varieties reflect varying resistance of the varieties fed upon by *S. zeamais*.

Basically if many maize varieties with genetic variations for enhanced resistance to insect storage pests are identified, the information there from could provide materials for breeding more resistant varieties as a contribution to the existing pest management strategies. Consequently farmers will benefit from varieties that can be stored longer without economical damage. Rationally, it makes more sense and it is indeed economical to safeguard the crop that has been harvested instead of trying to make up for the losses through increased production. van Emden (1999), Chapman (2000) among others report that host-plant resistance as a pest control method is environmentally safe, economically acceptable to farmers and most compatible with other components in the Integrated Pest Management (IPM) initiatives. IPM, however, was directed to growing crops in the field while limited attention was paid to protect the crop in storage Chapman (2000). In this regard, the availability of varieties which are resistant to *S. zeamais* infestations up to storage stage can not be overemphasized.

Various methodologies for determining degrees of susceptibility and hence resistance of maize varieties to stored insect pests have been reported by McCain *et al*. (1964), Howe (1971), Dobie (1974), Urrelo *et al*. (1990). The SDS-PAGE of female vitellogenic *S. zeamai*s fat body proteins presents an alternative and indeed a new scientific technique for the assessment of intrinsic resistance of maize varieties to the insect pest damage. This technique employs gel electrophoresis to separate fat body proteins based on their primary structure of size or mass and not amino acid sequence. Sodium Dodecyl Sulphate (SDS) is an anionic detergent which denatures the protein by breaking the disulphide bonds and gives a negative charge to each protein in proportion

to its mass (Brewer *et al*. 1974, Ansari *et al*. 2009).

The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g protein, giving an approximately uniform mass to charge ratio for most proteins, whereby the distance of migration through the gel may be assumed to be directly related to only the size of the protein (Coligan *et al*. 2002). In essence SDS linearizes the proteins so that they may be separated strictly by molecular weight. Further, due to differential migration based on their size, the smallest protein molecules move through the gel further down faster, while larger molecules take longer and result in bands staying closer to the top of the gel. It is reported that molecular weights of vitellogenins are generally large, ranging from 500 to 600 kD (Keeley 1974).

The significance of this initiative rests, in part, to the fact that maize, *Zea mays* (L.) is a major cereal food and cash crop grown and stored in many areas in Tanzania (URT 2005). Currently, different maize varieties are produced in various breeding programmes the world over with the basic aim of addressing only field pest management challenges (Dent 2000). However, it is now known that some grain physical and chemical characteristics could affect their resistance to insect damage in store, a feature which has not yet been fully appreciated (Dhliwayo *et al*. 2001, Rugumamu 2011). Kramer *et al.* (2000) and Flinn *et al.* (2006) reported that avidin, a glycoprotein found in chicken egg white when present in maize at levels of about 100 ppm is toxic to and prevents development of insects that damage grains during storage.

It is anticipated that this new technique could also be used to monitor mass production and distribution of maize varieties for domestic consumption and trade by the ever-increasing number of seed companies. Further, it is worthy noting that various varieties produced by business seed companies may be of "unknown" storage qualities that could lead to losses of higher magnitude resulting into large scale famine. It is in this regard that the following methodology is advanced.

MATERIALS AND METHODS

Parameters for assessment of maize resistance to *S. zeamais*

Maize varieties whose intrinsic resistance to the weevil were tested were ZM 621, H 622, H 511, Situka 1, Situka M1, ZM 523. The varying levels of fat body vitellogenin (FVg) were assessed by SDS-PAGE of fat body proteins from vitellogenic female *S. zeamais* raised on the different varieties. F_1 *S. zeamais* adults emerging from the maize varieties and the weight losses of the varieties after F_1 emergence were determined in order to provide benchmarks or point of reference in relation to the Vg levels in fat bodies of the insects fed on the varieties.

