Mechanism of Resistance in Improved Mungbean Lines against Bruchids

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Abstract

The purpose of this study was to identify the morphological and biochemical traits in mungbean seed responsible for resistance against bruchids. Seeds were tested for resistance by homogenizing mungbean seed and extracting compounds from the seed using techniques such as HPLC. Seed hardness was determined using a Pentrometer. This research project will impact mungbean production by reducing the amount of pesticide used to control Bruchids. Legumes are the second most important group of crops after cereals, particularly in developing countries. They are the cheapest source of dietary proteins (25-40%), carbohydrates (50-60%), fat, minerals, vitamins and amino acids such as lysine and tryptophan (Lambrides and Godwin, 2007). Mungbean also known as green gram is an important short duration legume grown widely in South and South East Asia. Bruchids are the important pest of mungbean both in field and the storage. The primary infestation occurs in the field, where female lays eggs on mature pods near the seeds. The eggs hatch and the larvae the seed testa and feed on internal content. Bruchids take a heavy toll on yield and storage losses are significant and sometimes total losses occur within 3–6 months (Somta et al., 2007; Tripathy, 2016). Biochemical compounds in seed and seed coat confer resistance to Bruchids in mungbean, but the basis of the resistance is complex and ambiguous. These factors influence egg hatching and effect larval growth and development. The resistance in *Vigna* species is either a result of a single component or a combination of chemicals.

Purpose

Vigna radiata commonly named mungbean or green gram is an important pulse crop in Southern and Eastern Asia. Mungbean is typically grown in rotation between two main grain crops such as rice. The seeds are consumed as sprouts or whole seeds and used in various dishes such as soup. Mungbean seeds are a good source of digestible protein that exhibits low flatulence. The lifecycle of mungbean is typically completed in approximately sixty days. As a legume, mungbean can utilize nitrogen fixation in symbiosis with soil rhizobia allowing the plant to grow well in nitrogen poor soil. Mungbean has excellent drought tolerance and can perform well in areas that are primarily rain-fed.

Major pests that affect mungbean include pod borer complex (*Maruca vitrata* and *Helicoverpa armigera*), Thrips spp., whitefly (*Bemisia tabaci*), cowpea aphid (*Aphis craccivora*), bean fly (*Ophiomyia (Melangromyza) phaseoli*) and bruchids (*Callsobruchus* spp.). Bruchids can infest mungbean in both the field and in storage but are primarily storage pests with heavy or total losses occurring within 3-6 months (Tripathy, 2016). Bruchid infestation in mungbean seed can result in weight loss, low germination, and nutritional changes which reduces the nutritional and market value possibly rendering it unfit for human consumption, agricultural and commercial uses (War et al., 2017). Female bruchids use various tactile, chemical, and physical cues to choose suitable egg-laying substrate. These include multiple sensory modalities, egg-marking pheromone, and larval feeding vibrations from the seed (Oshima et al., 1973; Ignacimuthu et al., 2000; Guedes and Yack, 2016). Current storage control methods of bruchids used include seed treatments which utilize chemicals such as carbon disulfide, phosphine, or methyl bromide. Seed quality is affected when the seed is coated in the seed treatment. Chemicals used pose a high food safety risk, severe illness has been reported when seeds with chemical residue were consumed. There are plant-based bruchid control methods which utilize plant derived extracts such as soy oil, maize oil, neem oil, hot pepper powder, custard apple and banana plants (Koona and Dom, 2005; Swella and Mushobozy, 2007). Plant-based control methods are slow to react, highly degradable and affects seed germination. (Yusuf et al., 2011). Currently, possible unintended effects on non-target organisms from the utilization of plant-based control methods cannot be ruled out (Sharma et al., 2012). Dust and wood ash has been shown to provide some protection against bruchids, but the high cost and laborious application process restricts its use and is uneconomical for resource-poor farmers (Tripathy, 2016; War et al., 2017).

