CHAPTER V
DISCUSSION

Host plant resistance can play a pivotal role in pest management in grain legumes. Resistance to insect pests should be one of the major criteria in development and release of new crop cultivars, in order to ensure prolonged cultivar life and cost effective production (Sharma and Crouch, 2004). The "built-in protection" in resistant plants disrupts their normal association with insect pests (Van Emden, 1997). Bruchid resistant cultivars have considerable potential for minimizing storage losses (Dongre *et al*., 1996). However, work on development of bruchid resistant cultivars is very limited; hence all the commercial mungbean varieties grown in India are susceptible. Understanding the mechanisms underlying bruchid resistance is essential for developing appropriate breeding strategies. Therefore, to encourage pulse breeding, production and storage, this study was aimed at identifying new bruchid (*Callosobruchus maculatus*) resistance source(s) from various seed samples, mechanisms of resistance and to identify markers associated with the resistance traits. Results obtained from the present study are discussed in the light of existing literatures available.

5.1. *Callosobruchus maculatus* as the test insect

The species of *Callosobruchus* that were encountered in the present investigations on infested mungbean and other legumes were identified as *C. maculatus* and *C. chinensis* based on morphological characters observed, which were validated from the standard key characters [Southgate *et al*., 1957; Borowiec, 1987; Beck and Blumer, 2007; CABI (2014a and 2014b)]. The genus *Callosobruchus* includes approximately 20 species, about three quarters of which are from Asia (Borowiec, 1987). They are cosmopolitan pests of stored legumes (Fabaceae) of the genera *Vigna*, *Phaseolus*, *Glycine*, *Lablab*, *Vicia*, *Pisum*, *Cicer*, *Lens*, *Cajanus*, etc. Raina (1970) reported that of the 20 species recorded, three are commonly found in India which includes *C. maculatus*, *C. chinensis* and *C. analis*. *C. maculatus* is the most widespread species, occurring in Africa, Asia and Australia (Howe, 1971; Southgate, 1979) and the most predominant species infesting legume seeds in Southern states (Srinivasan and Durairaj, 2007) including Tamil Nadu where the study was conducted.

Damage potential and other fitness parameters evaluated for *C. maculatus* and *C. chinensis* indicated that the former species is more dominant and destructive than the latter since it proved to be superior in all developmental aspects. This is in corroboration with various

studies reported earlier [Bhubaneshwari and Victoria, 2014(c)]. For instance, Raina (1970) reported that *C. chinensis* was less fecund than *C. maculatus* and asserted that *C. maculatus* was the dominant species on mungbean. In addition, more adult emergence, short mean developmental period (MDP) and high index of suitability substantiated the fact that *C. maculatus* is more competitive than *C. chinensis.* This study is also in accordance with Edwards and Singh (2006) who indicated that prolonged mean developmental period led to decreased index of suitability which was evident in the case of *C. chinensis*. However, there exists a slight deviation from the findings of Raina (1970) who reported a mean developmental period of 27 days for *C. chinensis*.

5.2. *Callosobruchus maculatus* development *vis-à-vis* population source, rearing host and rearing condition

The first reasonably comprehensive account of the biology and pest status of *Callosobruchus maculatus* was presented by Paddock and Reinhard (1919), who summarized information available to that date. Since then, various reports were available on how environmental factors affected development, reproduction and ultimately the growth of this insect populations (Howe and Currie, 1964). In this study, number of eggs oviposited per 50 seeds significantly varied with host species (mungbean and black gram) and insect population sources (Thanjavur and Coimbatore) under two distinct rearing conditions (incubator and room temperature). These finding are in agreement with Credland (2006), who stated that many factors affect number of eggs laid by *C. maculatus* females (fecundity) such as number of seeds available, rearing conditions and bruchid strain. In addition, Appleby and Credland (2003) revealed that populations from different areas vary in their fecundity on same number, species and cultivar of host seeds which supported the present findings on bruchid populations collected from two geographical areas of Tamil Nadu. The controlled conditions inside the incubator (30°C temperature and 70 % relative humidity) in the present study might have been found to be more favourable for the bruchid development than the room temperature since oviposition and development of Thanjavur *C. maculatus* population on mungbean were higher.

Earlier, Mookherjee and Chawla (1964) reported that adult females produced an average of 25-61 eggs throughout their lifespan, and the highest number of eggs/day/female was achieved at 30°C. However, *C. maculatus* can lay up to 115 eggs/female in its life span compared to 65 eggs by *C. chinensis* (L.) female [CABI, 2014(a) and (b)]. Similar results were obtained for

Thanjavur population in the present investigation, where fecundity per female was highest at 30^oC (controlled) (113.6 ± 3.93 eggs/50 mungbean seeds). These findings are also in conformity with Giga and Smith (1983), who suggested similar oviposition pattern where these variations reflect differences in insect geographical populations. *C. maculatus* can easily be raised in laboratories and has been used as a model organism in a number of ecological studies. Nevertheless, its development is strongly influenced by temperature and humidity (Xu, 1999), host substrate and population source (strain or biotype; Messina and Slade, 1999), which supported the present findings. The mean incubation period of egg ranged from 4-5 days and 5-6 days (data not provided) at controlled and uncontrolled conditions respectively during the present study, which is in agreement with Deng *et al*. (2002) who reported similar findings. Hence, it can be suggested that the extent of crop losses may vary depending on location, bruchid species, storage conditions, and period of storage.

From this investigation, mungbean was more suitable as bruchid host when compared to black gram. This is in accordance with Giga and Smith (1985), who observed that mungbean was the most suitable host for *C. maculatus* among other legumes in terms of oviposition, growth and development. Moreover, the report that mungbean is more nutritious than black gram in terms of vitamins and minerals (Dahiya *et al.,* 2015) corroborated the present results. The food influence in this study may suggest an ancestral cause or fitness cost depending on the species (Gbaye *et al*., 2011). Although the use of cowpea seeds has been suggested as the most favourable host for rapid bruchid mass-culturing by many workers (Strong *et al*., 1968; Jackai and Asante, 2003; Swella and Mushobozy. 2009), this study demonstrated the feasibility of using mungbean seeds as equally suitable bruchid rearing host taking into account of the comparatively shorter developmental period (24 days). Hence, both cowpea and mungbean can be used for rapid mass-culturing and long-term maintenance of bruchid generations for any behavioural, physiological, management and resistance studies under laboratory conditions.

Crop damage caused by any insect species depends on the rate of population increase of the species, which is influenced by factors like temperature, humidity, nutritional quality of host species and insect geographical location (Howe, 1965). Although various studies on the temperature and humidity effects on bruchid biology have been conducted (Mookherjee and Chawla, 1964; Xu, 1999; Deng *et al*., 2002), studies on their interactive influences on the life processes of *Callosobruchus* species are meagre and little studies have been carried out only for

one species, *C. chinensis* (Mainali *et al*., 2015), whereas it has not been conducted for *C. maculatus*. Moreover, interaction effects of the three factors under study have not been performed and reported as far as any bruchid species is concerned. Results on the interaction effects indicated non-significant results on *C. maculatus* oviposition and seed damage, whereas significant result was obtained in case of mean developmental period (MDP). This may be explained from the fact that MDP (in days) calculation depends on daily adult emergence till its cessation which mainly depends on rearing host, conditions and insect population source. Mainali *et al*. (2015) studied the interactive effects of temperature and relative humidity on oviposition and development of *C. chinensis* on azuki bean. They were also of the view that rather than the interactive effects, their individual effects on *C. chinensis* were more profound, which corroborates the present findings and suggested that this could be just a pure chance event. Gbaye *et al*. (2011) suggested that the growth or storage of more than one type of legume in close proximity could be problematic where bruchid infestation is high.