Raising *S. zeamais* F_1 and determination of maize weight losses

A susceptible variety UCA (Rugumamu 2005) that was not used in the resistance experiments was infested with *S. zeamais* cultured in the Department of Zoolgy and Wildlife Conservation. Emerging adults (parents) *S. zeamais* of between one and seven days old were sexed (F: M = 2:1) and conditioned to the 30 g of disinfested test varieties namely, ZM 621, H 622, H 511, Situka 1, Situka M1, ZM 523 at favourable conditions (29 ± 2^o C and R.H. of 60 - 70%) for seven days (Throne 1994).

Thereafter, six replicates of the conditioned 18 parent *S. zeamais* between 7 and 14 days old (peak of oviposition) were placed in fresh 30 g maize samples of each of the six varieties. The maize and insect samples were confined by wire gauze in 130 x 60 mm bottles and incubated for seven days after which these parent *S. zeamais* were removed from the test samples. Then the emerging F_1 adults were

collected and numbers recorded daily. Sample weight method was used to determine maize variety weight loss after F_1 emergence. Control maize sample replicates (un-infested) were used for correcting moisture content changes in calculating weight losses due to insect feeding.

Determination of vitellogenin levels in fat body cells of F_1 adult *S. zeamais*

Abdomens of the raised F_1 vitellogenic *S. zeamais* females of between 7 and 14 days old and that of males reared on the maize varieties were dissection along the ventral axis in order to sample fat bodies from the haemocoel. Fat body samples were placed in Eppendorf tubes containing Phosphate Buffered Saline (PBS) of pH 7.2 and crushed to obtain 80ul sample solution. Equal volumes of blue sampling buffer solution were added to the labeled sample and centrifuged at 10,000*g* for five minutes at 5^{o}C, then boiled in water bath for 3 minutes and cooled on ice. Bromophenol blue, a tracking dye was added to the sample. The dye was to track the progress of the protein through the gel during the electrophoretic run. The different fat body sample solutions were stored at -20^{o}C for later use.

The labeled fat body samples in aliquots of 10 µl were loaded on the gels for separation by SDS – PAGE. A current of 10 mA (per slab) was applied carefully for one hour after which the current was increased to 20 mA to run for two hours. When the blue colour was seen in the electrophoretic buffer, the gels were removed from plates and their sides marked for sample identification. Then staining of gels with Coomasie Brilliant Blue R - 250 was carried out for one hour on a shaker before being repeatedly de-stained for ease of visualization and identification of the separated protein bands.

The gels scanned on a Chromoscan did reveal distinct bands in female and male lanes. In the absence of a protein molecular weight marker, the FVg bands in females were identified by comparing female and male lanes (Dhadialla *et al.* 1991, Fleig 1997). Estimation of different amounts of female *S. zeamais* FVg, was done by using the deepest stained band in each female lane of the sample gel as a standard band and equated to 100% (Fleig 1997). Then FVg bands in separate lanes were therefore compared with their corresponding standard bands.

Data Analysis

Analysis of Variance by Kruskal-Wallis test was used to first, analyse Vg levels in fat bodies of *S. zeamais* reared in different maize varieties, second, F_1 *S. zeamais* populations from the tested maize varieties and third weight loss of the varieties after F_1 emergence. Correlations were performed on FVg and F_1 (Fowler *et al*. 1999, Sokal *et al*. 1998).

RESULTS

Number of F_1 *S. zeamais* adults from the maize varieties

The mean numbers of F_1 *S. zeamais* which emerged from the six varieties of maize ranged from 280.00 ± 4.761 to 176.33 ± 1.87. The mean progeny production of *S. zeamais* on variety ZM 523 was highest and that on ZM 621 was the lowest of the six tested varieties (Table 1). ANOVA indicated significant differences of the emerged F_1 among the varieties at $P < 0.0001$.

Weight loss of the maize varieties after F_1 *S. zeamais* emergence

Calculated weight loss varied for each of the six maize varieties after F_1 emergence. Variety ZM 523 lost the greatest percent weight of 5.46 ± 0.13 while the loss in ZM 621 was 2.83 ± 0.02 and hence the lowest (Table 1). The trend of weight loss was as follows: ZM 621<H 622< H 511< Situka 1< Situka M1 < ZM 523. The P value after a one way ANOVA was <0.0001, showing extremely significant differences of weight losses among the maize varieties.