Host plant resistance is an important component of pest management program against insect pests. Plant defenses against insect pests can be categorized into antibiosis; a direct effect on insect growth and development, antixenosis; a non-preference of the insect pests and tolerance; the ability for a plant to compensate for the loss of damage caused by a pest. The legume-bruchid interactions are highly specific, as one insect species feeds on a very few seed species (Somta et al., 2007).

Morphological and biochemical traits of mungbean seed contribute to insect resistance against Bruchids. Physiological and biochemical mechanisms affect the insect's cellular processes, growth and development (Edwards and Singh 2006). Morphological traits include spines, trichomes, seed color, seed texture, seed hardness, and seed size. These traits either deter the female beetle from laying eggs on the seed or prevent the larva from burrowing into the seed. Plant secondary metabolites as phenol content, condensed tannin content, and plant defensive proteins can have toxic effect on insect pests, thus, reducing their growth and development, thereby preventing predation of bruchids on the mungbean seed. The production of secondary metabolites and anti-nutritional compounds can cause anti-metabolic activity in bruchids leading to their death (Singh 2002). Biochemical traits studied for resistance to bruchids include phenol content, condensed tannin content, soluble protein content, total starch content and soluble sugar content. In total, 43 accession lines of Mungbean were screened for these traits and compare against a commercially susceptible line and a commercially resistant line.

The main aim of this study was to identify the morphological and biochemical traits in mungbean seed responsible for resistance against bruchids. These traits would form an important component for breeding bruchid resistant mungbean and other pulses as well.

The main objectives were: Screening of improved mungbean lines against *Callosobruchus* maculatus, identify the morphological (seed colour and seed hardness), study biochemical traits (phenol content, condensed tannin content, soluble protein content and total starch content) conferring bruchid resistance in mungbean and identify phenolic compounds through HPLC.

Materials & Methods

Chemicals: The chemicals used in this study were of analytical grade. Bovine serum albumin (BSA), tannic acid, vanillin, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium carbonate (Na2CO3), acetone and vanillin were obtained from Sisco Research Lab., Mumbai, India. Bradford reagent, Folin-Ciocalteau reagent

Instruments: The Centrifuge (5430-R) and UV Vis-spectrophotometer (Biospectrometer-basic) used for the estimation of biochemical parameters were from Eppendorf, (Hitachi, Japan). Seed hardness was determined using the Texture Analyzer-Penetrometer(Model: TR Turoni, Italy). Solvents were concentrated by Buchi Rotovapor R-205 (Buchi, Switzerland). The HPLC system used was of Waters Series consisting of a Separation module (2695) with Controller (600) and equipped with photodiode array detector (2996).

Screening of Improved Mungbean Lines Against *C. maculatus*: AVMU lines (44) along with resistant (V2802) and susceptible (NM 94) checks were screened against *C. maculatus.* Fifty seeds of each line were taken in separate vials and five pairs of adults of bruchids (1-2 days old) were transferred into the vials. Three replications were maintained for each line. After seven days of release, the test insects were removed from the vials and the total number of eggs laid on the eggs was counted. Also, the number of seeds with eggs was counted. Eggs laid on the walls of the vials were not considered. From 30 days onwards to 45 days, number of damaged seeds and the number of adults emerged were recorded.

Morphological Traits Against Bruchids

Seed Colour and Lustre: Seed colour and seed lustre were determined visually and the seeds were grouped into shiny green, dull green and brown colours.

Seed Hardness: Seed hardness was determined using the Texture Analyzer –Penetrometer (Model: TR Turoni, Italy). The hardness of grains was measured with comparison test. Pressure was exerted on an individual grain until it cracked and the cracking point was recorded in newtons.