In view of the above results and considering the damage potential with comparatively shorter MDP compared to *C. chinensis*, Thanjavur *C. maculatus* population was selected for resistance evaluation and resistance mechanism studies on various seed samples of rice bean, mungbean and one of their inter-specific populations. This considers the suggestions of Credland (1994), who had rightly pointed out the fact that reliable bioassays conducted for bruchid resistance evaluation must always employ a known, identified biotype of the insect.

5.3. Fecundity, survival and developmental pattern of Thanjavur *Callosobruchus maculatus* populations on mungbean

Present studies on fecundity, survival and developmental pattern of Thanjavur *C. maculatus* population through twenty-five generations on VRM(Gg)1 seeds indicated that bruchids completed their immature and pre-adult stages in 23.87 days (21st generation) during June, 2016 within the thermal ranges of 24.29^{o}C (mean minimum) to 33.58^{o}C (mean maximum) and 84.48 % RH. Ouedraogo *et al.* (1996) recorded that low temperature in December and January reduce the development of immature stages of bruchid in mungbean seeds, while the most suitable months for the development of pre-adult stages was July to October . Similar observations were recorded in this study. Thanthianga and Mitchell (1990) earlier found that, larval competition at initial stages tend to increase the developmental period, which is in agreement with the present findings. Population count and percentage seed infestation was

directly proportional to the level of *C. maculatus* infestation. Oviposition potential of *C. maculatus* females reached a peak within 2 days after its commencement and then declined with time [Fig.6(a)]. This finding may be explained from the reports of Howe and Currie (1964), Booker (1967), and Tun (1979), who suggested that the short period for egg laying potential in this bruchid species may be an inherent survival mechanism for perpetuation of generations given the correspondingly short period of adult longevity. This could also ensure rapid multiple re-infestations of pulse seeds in storage with consequent rapid population buildup since they oviposit continuously without feeding after their emergence from the infested seeds, which was noticed during this investigation for twenty-five generations and during sub-culturing process. Moreover, this behaviour is particularly important because adult bruchids do not feed and have to depend on the energy and other essential nutrients reserve within their body while developing inside the host seed, which may also explain the decline in oviposition with time.

With regard to longevity of adult females, low mortality rate was noticed from the first to the third day of commencement of oviposition [Fig.6(e)]. This was followed by drastic increase in their mortality rates particularly between the third and fourth day of oviposition. It was also observed that all the female bruchids died before reaching 11th day after the commencement of oviposition. This clearly indicated the shorter lifespan of *C. maculatus* females than males, which is in conformity with Tun (1979). On the contrary, Mandal and Konar (2006) reported that adult longevity of female was more than male and the sex ratio was more in favour of male when they studied the biology of *C. chinensis* for eight generations. This may be due to difference in bruchid species studied. Adults were segregated into males and females after their emergence to work out the sex ratio by keeping the constant value of male as one. Data on number of emerged males and females and sex ratio of *C. maculatus* from susceptible mungbean variety [VRM(Gg)1] showed that sex ratio was in favour of females in all the subsequent generations studied except few developmental generations, *viz.* F_7, F_8, F_{11}, F_{20} and F_{22} [Table.31(b)], where it was equivalent to that of males. Similar trend was observed by Fox *et al*. (2006) who reported that sex ratio of emerging adults were significantly female biased. The genetics of sex determination is unknown and have not been studied in bruchids. However, this biased emergence trend may be explained with the assumption that female sex determination might have taken place during embyonic development since females play a more important role (egg laying/offspring production) for their continuous perpetuation.

5.4. *Callosobruchus maculatus* resistance determination on rice bean (*Vigna umbellata*) landraces and mungbean (*Vigna radiata*) varieties and their relationships with seed characteristics

Variation in seed colour was observed among rice bean landraces, which is supported by Mohar *et al*. (2005) who described seed coat colour differences in rice bean accessions collected from Manipur and stated that several accessions had black, red, cream, violet, purple, maroon, brown and mottled grains with greenish, brownish and ash grey background. Seed coat colour difference was also reported by Tian *et al*. (2013) when they investigated the genetic diversity of 472 rice bean accessions (388 cultivated, 84 wild) collected from 16 Asian countries including India. Quantitative seed parameters like seed length (SL), seed breadth (SB), hundred seed weight (HSW), seed coat/testa thickness (SCT or STT), seed hardness (SH) have not been reported as far as these landraces are concerned. Significant differences (at p = 0.05) were noticed in these parameters. Tian *et al*. (2013) also reported similar findings among seventeen rice bean accessions from Thailand with highest HSW of 12.57 g. Furthermore, Srinivasan and Durairaj (2007) conducted similar studies on wild *Vigna* species including seventeen *V. umbellata* accessions from Tamil Nadu and recorded highest HSW of 7.51 g in "LRB 40-1" accession. Among the landraces in the present study, LR(M)4 and LR(M)1 exhibited maximum HSW of 34.68 g and 34.65 g, respectively. This reveals that rice bean found in Manipur possess the largest seed size of all the accessions reported so far.

Hard-seededness is common in this legume crop, although this trait does not appear to be consistent within varieties (Khadka and Acharya, 2009). Similar trend in seed-hardness consistency was recorded in this study. Maximum and minimum seed coat thickness were recorded in LR(M)2 (0.135 mm) and TNAU Red (0.088 mm) respectively and others ranged between 0.093 and 0.133 mm. Reports on rice bean seed coat thickness are scarce. Rice bean landraces under present study recorded seed coat thickness in the range of 0.088 to 0.135 mm. Srinivasan and Durairaj (2007) reported thickness of Tamil Nadu rice bean seed coat ranging from 0.047 to 0.070 mm, which deviates from the current findings. This suggests thicker seed coat in Manipur rice bean compared to other accessions reported till date. 100 seed weight (HSW) among different mungbean varieties ranged from 3.636 g to 5.600 g which finds support from the results of Tomooka *et al*. (2000) who reported HSW of 5.9 to 6.8 g in Thailand mungbean accessions. Marconi *et al*. (1997) reported that HSW for four bruchid resistant

accessions of *Vigna vexillata macrosperma* was found to vary from 4.0 to 5.7 g which coincides with the moderately susceptible mungbean varieties (4.219 g and 4.701 g in VBN-1 and IPO-2-14) used in present investigations.