Amount of fat body Vitellogenin in *S. zeamais* reared on different maize varieties

The SDS – PAGE technique revealed varied band thickness of fat body Vg of females *S. zeamais* reared on different maize varieties as shown in the sample gel (Fig. 1).The estimated mean percentage of FVg was highest (83.33 ± 3.33) in *S. zeamais* raised in variety ZM 523 and lowest (43.33 ± 3.33) in insects that infested ZM 621 (Table 1). One way ANOVA indicated extremely significant difference among the levels of FVg, at $P < 0.05$.

Table 1: *S. zeamais* F_1 numbers, maize weight losses and *S. zeamais* fat body Vg levels (FVg) (means of six replicates)

Variety	F_1 nos.	% wt. Loss	% FVg
ZM 523	280.00 ± 4.76	5.46 ± 0.13	83.33 ± 3.33
Situka M1	260.33 + 4.37	4.11± 0.08	80.00 ± 5.77
Situka	251.33 + 3.49	4.01± 0.01	70.00 ± 0.00
H 511	241.00 ± 3.25	3.61 ± 0.01	66.66 ± 3.33
H 622	224.83 ± 2.68	3.52 ± 0.03	60.00 ± 0.00
ZM 621	176.33 ± 1.87	2.83± 0.02	43.33 ± 3.33

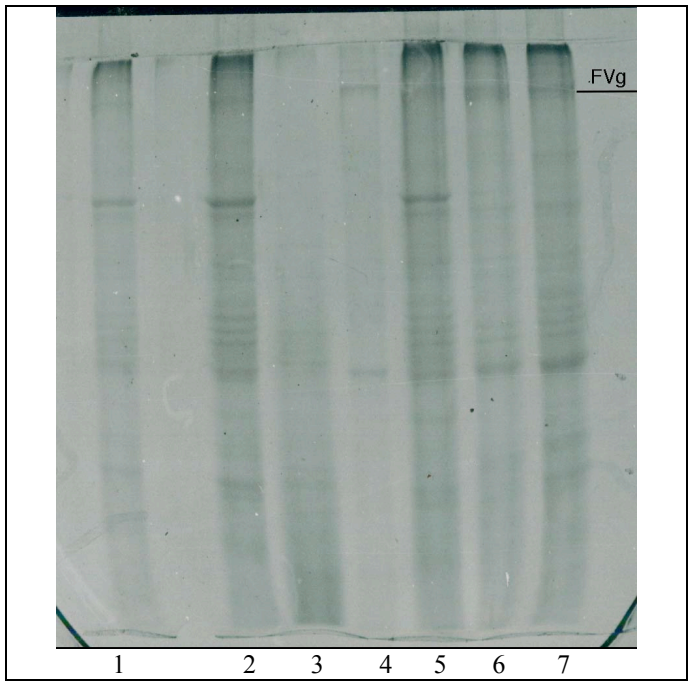

Figure 1 Sample gel image of fat body solution of vitellogenic *S. zeamais* females reared on different maize varieties after an electrophoretic run. The arrow shows positions of fat body Vitellogenin (FVg) in the numbered lanes.

From left to right:

Lane 1	FVg of females from ZM 621
Lane 2	FVg of females from H 622
Lane 3	Males' fat body solution
Lane 4	FVg of females from Situka 1
Lane 5	FVg of female from H 511
Lane 6	FVg of femalse from ZM 523
Lane 7	FVg of females in Situka M1

Relationship of FVg amounts in insects, F_1 *S. zeamais* numbers and maize weight losses

The F_1 *S. zeamais* numbers from the maize varieties correlated positively with the trend of Vg percentage in the insects reared on different maize varieties, $r = 0.98$, $P < 0.05$. Further, the amount of weight loss due to *S. zeamais* attack positively correlated with the Vg level in insects which emerged from the maize varieties, $r = 0.74$, $P < 0.05$.