Materials & Methods Continued

Evaluation of Biochemical Traits of Seed

Estimation of Phenol: For extraction of total phenol, 0.5 g of mung bean seed was homogenized with 3 ml of 99% methanol with a pestle and mortar and centrifuged at 10000 rpm for 20 min. The supernatant was collected and the residue was re-extracted with same extraction described earlier. The supernatants were pooled together in a test tube and evaporated to dryness in hot water bath. The residue was dissolved in 5 ml of distilled water and the extract used as a source of total phenol (Malik and Singh, 1980). Total phenolic contents were estimated spectrophotometrically by Folin- Ciocalteau reagent method with slight modification. Different aliquots of samples (0.5 mL) were pipetted out into test tubes. 0.5 mL of Folin-Ciocalteau reagent was added. The solution was allowed to stand for 3 min and 2 mL of 20% Na2CO3 solution was added to each tube. The contents were mixed thoroughly. The tubes were kept in boiling water for exactly one min, cooled and the absorbance was measured at 760 nm against a reagent blank. Prepare a standard curve with different concentrations of Gallic acid. The results are expressed as µg of gallic acid equivalent (GAE/ µg) per gram of the extracts.

Estimation of Condensed Tannins: Condensed tannins content was estimated by following the method of Lattanzio et al. (2005) with slight modifications. For extraction of tannin, extract 100 mg seed powder with 5ml of acetone: water (70:30 v/v) mixture in a water bath at 30 0C for 30min and vortexed at frequent intervals. Centrifuged the sample at 10,000 rpm for 15 min and collect the supernatant. Repeat the extraction, combine supernatants and evaporate to dryness at 80 ^{0}C, make the extract to 2 ml with distilled water. Condensed tannin content was estimated by Folin Ciocalteau method and the results are expressed as tannic acid equivalents from a standard graph prepared from various quantities of tannic acid. To 0.5 ml of extract, 0.5 ml of Folin Ciocalteau reagent (1:1 diluted with distilled water) was added followed by the addition of 1 ml of 20% sodium carbonate. The solution was made upto 10 ml with distilled water and incubated at 25- 30 °C for 40 min. The absorbance of the blue color developed was read at 725 nm in using UV-VIS spectrophotometer.

HPLC Analysis of Phenols: Methanol extracts were vacuum evaporated and filtered through a polyvinyl difluoride filter (PVDF; Millipore, Millex-GV, filter 0.22 diameter) membrane. Separation of the compounds was performed on an Atlantis C18 column (4.6 mm × 250 mm) at a flow rate of 1 mL minute-1 for 40 minutes with 20 µL injected volume of the extract. The column was used at ambient temperature. The mobile phase was water (A) and acetonitrile (B) (v/v) containing 1% orthophosphoric acid. The mobile phase was filtered through a 0.45 µm membrane filter and deaerated using a sonicator (D-Compact, 443). The elution profile used was 0–5 minutes, 65% A, 35% B (isocratic); 5–12 minutes, 35%–40% B in A (linear gradient); 12–20 minutes, 40%–45% B in A (linear gradient); 20–30 minutes, 55% A, 45% B (isocratic); 30–35 minutes, 45%–35% B in A (linear gradient); and 35–40 minutes, 65% A, 35% B. All compounds were identified by comparing their HPLC retention times to those of authentic standards. The peak area of each identified compound was transformed into quantities of the compounds and was expressed in nanograms using internal standard peak areas.

Results & Discussion

Screening of Mungbean Improved Lines Against *C. maculatus*: Improved mungbean lines AVMU1601, AVMU1602, AVMU1603, AVMU1604, AVMU1605, AVMU1606, AVMU1609, AVMU1610, AVMU1611, AVMU1612, AVMU1613, AVMU1614, AVMU1616, AVMU1617, AVMU1618, AVMU1619, AVMU 1620, AVMU 1621, AVMU1622, AVMU1623, AVMU1624, AVMU 1625, AVMU1626, AVMU1627, AVMU1628, AVMU1629, AVMU1630 showed no damage (%) in the seeds (Table 1). Although the eggs were laid on these lines, there was no hatching of the larvae. This can be attributed to either physical traits such as seed hardness of the biochemical traits that could either kill the eggs of the first instar larvae emerging from them (Van Huis and De Rooy, 1998; Lattanzia et al., 2005; Soumia 2015). Heavy damage was observed in the susceptible check, NM 94 (98.4%) followed by AVMU 1659, AVMU 1657 and AVMU 1651 (64% each).