In search for better source(s) of resistance to the economically abundant and important bruchid (*C. maculatus*) species in India, Manipur rice bean landraces and mungbean varieties from various agricultural stations in Tamil Nadu were screened under "no-choice" test conditions. Resistance evaluation revealed that no seeds were rejected for egg-laying by *C. maculatus* but number of eggs laid per 20 seeds differed significantly between the two *Vigna* species. This finding is supported by Sandhu *et al*. (1986), who observed significantly higher oviposition response per 20 seeds of different rice bean landraces than mungbean varieties under no-choice test. This might be due to differences in the level of compounds present in the seed coat or differences in seed size for oviposition, which is also supported by Kaur (2013). Credland and Wright (1988) demonstrated that extracts obtained from seed coats of pulse varieties, irrespective of their qualitative characters, are required to stimulate oogenesis and oviposition by *C. maculatus*. The present investigations were also supported by Srinivasan and Durairaj (2007) who reported that all the *V. umbellata* accessions and other *Vigna* species used during their study were almost equally preferred for oviposition by South Indian *C. maculatus*. Kashiwaba *et al*. (2003) observed that *C. maculatus* oviposition on seeds of 8 cultivated rice bean accessions varied from 11.3 to 53.0 eggs/10 seeds. The highest number of eggs laid was in close agreement with the present findings.

Rice bean cultivation is not very common in Southern states of India except hilly tracts of Western and Eastern Ghats (Arora *et al*., 1980) and few places such as Karnataka, Tamil Nadu (Pavithravani, 2012). Till date, no literature is available on bioassay and development of *C. maculatus* on various rice bean landraces cultivated in Manipur. Present investigations on bruchid resistance evaluation showed that among eight landraces collected from Manipur, three entries *viz*. LR(M)3, LR(M)4 and TNAU Red were found to be completely resistant to bruchid attack. This is in conformity with Tomooka *et al*. (2000) and Kashiwaba *et al*. (2003), who suggested that certain wild and cultivated sources of rice bean are known to have resistance to *C. maculatus* under storage conditions. Vir (1980) also indicated that *V. umbellata* accessions failed to support normal adult emergence and actualized that mere ovipositional preference for a

variety does not seem to have any relation with suitability of the seed for proper bruchid development.

All the commercial mungbean varieties developed and grown in India are susceptible to bruchid. Among the fifteen improved varieties of mungbean evaluated in this study, no bruchid resistance source was identified. However, two of them (VBN-1 and IPO-2-14) showed reduced seed damage per cent with high mean developmental period (MDP), whereas other varieties were susceptible. On the other hand, bruchid MDP on these susceptible varieties were extended from that observed in the susceptible check, VRM(Gg)1 (28 days), by 1 to10 days. High susceptibility of different mungbean varieties to bruchid attack was also stated by Bajiya *et al*. (2011), which holds true with the current findings. Karthikeyan *et al*. (2008), in their study on bruchid susceptibility of genetically improved mungbean varieties, reported that more number of adults emerged from VBN-1 seeds suggesting its high susceptibility, whereas VBN-2 demonstrated no bruchid emergence and zero seed damage, which is contrary to the above findings. This may be attributed to differences in the environment for resistance evaluation, *C. maculatus* population used and/or host seed used for rearing, cowpea in their case. This study confirmed the varied suitabilities of *C. maculatus* life performances on different mungbean varieties. In addition, number of mungbean seeds with larval penetration hole (hatched eggs) was significantly higher when compared to rice bean. Seeds without penetration hole (unhatched eggs) might be due to non-viability of eggs rather than due to resistance offered by mungbean seeds for larval entry. Even if more number of eggs were laid per seed, only one adult emerged from individual mungbean seed [Plate. 22(d)], which is supported by Mitchell (1991) and Dabi *et al*. (1978) suggesting only one adult emergence per seed for South Indian *C. maculatus* strain. This finding is also in accordance with Edwards and Singh (2006), who indicated that prolonged mean developmental period led to decreased index of suitability.

In the present investigations, shortest mean developmental period (MDP) for *C. maculatus* was observed in VRM(Gg)1, 28.21 days, as compared to rice bean landraces which took the longest developmental period of 43.89 days on LR(M)6. Ponnusammy *et al*. (2014) reported MDP in the range 26.1 to 31.5 days on mungbean accessions which are in trend with the present finding on mungbean. Results on rice bean are in conformation with Pavithravani *et al*. (2013), who reported bruchids took 33.5 and 33.5 days respectively on resistant rice bean genotypes, LRB238 and JP100304. Srinivasan and Durairaj (2007) also reported the longest

MDP on three rice bean accessions, which varied from 32.33 to 38.67 days. Rice bean germplasm collected from Manipur are known to possess several desirable traits such as tolerance to several biotic (disease and insect) and abiotic stress like drought tolerance (Mohar *et al.*, 2005), which is in light of the present result on *C. maculatus* resistance. Host seeds with low index of suitability (IS) could be regarded as being poor or non-host of *C. maculatus*, whereas with high IS, as suitable host (Swella and Mushobozy, 2009). The calculated values of Howe's index of suitability was highest (0.065) for VRM(Gg)1 among the infested mungbean varieties thus supporting that it is highly susceptible and can be used as a suitable host for *C. maculatus* rearing.

On the other hand, there exists a knowledge gap in regard to information on the role of certain biophysical and biochemical parameters in imparting resistance to bruchid in *Vigna* species and this knowledge may help to find the solution for *C. maculatus* management. Hence, all these gaps have necessitated the need for a comprehensive study on biophysical as well as biochemical factors responsible for resistance to *C. maculatus* in the two *Vigna* species under storage conditions. In order to determine the relative influence of seed biophysical characters in conferring resistance to South India *C. maculatus*, regression and correlation analysis were performed. Results demonstrated non-significant relationships ($p > 0.05$) in all cases for both the *Vigna* species. Earlier reports suggested that oviposition is directly proportional to seed size (Southgate, 1979; Sulehrie *et al.*, 2003). However, the present findings did not match with those of the previous workers since TNAU Red (a completely resistant landrace) exhibited high number of eggs laid (189.00 and 208.67 eggs / 10 seeds) despite of its smallest size (4.490 g). This proves that oviposition alone can not be considered for determining bruchid resistance. Dasbak *et al*. (2009) considered thousand grain mass (TGM) and testa thickness to be the most important factors responsible for resistance to bruchid in pigeon pea. Many authors emphasized seed resistance to differences in seed size and asserted that larger seeds supply more food and space for insect growth (Khare and Agrawal, 1963). However, it did not hold true for rice bean landraces studied. Jason and Fox (2003) suggested that female *C. maculatus* are better at judging seed mass and distribute their eggs in a manner that maximize the amount of resources for their offspring. This pattern was observed in few landraces. Thus, considering the seed size and hatching per cent in the current study, rice bean seeds displayed sufficient resources for bruchid growth and the grubs penetrated the seed coat and reached cotyledons, yet fewer or no adult

emergence was recorded in spite of more number of eggs laid per seed in all the landraces. Earlier reports also stated that there is no relationship between level of bruchid (*C. chinensis* and *C. maculatus*) resistance and seed size (Tomooka *et al*., 2000; Srinivasan and Durairaj, 2007), seed coat thickness and seed hardness (Srinivasan and Durairaj, 2007). Chatterjee and Dana (1979) and Sandhu *et al*. (1986) also revealed that bruchid larvae were successful in penetrating the seed coat but failed to develop inside the cotyledon of some rice bean landraces in their experiment, which backs the present findings on egg hatchability and failure for adult emergence from some landraces. This is also supported by Kashiwaba *et al*. (2003), who reported complete inhibition of *C. maculatus* emergence from several *V. umbellata* accessions and suggested that in resistant samples, bruchids died during first and second grub instar stages.