DISCUSSION

Amount of Vitellogenin in fat body of female *S. zeamais* determined by SDS-PAGE

Findings revealed that the fat bodies of *S. zeamais* which fed on the most resistant variety synthesized the least amount of Vg while the highest amount of Vg was produced by the insects that fed on the most susceptible variety of them all. Following from the above and according to Chapman (1998), the insect reproductive success could be explained in terms of the amount and quality of food available to it during preparation for vitellogenesis. In this regard, ZM 621 being the most resistant of the six varieties provided least materials for the fat body to synthesize the proteins for *S. zeamais* vitellogenesis. It is further reported by Wheeler (1996) that the amount of nutrients available controls the whole process of oogonia development into oocytes. Furthermore, Raikhel (1992) reported that when the oocyte in mosquito or *Blaberus discoidalis* initiates vitellogenesis following feeding the follicle cells shrink and separate to allow the Vg to flow into the oocyte. The more resistant the maize variety to the insect pest damage, the less the amount of the Vg synthesized in the fat body of the insect fed on a particular variety.

Emerged number of F_1 *S. zeamais* from the maize varieties

S. zeamais parents that fed on the different maize varieties produced varying numbers of F_1 progeny and it was observed that, out of the six maize varieties, the fewest numbers of F_1 *S. zeamais* emerged from ZM 621 reflecting a more resistant variety whilst ZM 523 gave rise to most F_1 individuals and therefore more susceptible to the insect pest. A similar observation was reported by Okelana *et al*. (1985) regarding the influence of humidity on *S. zeamais* and also by Appleby *et al*. (2004) with respect to the environmental conditions affecting the response of West African *Callosobruchus maculatus* populations to susceptible and resistant cowpeas.

In this study the findings indicate a positive relationship between the amount of Vg synthesized in the fat body of parent *S. zeamais* and the number of F_1 emerging from the maize variety. It is therefore evident that the amount of Vg synthesized by an insect fat body denotes its reproduction capacity and hence infestation levels of the insect pest to a particular maize variety. As advanced by Murata and Imanura (2008), it is worth noting here, therefore, that, resistant maize varieties negatively affect *S. zeamais* fecundity and hence less infestation levels.

Weight loss of the varieties after F_1 *S. zeamais* emergence

Dobie (1974), Brent and Traniello (2002) reported that susceptibility of maize varieties and other cereals to insect pests attack when determined by weight losses were variable.

The trend of observation from this study shows that the more the number of F_1 *S. zeamais* developed in a maize variety, the greater the amount of weight loss by the variety. Therefore the six maize varieties exhibited varying resistance to *S. zeamais* as expressed by weight losses. The losses were positively related to fat body Vg levels in vitellogenic female *S. zeamais* reared on the varieties. In this regard the greater the amount of the Vg synthesized in the fat body of the insect fed on a variety, the more the F_1 insect numbers the greater the weight loss of the variety and indeed the least intrinsic resistance of the variety to infestation by *S. zeamais*.

CONCLUSION AND RECOMMENDATION

The SDS – PAGE technique showed fat body Vg levels which varied among female *S. zeamais* reared on the different maize varieties. It is therefore concluded that SDS – PAGE of fat body from vitellogenic female *S. zeamais* reared on different maize varieties is a useful and robust technique for the assessment of fat body Vg levels in an insect pest and hence its reproductive potential. The technique in turn leads to the identification of resistant maize varieties to *S. zeamais* infestation. Since the insect reproduction after feeding on a variety depends on the amount of Vg synthesized by fat body, it follows automatically that the amount of Vg in the fat body of a vitellogenic female *S. zeamais* is a measure for the intrinsic resistance of a variety to the insect pest. The results therefore, do form the basis for the management of *S. zeamais* infestations and indeed a contribution to the biological components of IPM.

Given the rigor of the technique, it is recommended that further studies using SDS – PAGE of insect pest FVg levels could be directed to the assessment of many more maize varieties available for cultivation by farmers in order to identify their resistance to *S. zeamais* and other insect pests in lead time. Further, a proximate study on the identified resistant varieties is recommended in order to avoid compromising the nutritive value and palatability of the varieties with their resistance to insect pest attack.

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