Seed Color: In mungbean, seed color contributes to the resistance/susceptibility to bruchids (Asian Vegetable Research and Development Center [AVRDC], 1981; Appleby and Credland, 2003; Lattanzio et al., 2005; Somta et al., 2007). It is considered as an important factor for bruchid oviposition. Our results showed that most of the shiny green colored seeds are susceptible to bruchid damage except AVMU1601, AVMU1605 and AVMU1606. Among dull green seeded improved mungbean lines, AVMU1603, AVMU1604, AVMU1609, AVMU1612, AVMU1613, AVMU1618, AVMU 1620, AVMU 1621, AVMU1622, AVMU1623, AVMU1624, AVMU 1625, AVMU1626, AVMU1627, AVMU1628, AVMU1629 and AVMU1630 were resistant to bruchid damage. Further, some brownish seeded lines such as AVMU1602, AVMU1610, AVMU1611, AVMU1614, AVMU1616, AVMU1617 and AVMU1619 also showed no damage by bruchids. It has been reported that yellow and green shiny colored seeds are preferred to green rough or black seeds for oviposition and bruchid development (War et al., 2017). Seed coat or testa plays an important role in oviposition stimulation (Asian Vegetable Research and Development Center [AVRDC], 1988). The seed color also influences egg laying behavior with smooth seed coats being preferred compared to the rough seed coat type. However, Kapila and Pajni (1989) did not find any correlation between seed size and seed color with bruchid resistance in French bean.

Seed Hardness: Some of the bruchid resistant lines showed higher hardness than some of the susceptible lines (Fig. 4). The highest seed hardness was observed in AVMU 1625 (54.12 N), V2802 (53.99 N), AVMU 1652 (52.71 N), AVMU 1619 (51.70) and AVMU 1630 (51.43). The lowest seed hardness was observed in AVMU 1655 (17.21 N), AVMU 1617 (16.90 N) and AVMU 1649 (15.95 N). Seed coat hardness is an important trait for bruchid resistance in mungbean (Van Huis and De Rooy, 1998). The hard seed coat prevents the larvae to penetrate into the seed and the roughness render seeds unfit for oviposition by bruchids in mugbean, cowpea, chickpea and other pulses (Nwanze and Horber, 1976; Brewer and Horber, 1983; Messina and Renwick, 1985; Ahmed et al., 1993; Landerito et al., 1993; Shade et al., 1996).

Phenols: The phenol content of some of the AVMU lines was significantly higher than that of V2802 and NM 94. Phenols are the important plants secondary metabolites involved in resistance against insect pests (War et al., 2012). They are directly toxic to insects and/or act as feeding deterrents (War et al., 2013; Dixit et al., 2017). It has been reported that phenols reduce the growth index of *C. chinensis* in mungbean (Soumia 2015). Strong negative correlation has been reported between phenol content of the seed and adult emergence of bruchids (Sowmya 2015). Phenols in legume seed reduce the penetration of neonate larvae of bruchids (Bhattacharya and Banerjee, 2001). Further, phenols in seed increase the larval developmental period and reduced percentage of adult emergence, thereby, higher the resistance to storage insect pests (Patel 2002; Misal et al. 2008; Lazar et al., 2014). Further, Ghosal et al. (2004) attributed the resistance or susceptibility of legume seeds to *C. chinensis* to the phenol content. Resistance or susceptibility of pulse beetle is attributed to bio chemical content of seeds such as phenol (Deshpande et al., 2011).