Therefore, in view of all the above information described, no antixenosis is evident in rice bean seed resistance to bruchid and hence it cannot be considered as a resistance factor. Kashiwaba and Tomooka in 2002 (US Patent 6,770,630B2) identified the chemicals responsible for bruchid resistance in *V. umbellata* (a Japanese cv. "Menaga") from seed cotyledon, which are derivatives of the flavonoids naringenin and quercetin. A recent study by Chotechung *et al*. (2016) revealed the possible function of a gene for bruchid resistance in mungbean, which encodes for a polygalacturonase-inhibiting protein (PGIP) useful in plant defense against many diseases. Therefore, the above results clearly indicates and provides evidence that antibiosis alone is responsible for imparting bruchid resistance in rice bean.

Yadav and Pant (1974) reported that *Callosobruchus* spp. will oviposit on any available seed, even though the seed may not be suitable for their development. Host association of this insect is solely determined by factors favouring oviposition of the seed varieties (Karthikeyan *et al*., 2008). The level of chemical composition (deterrent, attractant or stimulant) present in the seed coat may well explain why eggs were not equally distributed among seeds of the different varieties used. Present result on VBN-2 showed 29.36% adult emergence and MDP of 32.31 days. This is contrary to the findings of Karthikeyan *et al*. (2008), who observed 0 % and 0 days for both the studied parameters. This may be attributed to the differences in *C. maculatus* strain used. The number of emerging adults determines the extent of damage, and consequently, seeds permitting more rapid and higher levels of adult emergence will be more extensively damaged (Singh *et al*., 1985). The extended developmental period may reveal difference in antibiotic

resistance factor(s) of mungbean varieties tested which is indicated by diminished growth performance of *C. maculatus* on infested seeds.

In addition, present studies on bruchid infestation effects revealed negative influence on mungbean seed qualities as evident from high seed weight loss, reduced seed protein content and reduction in germination percentage. Patro *et al*. (2007) reported that germination percentage and vigour index decreased with increase in *C. maculatus* infestation level which supported the present findings. The infested seeds led to failure of seedling establishment which, otherwise, will subsequently result into low productivity of the crop. Result on seed weight loss is in conformity with Soumia *et al*. (2015), who reported loss in mungbean seed weight of up to 46.46 % due to *C. analis* infestation and suggested that percentage weight loss had positive correlation with growth index. Tripathy (2016) stated that bruchid infested seeds show poor germination and become nutritionally poor due to protein and carbohydrate degradation and attract low market value, which was observed in this study that showed changes in total seed protein content.

5.5. Screening and evaluation of South Indian *Callosobruchus maculatus* resistance in VRM(Gg)1, TNAU Red and their F_9 recombinant inbred lines (RILs)

Results on preliminary resistance evaluation suggested extreme reaction responses to South Indian *C. maculatus* strain by the two *Vigna* parents, which also confirmed that VRM(Gg)1 (*V. radiata*) is highly susceptible whereas TNAU Red (*V. umbellata*) is completely resistant to *C. maculatus* (Table. 42). The aim of any cross-breeding and its subsequent advancement is to increase consistency in performance of the seeds with respect to its vigour and reaction to biotic and abiotic stresses. Plant breeders are interested in increasing bruchid resistance in cultivated *Vigna* species and understanding resistance mechanisms operating in wild *Vigna* species. Wild plants belonging to the genus *Vigna* are a potent source of resistance as evidenced by several authors. Marconi *et al*. (1997) observed several species of *Vigna* including *V. vexillata*, *V. reticulata* and *V. luteola*, as resistant to bruchids. In mungbean, wild relatives have better resistance than the cultivated species (Tomooka *et al*., 1992; Somta *et al*., 2008a). Several bruchid resistant mungbean have been developed using resistance gene from TC1966 (Tomooka *et al*., 1992) and the resultant varieties were released to farmers in many countries. However, since the resistance comes from only a single source (TC1966), which is a wild mungbean variety (*V. radiata* var. *sublobata*), there are reasons to believe that bruchids can soon evolve to break the resistance. Shade *et al*. (1996) reported that *C. maculatus* was able to develop

a biotype to overcome Tvu2027 (a resistant cowpea accession), after selection on resistant cowpea seeds for over 53 generations. Thus, new sources of resistance are necessary for developing multiple resistant cultivars for durable resistance.

The level of resistance in some of the mungbean varieties released for cultivation are low to moderate while high levels of bruchid resistance have been reported in wild relatives of several *Vigna* crops (Marconi *et al.*, 1997; Tomooka *et al.*, 2000) such as *V. umbellata* (Somta *et al.*, 2006a; Kashiwaba *et al.*, 2003). Hence, resistance genes from the wild relative need to be transferred into the cultivated species for developing high yielding varieties with acceptable agronomic backgrounds. Considerable amount of literatures citing the resistance evaluation of bruchid (*C. maculatus*) on various legumes are available. However, bruchid resistance evaluation on inter-specific populations of different crop species is meager. Bruchid resistance screening on inter-specific populations between *Vigna umbellata* and *V. nakashimae* was reported by Somta *et al.*, 2006(b) and Sudha *et al.* (2013) reported disease (mungbean yellow mosaic virus, MYMV) resistance screening on inter and intra-specific crosses of mungbean (*V. radiata*). However, bruchid bioassays on *V. radiata* and *V. umbellata* cross populations have not been conducted and reported till date. In the present study, recombinant inbred lines (RILs) of VRM(Gg)1 (highly bruchid susceptible) and TNAU Red (completely resistant) were evaluated with an aim to identify bruchid resistance source(s), following standard resistance screening procedures (Tomooka *et al.*, 2000; Gibson and Raina, 1972) with both free-choice and no-choice tests.

Bioassays are indispensable in many host plant resistance studies. The design and implementation of insect resistance bioassay/screening strategies are issues of considerable attention (Kavitha and Reddy, 2012) and development and standardization of methods for insect resistance screening is the key for an effective resistance-breeding program. Resistance breeding programmes are in progress for only a few pests because of the difficulties involved in screening and selection of test material under uniform insect infestation across seasons and locations. In case of storage insects like *C. maculatus*, reliable screening can be performed only under laboratory conditions. Methods for screening resistance involve infesting seeds artificially with insects for oviposition, duration of development on test samples; adult emergence pattern and calculating seed damage per cent, susceptibility indices from these observations (Seram *et al.*, 2016b; Dobie, 1974; Jackai and Asante, 2003). Semple (2016) stated that "free-choice" test is more practical when large number of samples is to be tested, which can be conveniently used to

eliminate obviously susceptible samples. Whereas "no-choice" technique has been widely used to complement "free-choice" procedures to both identify and confirm the presence of resistance. In view of these points, an "innovative model" was designed and developd for screening 187 RILs under free-choice condition. All the lines were subjected to host preference test in this newly designed model. Results showed wide variation among the RILs for bruchid resistance/susceptibility thus providing an opportunity for selection. Few RILs displayed moderate to high (score 5, score 3) resistance levels but a greater number of tested RILs were highly susceptible (score 7) to this South Indian bruchid strain (Annexure. V). Performance of most of the RILs was not consistent suggesting that resistance evaluation could not be reliable and that free-choice screening should always be confirmed and validated by "no-choice test". Erler *et al*. (2009) conducted resistance evaluation of chickpea against *C. maculatus* under free-choice and no-choice tests and reported reliable results. TNAU Red, the resistant parent used as check, was completely resistant despite more number of eggs laid per seed whereas the susceptible parent and check, VRM(Gg)1, was highly susceptible with maximum 9.00 adults emerged from 10 seeds and exhibiting 94 per cent seed damage.