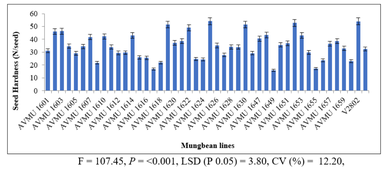
Condensed Tannins: AVMU lines (AVMU 1602, AVMU 1607, AVMU 1615 and AVMU 1618) showed higher levels of condensed tannins compared to the checks. Tannins in stored seed are involved in resistance against bruchids (Deshpande 1992; Lale and Makoshi 2000; Lattanzio et al. 2005). They have an astringent (mouth puckering) and bitter taste, which deters the insect pests. Tannins bind to the proteins and digestive enzymes in insect midgut and precipitate them through hydrogen or covalent bonds, thereby, limiting their availability to the insect pests and ultimately reducing the insect growth and development (Peters and Constabel 2002; War et al. 2012). In cowpea, tannins are effectively involved in the resistance against bruchids, which deter insects from oviposition (Lattanzio et al. 2005). Lale and Kolo (1998) showed that in bruchid resistant cowpea, biochemical factors in the testa/seed coat effect the oviposition and survival of pulse beetle eggs. Tannins in seed determine the resistance or susceptibility to the pulse beetle (Deshpande et al., 2011). Significantly positive correlation between tannin content in the seeds with incubation period, larval-pupal period and total developmental period of *C. maculatus*, and strongly negative with the adult emergence (Misal et al. 2008; Lazar et al., 2014). However, Desroches *et al.* (1994) did not observe significant effect of tannins on the penetration of bruchid larvae into *Vicia faba* seed.

Proteins: Differential amounts of proteins were observed across the mungbean lines. The highest protein content was observed in V2802, AVMU 1650, AVMU 1656 and AVMU 1649 (191.4, 190.20, 187.65 and 185.4 mg g^{-1} seed, respectively). The lowest protein content was observed in NM94 (154.50 mg g^{-1} seed). Seeds with high protein content have been found less susceptible to insect pests (Singh et al., 1995; Sowmya 2015). Proteins including chitinase, *b*-1,3-glucanase, and peroxidase in mungbean are involved in bruchid resistance (Khan et al., 2003). Further, bruchid resistance in a NIL VC6089A has been reported to occur due to the BURP (BNM2, USP, RD22, and PG1b) protein family (Lin et al. 2016). They observed that bruchid resistant lines, such as VC6089A, TC1966, and RIL59 showed higher expression of g39185 (resistant-specific protein), g34458 (gag/pol polyprotein), and g5551 (aspartic proteinase) than the susceptible ones (VC1973A and NM92). However, the bad taste or toxicity of these chemicals to non-target organisms has posed a great challenge to scientists to minimize their effects but to stabilize the resistance.

Results & Discussion Continued

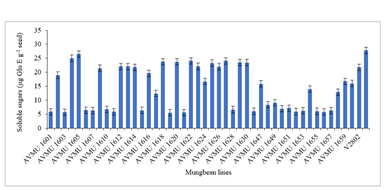
Soluble Sugars and Starch Contents: The soluble sugar and starch contents of AVMU lines different across the mungbean lines (Figs. 5 and 6). High amounts of soluble sugar content were observed in AVMU 1604, AVMU 1605, AVMU 1622, AVMU 1627 and NM94 (24.97, 26.5, 24.05, 24.08 and 27.8 Glu E g^{-1} seed, respectively). Reduced sugar content was observed in AVMU 1601, AVMU 1603, AVMU 1611, AVMU 1619, AVMU 1621, AVMU 1652 and AVMU 1656 (5.87, 5.75, 5.89, 5.57, 5.61, 5.95 and 5.82 g Glu E g^{-1} seed, respectively). High starch content was recorded in AVMU 1654, AVMU 1647, AVMU 1602 and AVMU 1607 (199.4, 198.1, 179.1, 178.9, and µg Glu E g^{-1} seed, respectively), while as reduced starch content was recorded in AVMU 1610, AVMU 1624 and AVMU 1629 (76.5, 76.5 and 75.2 µg Glu E g^{-1} seed, respectively). Seeds with higher soluble sugars and starch content are more susceptible to insect pests including bruchids (Lazar et al., 2014). The total soluble sugar content in the seed are negatively correlated to the incubation period, larval-pupal period and total developmental period, while positively correlated to the adult emergence percentage (Lazar et al., 2014).