Callosobruchus maculatus is known to be fairly plastic when it comes to host use (Rova and Björklund, 2011). Oviposition response revealed that Thanjavur *C. maculatus* preferred egg-laying on all RIL seeds, while differences in other growth parameters were observed under free-choice test. Of the total RILs, 136 RILs registered less than 30 eggs per 10 seeds. However, low number of eggs laid on different lines did not reflect the level of resistance. This could be visualized from fact that RIL 46 and RIL 118 which recorded only 8 and 9 eggs per 10 seeds was found to be highly susceptible (score 7) with 70 % and 80 % seed damages respectively. In contrast, RIL 125 registered low seed damage of only 20 %, which had 101 eggs per 10 seeds. The reason might be that, only one adult will emerge from a seed, though more number of eggs was laid per seed. Hence, in bruchid screening technique, level of resistance for a sample cannot be ascertained in terms of oviposition alone and may not have any significant role on seed damage and host suitability, which is in accordance with the findings of Dabi *et al*. (1978). Moreover, Dick and Credland (1984) observed that oviposition preference is influenced by host availability to a greater extent and has nothing to do with the actual resistance nature of an accession.

From 187 F_9 RILs evaluated, *C. maculatus* emerged from 181 RILs and no adult emergence was noticed from remaining 6 RILs during resistance evaluation [Fig. 9(g)]. However, some of the RILs were more or as susceptible as VRM(Gg)1 (susceptible parent). This study demonstrated the presence of adequate genotypic variation among the inter-specific populations of mungbean and rice bean on their resistance to *C. maculatus*. Variations in number of *C. maculatus* adults emerged (exit holes) and percentage damaged seeds on RIL populations at different days may indicate the presence of anti-nutritional compounds in seed cotyledon, which hindered normal bruchid development. The duration corresponds to the time on which the Thanjavur bruchid species (a) completely damaged seeds of the susceptible parent [VRM(Gg)1] and (b) adults stopped emerging from these seeds. The experiment was terminated at 60 days, when no adults had emerged for more than one week. RILs were grouped into 5 categories by following resistance criteria based on seed damage given by Weigand and Tahan (1990) and Sun *et al*. (2008). Frequency distribution of percentage seed damage by *C. maculatus* showed a skewed distribution towards the susceptible parent.

Hatched eggs were characterized by the presence of white frass, which indicated successful larval entry through seed coat (Raina, 1970) or presence of white dot, when eggs are scrapped off. Number of damaged seeds for calculating seed damage percentage was characterized by one exit hole per individual seed of VRM(Gg)1 and RILs (due to adult emergence), which is supported by Mitchell (1991). Desroches *et al*. (1995) defined mean development period (MDP) as the number of days from day 5 of oviposition to 50% emergence of progeny. Resistant RILs recorded significantly higher MDP compared to others. If no adult emerged over the test period, index of suitability value was equal to zero (IS=0), which was observed in the resistant RILs. Bruchids were counted daily until no more adults emerged, at roughly 60 days after infestation. Ofuya and Agele (1990) concluded that resistant hosts delay bruchid development inside the seeds, which in turn would reduce the rate of multiplication over generations. Sulehrie *et al*. (2003) further stressed that delay in developmental period alone may lead to a considerable reduction in seed loss during storage even though higher mortality is not realized in a resistant variety. Larval competition at initial stages may also contribute to the delay in developmental period. The larval stage is the only feeding stage in case of seed beetles and it is a measure of both the physiological and usefulness of the food and total amount of food ingested (Hovanitz and Chang, 1962). Thanthianga and Mitchell (1987) observed that when

more than one larva enters into a seed, one larva feed normally, while the other gets inhibited. But, only when the burrows made by the two larvae intersected, the younger larva dies. On the other hand, if the older larva pupates or dies without the burrows intersecting, the inhibited larva then feeds and matures. If the latter happens, an extended mean developmental period would be the result. A similar kind of phenomenon might have existed in some susceptible RIL seeds which exhibited prolonged mean developmental period than VRM(Gg)1 (susceptible check), other than due to antibiosis mechanism.

Confirmation of *C. maculatus* resistance to the selected 88 RILs from free-choice test was conducted under "no-choice test" laboratory infestation. Results confirmed the resistance of few RILs, *viz*. RIL 158, 165 and 169, which consistently showed no emergence or seed damage in both the tests. High mean developmental period (days) was also considered for resistance confirmation. Mean developmental periods were significantly different in some of the RILs during resistance confirmation bioassay. This study also suggested that resistance of *Vigna* crops to bruchids is a complex one as it is governed by several factors.

Present studies also highlighted the use of high infestation level (e.g. five pairs per ten seeds or twenty seeds) for effective bruchid resistance determination since low infestation (e.g. two pairs) did not give reliable results in case of rice bean landraces. Laboratory experiments using excessive numbers of females on few seeds or few bruchids on large number of seeds are also unrealistic. For example, Bellows (1982) used up to 120 pairs of bruchid beetles in a 4 cm dish with 14-16 seeds only. Sarwar (2012) used up to 25 g seeds per replication for resistance assessment on chickpea genotypes against *C. maculatus*. Moreover, Sharma and Thakur (2014) carried out varietal screening of cowpea against bruchid using 250 g seeds with only two pairs of bruchid, which is inaccurate, not reliable and may give misleading results. The use of five bruchid pairs per ten seeds under "no-choice test" with replications may be regarded reliable and suggested in resistance screening since parameter for resistance categorization depends on seed damage percentage, which is calculated at the end of the experiment, irrespective of seed count and lesser the seed number, the more precise and consistent will be the bioassay. Tomooka *et al*. (2000) and Kashiwaba *et al*. (2003) also considered ten seeds infested with five pairs of freshly emerged bruchid adults for evaluating the effectiveness of wild *Vigna* species for bruchid resistance, which backs the present study.

In a nutshell, results on this study emphasizes the availability of resistant genes in the underutilized *Vigna* species (*V. umbellata*) and few RILs of mungbean and rice bean, which can be used in conventional breeding programmes as well as molecular breeding techniques to develop resistant cultivars of pulse crops. Current bioassay with 187 RILs also suggests that expression of bruchid resistance gene could be modified by some environmental factors. Furthermore, after few RILs expressed consistently high levels of bruchid resistance under both the tests with artificial infestation, it was considered necessary to carry out separate studies to validate the observed resistance. Therefore, resistant rice bean landraces, selected ten extreme RILs (resistant and susceptible) of VRM(Gg)1 and TNAU Red were re-subjected to *C. maculatus* for re-confirmation of resistance, which also included different studies such as role of seed coat on resistance evaluation and ovipositional preference by antennae ablated and normal *C. maculatus* females, discussed in the later section.

5.6. Seed damage percentage as the most reliable parameter for bruchid resistance determination

Simplified visual plant damage ratings and indices have been established by breeders for resistance screening against important insect pests (Singh and Singh, 1990). These damage ratings facilitate quick and easy screening of lines that does not require tedious count measurements. However, there are inconclusive reports on bruchid resistance ratings based on index of suitability. Therefore, based on bioassay results from the present investigation, seed damage percentage, which is quick and easy to calculate, was considered as the most reliable parameter for bruchid resistance determination. The supporting statements and references for this are discussed below.

Commonly used resistance parameters such as per cent adult emergence (per cent survivality), mean developmental period (MDP), per cent seed weight loss (Jackai and Asante, 2003; Badii *et al*., 2013) and Howe's index (Howe, 1971) present problems in describing and comparing the results of different bioassays. Moreover, reliance on these parameters individually may lead to misleading interpretations. For example, index of suitability (Howe's Index) is commonly used to determine bruchid resistance/susceptibility of a seed sample. However, it can not be used for those seed samples that do not have acute toxicity but disturb insect growth by inhibiting feeding or altering normal development. Similar observations were noticed in the present study, which is evident from bioassay results on rice bean landraces and few RILs.

Alternatively, parameters such as effects of allelochemicals on weight gain, pupal weight, and duration of development, daily survival or mortality have been used to study and determine bruchid resistance. These parameters, however, vary with insect species and even with instars within the same species.

A range of investigations by several workers have stressed on suitability index as the standard criterion in bruchid resistance evaluation (Badii *et al*., 2013; Dasbak *et al*., 2009; Ponnusamy *et al*., 2014; Soumia *et al*., 2015; Sulehrie et al., 2003). Based on this parameter, accessions can be categorized as resistant (<0.05), moderately resistant (0.051-0.060), moderately susceptible (0.061-0.070) and susceptible (>0.081). Among the rice bean evaluated, the five damaged landraces exhibited growth index <0.05 (IS = 0.015, 0.006, 0.024, 0.009, 0.012) [Table. 34(b)] in which they can be placed under resistant category despite of high or moderate seed damage. This was not desirable since the present study was aimed at resistance source(s) identification with complete immunity for effective resistance breeding. On the other hand, scoring based on seed damage (Weigand and Tahhan, 1990) showed these landraces as moderately susceptible to *C. maculatus*. Similarly, in terms of 187 RIL seeds, 58 RILs registered index of suitability below (<) 0.05 [Fig. 9(h)], in which they can be categorized as resistant. However, results on resistance categorization based on seed damage per cent showed otherwise. Furthermore, importance should be given on damage percentage since suitability index is dependent on survival and mean developmental period (MDP), and MDP considers only 50 per cent of adult emergence. Also, all these parameters ultimately inflict damage to seeds. In addition, damage percentage is based on the total number of seeds damaged at the end of the experiment, irrespective of the number of adults emerged which is accurate, reliable and provide effective results. Seed damage per cent was considered for evaluating the effectiveness of wild *Vigna* species for bruchid resistance by Tomooka *et al*. (2000) and Kashiwaba *et al*. (2003). Moreover, Sun *et al*. (2008) and Somta *et al*. (2008a) demonstrated resistance evaluation based on seed damage per cent for QTL mapping and identification of molecular markers linked with bruchid resistance in mungbean (*V. radiata*) and wild species, *Vigna nepalensis*. On the contrary, genotypes with a high suitability index were considered susceptible and those with a low index as resistant by Kananji (2007). This is based on the assumption that a few insect progenies would emerge out of a resistant genotype and insect progeny development would take a longer time in a resistant than in a susceptible genotype. This parameter was not considered in the present

investigations since it did not give reliable results on bruchid resistance determination, as explained earlier.

5.7. Relationships between different bruchid biological parameters in F_9 RIL populations

Analysis performed between different bruchid biological parameters revealed highly significant correlation between parameters like number of adults emerged, per cent survival, MDP and per cent damage, and suitability index (IS). However, the correlation was more with per cent damage (r = 0.999**, 0.633**, 0.269**) than IS (r = 0.670*, 0.862*, 0.093^{NS}) at p = 0.01 and p = 0.05 levels of significance (Table. 45). This further supported the selection for damage per cent over index of suitability for resistance determination. This is in corroboration with the findings of Soumia *et al*. (2015), who reported significant (at p = 0.05 and p = 0.01 level of significance) relationships among all the variables measured for bruchid resistance such as oviposition, developmental period, percentage adult emergence and growth index. However, Soumia *et al*. (2015) and Ponnusammy *et al*. (2016) suggested strong correlations between bruchid parameters with Dobie's suitability index and adult bruchid emergence, which were used to classify resistance. This slightly deviates from the current findings.

5.8. Mechanisms of resistance

The two common mechanisms employed by host plants to resist insect damage are antixenosis and antibiosis. In case of seed resistance to bruchid, seed size, seed coat thickness and hardness may possess possible antixenotic properties in relation to oviposition response, hatching percentage or developmental period whereas biochemical compounds, enzyme inhibitors found in seed cotyledon can have important role in antibiosis mechanism. In many cases, it is obvious that physical characters of seed and biochemical constituents such as phenols, tannins, trypsin inhibitor and amylase inhibitor are important in conferring resistance to bruchids. Antibiosis is considered as an effective defense strategy employed by legumes against stored seed insect pests (Edwards and Singh, 2006). In general, antibiosis mechanism in insects is manifested by reduced fecundity, abnormal growth, sub-sized adult, etc. (Painter, 1951). Several studies were conducted worldwide on the resistance mechanisms to bruchids in different pulses and few have pointed out antixenosis, in addition to antibiosis. But these, however, have been inconclusive (Murdock *et al*., 1990). Hence, present investigation was taken up to understand the physical and biochemical basis of resistance in different samples (rice bean, mungbean and their RILs) to *C. maculatus*.

Callosobruchus maculatus oviposition was higher on drum shaped seeds. However, bruchid seed damage recorded equally, irrespective of seed shape. This is substantiated by Szentesi and Jermy (1994) who suggested that seed shape appears to be a plant species character independent of bruchid infestation. Seed colour and seed dimension had no effect on the susceptibility of different samples used in the present study. Seed colour trait is maternally inherited, so the seed phenotypes reflect the genetic constitution of the parent plant and has nothing to do with insect attack (Isemura *et al.,* 2012), which was observed in the present investigation. Earlier, Booker (1967) observed that in multiple-choice test, smooth seed surface was preferred for oviposition by bruchids than rough seed surface in similar sized seeds but preference for smooth seed surface with small seed size could not surpass the preference for large seed size in spite of its rough seed coat. Sandhu *et al*. (1986) studied on rice bean landraces with different size, seed coat texture and colour for oviposition preference by *C. maculatus* as a component of susceptibility and reported that larger size, smooth and well-filled seeds were preferred for oviposition.