HPLC Fingerprinting: The number of peaks varied across the bruchid resistant and susceptible improved mungbean lines (Fig. 6). The number of peaks was greater in the bruchid resistant lines than the susceptible lines and the susceptible check, NM94 (Fig. 7). AVMU 1601, AVMU 1609 and V2802 had 15, 11 and 11 peaks, respectively, while as the susceptible check NM94 had 10peaks. Each peak determines a single compound. Thus the compounds shown by the additional peaks could be attributed to the bruchid resistance in these lines. However, the susceptibility of some of the compounds cannot be ruled out in the resistant lines as well. The identification of compounds is in progress.



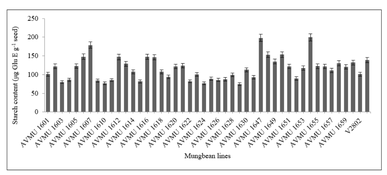
F = 107.45, *P* = <0.001, LSD (P 0.05) = 3.80, CV (%) = 12.20,

Figure 4. Seed hardness of improved mungbean lines



F = 44.32, *P* = <0.05, LSD (P 0.05) = 3.74, CV (%) = 10.89,

Figure 5. Soluble sugars (µg Glu E g^{-1} seed) of seeds of the improved mungbean lines



F = 83.09, *P* = <0.01, LSD (P 0.05) = 7.98, CV (%) = 14.88,

Figure 6. Starch content (µg Glu E g^{-1} seed) of seeds of the improved mungbean lines

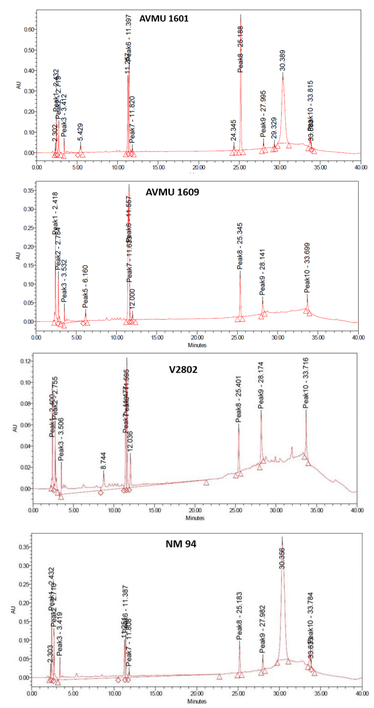


Figure 7. HPLC chromatograms of AVMU 1601, AVMU 1609, V2802 and NM94

Conclusion

AVMU1601, AVMU1605, AVMU1606 with shiny green seed are highly resistant to *Callosobruchus maculatus.* Some of the bruchid resistant lines showed higher hardness than some of the susceptible lines. Phenol content of some of the AVMU lines with reduced bruchid damage was significantly higher than that of V2802 and NM 94. AVMU 1602, AVMU 1607, AVMU 1615 and AVMU 1618 showed higher levels of condensed tannins compared to the checks. Soluble protein content was significantly different between bruchid resistant and the susceptible lines. The soluble sugar and starch contents of bruchid resistant mungbean lines were significantly lower than the susceptible lines and the susceptible check, NM94. HPLC chromatogram showed differential peaks among the bruchid resistant and bruchid susceptible AVMU lines and V2802 and NM 94. Thus, the higher amounts of phenols, tannins and proteins, and the lower contents of total soluble sugars and starch can be attributed to the resistance and/or susceptibility to *C. maculatus* in mungbean. However, in-depth studies are needed to elucidate the effects of these compounds on bruchid growth and development to confirm their role in resistance/susceptibility of mungbean to bruchids.

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