Messina and Renwick (1985a) reported that rough seed coat provides difficulties for *Zabrotes subfasciatus* because it glues its eggs on the seed testa. A positive correlation between smooth seed coat texture of cowpea and *C. maculatus* oviposition was also reported by Messina and Renwick (1985b). Ovipositional preference for smooth seed coat have been reported in mungbean (Lambrides and Imrie, 2000; Sulehrie *et al.*, 2003), cowpea (Nwanze and Horber, 1976; Oigianbe and Onigbinde, 1996) and chickpea (Rathore and Chaturvedi, 1997). These reports are in agreement with the present findings since smooth textured RIL seeds were more preferred for oviposition as seen in RIL 167 and RIL 170, which recorded 77 and 73 number of eggs per 10 seeds respectively [Fig. 10(c); Annexure.IV]. However, oviposition load of South Indian *C. maculatus* was highest on a rough textured RIL 125 with 101 eggs laid on 10 seeds, which deviates from the previous reports. Observations made by Akingbohungbe (1976) supported this result, who suggested oviposition preference for rough seeds may be due to "possible attractant" present in seed coat.

Bruchids are not able to colonise very flat seeds, the smallest dimension of which leaves no space for an adult (Center and Johnson, 1974). On the contrary, Szentesi and Jermy (1994) indicated that bruchid colonisation is strongly affected by seed morphological (size and shape) parameters. Also, they suggested that even if opportunity is provided to develop on a larger seed,

a bruchid would not grow larger because of possible advantages of larger body size within genetically determined limits. Seed size (Nwanze *et al.*, 1975; Dharmasena and Subasinghe, 1986) has also been shown to influence infestation by bruchid as large grain legumes provide more surface area for oviposition and larval development than small-size grains. Surprisingly, many plant species with large enough seeds are not infested however, which is also evident from the present study on Manipur rice bean landraces.

Hundred seed weight (HSW) in the present studies showed that it did not play any role in number of eggs laid. However, HSW as a factor for increased egg laying was discussed by several workers. Sulehrie *et al.* (2003) opined that greater seed weight and higher surface area in mungbean leads to higher oviposition. Similar conclusions were drawn by Mitchell and Thanthianga (1990), who reported that large seeded mungbean varieties induce bruchids to lay more eggs than on smaller seeds. However, Cipollini and Stiles, (1990) showed that smaller seeds were preferred to larger ones for oviposition.

It is clear from the present results on hatching percentage that *C. maculatus* were successful in penetrating the seed coat and reached cotyledons in most of the RIL seeds. This may indicate that seed coat thickness did not influence the entry of bruchids, contradicting the findings of Applebaum and Birk (1972), who observed that resistance in some broad bean varieties was solely due to difficulties encountered by hatching larvae in penetrating the seed coat. Messina and Renwick (1985c) also reported that hard/thick seed coat may prevent larvae from successfully penetrating the seed. However, Thiery (1984) was of the opinion that seed coat thickness is not important in conferring resistance to cowpea against *C. maculatus,* which is in agreement with the current finding.

5.8.2. Relationships between seed biophysical attributes and fitness of *C. maculatus*

Seed size has been reported to correlate with susceptibility of bean genotypes: varieties that are large-seed being more susceptible than small-seeded bean varieties (Schoonhoven and Voysest, 1991). Giga and Smith (1983) reported that physical factors such as seed coat hardness and seed coat roughness confer resistance to bruchids. However, from the correlation analysis, there was no evidence of the role of phenotypic traits in influencing resistance to bruchids and seed size did not influence *C. maculatus* resistance or susceptibility to any sample evaluated in the present study. The argument is that small seed size presents a barrier as mortality, size and

fecundity of bruchid progeny are strongly affected by overcrowding within seeds (Schoonhoven *et al.,* 1983; Cipollini and Stiles, 1990). This can be explained from the result that TNAU Red (*V. umbellata,* the resistant parent), which has a 100-seed weight of 4.490 g, can easily allow larval growth inside the seed. However, *C. maculatus* larvae could only penetrate through its seed coat and were unable to develop inside the seed. This is supported by the positive but non-significant correlation observed between seed size and oviposition preference by *C. maculatus* (r = 0.08). Although seed size of RILs and seed damage by *C. maculatus* was significant at p=0.01, the correlation (r = 0.192**) between these traits was weak to suggest any significant role of seed size in influencing resistance. Hardness of seed was found to have non-significant correlations with all the bruchid parameters *i.e.* egg hatchability (r = 0.113), adult emergence (r = 0.078), seed damage (r = 0.078), MDP (r = 0.034) and IS (r = 0.068) (Table. 48). Therefore, seed hardness did not seem to be a factor responsible for reducing susceptibility of the RILs to *C. maculatus* in the present study. These results are in conformity with Bhatnagar *et al*. (2001) who reported that there was no association between seed size of different legume varieties and *C. maculatus* oviposition preference. Dabi *et al*. (1979) also found positive but non-significant correlation between seed size and amount of food consumed while studying relative susceptibility of some cowpea varieties to bruchid. Kashiwaba *et al*. (2003) also suggested that the hard seed coat or chemical substances in the seed coat were not the main factors causing bruchid resistance in rice bean. The present findings are also in similar trend with Srinivasan and Durairaj (2007) who reported that seed coat thickness and seed hardness of wild *Vigna* species had a non-significant relationship with that of *C. maculatus* developmental parameters. Kaur (1997) and Kaur and Ramzan (2001) also reported that seed hardness did not attribute to delay in *C. maculatus* development on soybean as the correlation coefficient between these parameters was recorded to be negative and non-significant. However, Kaur (1997) found negative but significant correlation between thickness of soybean seed coat and the larval penetration holes by *C. maculatus*, which did not coincide with the present studies.

Non-significant relationships between seed characters and bruchid parameters indicated that seed morphology (antixenotic mechanism) does not play a role in imparting resistance to *C. maculatus* damage. Therefore, from the above discussions, it is obvious that biophysical factors like, seed size, texture, hardness and seed coat thickness had no role in conferring resistance to bruchids which indicates the possibility for the presence of antinutritional factors that made a

few RILs of *V. radiata* and *V. umbellata* less suitable to *C. maculatus*. Earlier, Southgate (1979) suggested the role of biochemical constituents in cotyledons for imparting resistance to bruchids.

5.8.3. Antibiosis mechanism manifested through insects

Antibiosis is the mechanism by which a colonised host is resistant, because it has an adverse effect on insect's development, reproduction and survival (Dent, 2000). It can also be manifested by the effect of larval environment on oviposition after host acceptance. For instance, the more an insect has acquired resources in the larval stage, the higher is usually its fecundity (Honek, 1993). Optimality models predict that high fecundity should lead to higher ovposition rates, and thereby lower selectivity, wider host range, and larger clutches. On the other hand, resource-rich larval life could also lead to longer life expectancy and higher searching efficiency which, in turn, could elevate acceptance threshold (Javois, 2005). Reduced or no adult emergence and extended larval development periods in resistant RILs suggests that antibiosis or anti-feedant activity may be the actual resistance mechanism against bruchids.

Observations and experiments such as determination of body size and weight of bruchid adults emerged from different RILs and fecundity check for such adults on VRM(Gg)1 seeds were conducted in order to determine and confirm antibiosis mechanism in resistant seed samples, which were demonstrated through insect responses and behaviour. Susceptible samples were included for comparisons. Body size of bruchid adults emerging from resistant lines (RIL 158, 165, 169) and rice bean landraces (moderately susceptible) were significantly reduced and there was complete absence or reduced of oviposition (female fecundity), when tested on susceptible parent, VRM(Gg)1 seeds. Moreover, weight of *C. maculatus* adults emerging from these resistant RILs and rice bean landraces were adversely affected when compared to normal weight of adults emerged from VRM(Gg)1 and susceptible samples. This may be explained from the fact that, for insects that develop inside discrete seed host, both host size and host qualities constrain offspring growth, influencing the evolution of body size and life history traits (Amarillo-Suarez and Fox, 2006). Szentesi and Jermy (1994) suggested that bruchid body size is a constant trait and the variability of body size in bruchids emerging from different host seeds is very small. Compounds present in resistant seed cotyledons interfere with normal growth and development of *C. maculatus*, insects mature at a smaller size, generating phenotypic variation in body size and development time within populations (Kirk, 1991). Body size variation in *C. maculatus* adults emerged from resistant and susceptible lines were highly significant indicating

that compounds present in seed cotyledons might have interfered directly with bruchid larval development/performance inside resistant seeds. This difference is likely a consequence of the presence of substances, which are not suitable for *C. maculatus* larval development. Clearly, it indicates antibiotic factors responsible for resistance in rice bean landraces and few selected lines *viz.* RIL 158, 165 and 169. Body size ratio (BSR) and body size differed among adults emerged from different RILs. Kashiwaba and Tomooka (2002) also indicated that antibiosis was a component of bruchid resistance in rice bean.

In addition, Cardona *et al.* (1989) further observed that even though seed testa may occasionally act as a physical barrier, factors responsible for resistance were chemical in nature and they were present in the cotyledon. Other chemical factors within the seed, such as phytohemagglutinin (PHA), have also been reported to confer resistance in dry beans (Ishimoto and Kitamura, 1989). They expressed resistance in terms of reduced oviposition, prolonged larval development and reduced progeny weight. In all these cases, growth and development of bruchid larvae feeding is inhibited or retarded indicating the importance of antibiosis as resistance factor, which is also supported by Gatehouse *et al.* (1979) and Baker *et al.* (1989) who suggested similar conclusions. Semple (1992) indicated that crop varieties can display variable resistance depending on the crop's geographical origin and agronomic cultural practices. This suggests that different production environments may result in differing levels of resistance and affect crop varieties' susceptibility to storage pests, which was also observed in case of rice bean and mungbean samples, which were collected from different geographical regions.

5.9. Biochemical analyses

Grain protein content is considered an important quality characteristic of grain legumes. Katoch (2011) evaluated thirty diverse rice bean genotypes from Palampur for their nutritional value and found variations in crude proteins ranging from 16.1-19.12 %. Protein in rice bean is rich in limiting amino acids, methionine and tryptophan (Carvalho and Vieira, 1996). Different studies by Buergelt (2009) and Zaman and Malik (1999) reported crude protein ranging from 18.3 - 32.2 % and 20.32 - 21.93 % (Pakistan) in rice bean genotypes. Crude protein in the range 14.62 to 21.52 % were observed in Manipur rice bean seeds determined in the present study, which corroborates with the earlier reports. Protein content in mungbean varieties were 19.27 - 22.61 %. Chavan *et al.* (2009) reported that dry rice bean seeds contained 56.58% starch and that the chemical composition of starch does not differ significantly from other edible legumes.

Natasha (2013) suggested that common bean resistance to bruchid species, *Acanthoscelides obtectus* damage is accompanied by reduced starch content, and high content of an acidic polysaccharide. Kumar (1992) reported that there was a significant and positive correlation between proteins with bruchid damage, whereas a negative correlation prevailed between phenols contents in seeds with seed damage. However, these reports deviates from the current findings, since relationship analysis performed between protein content (%) and bruchid biological characteristics on susceptible and resistant samples revealed non-significant correlations. On the other hand, Makanur (2010) suggested that protein content in seeds had no influence on susceptibility or resistance of cowpea seeds to *C. maculatus*. Similar results with regard to correlation were also reported by Kapila and Rajni (1989) in pea and Talekar and Lin (1992) in mungbean and black gram varieties, which are in support of the present finding.

Phenol concentration varies significantly between different plant parts and generally decreases with an increase in plant age (Kawsar *et al.*, 2008). The biochemical basis for resistance to *C. maculatus* in rice bean (*V. umbellata*) was estimated by Srinivasan and Durairaj (2007) and results revealed that amount of proteinase inhibitors were higher in resistant cultivars than susceptible ones. Pavithravani *et al.* (2013) also suggested significant role of alpha-amylase inhibitory activity in imparting bruchid resistance in rice bean. Proximate analysis of resistant seed samples were not carried out due to limited quantity of seeds. However, GC-MS analysis suggested the presence of some inhibitory compounds (e.g. phenol) in few seed samples, which are discussed in the later section.

5.10. Role of seed coat on *Callosobruchus maculatus* oviposition and development

The role of seed coat on ovipositional preference by *C. maculatus* has been discussed by many workers. In order to analyze exclusively the role and influence of antixenosis mechanism, an experiment was therefore set up to test the hypothesis that seed coat played a crucial role in conferring resistance to bruchid attack. Bioassays were conducted with decorticated and intact seeds. Results from this study demonstrated that when female *C. maculatus* have a choice for oviposition site, they preferred seeds with intact seed coats to decorticated seeds. Schoonhoven and Voysest (1991) reported that *Zabrotes subfasciatus* (a bruchid species) depends on the good surface area provided by seed coat for successful oviposition, where eggs are glued onto it, hence it may not easily lay eggs, when seed coat is removed. *C. maculatus* might have experienced a similar phenomenon during oviposition on decorticated seeds in the present investigation. This

may also be attributed to involvement of either an 'oviposition attractant' in seed coat or an 'oviposition deterrent' in seed cotyledon. Credland and Wright (1988) demonstrated that extracts obtained from seed coats of pulse varieties, irrespective of their qualitative characters (eg. seed coat texture, colour), are required to stimulate oogenesis and oviposition in mated bruchids, which supported the above justification. Evidence of seed coat as a possible physical or chemical deterrent to C. *maculatus* oviposition was also demonstrated by Kemal and Smith (2001). These reports could attribute to the varied oviposition performances on intact and decorticated seed samples evaluated in this study. However, rice bean seeds with seed coat removed also showed complete resistance.

Seed coat is a major barrier, which acts as an antixenotic factor and contributes significantly to the mortality of neonate larvae during seed perforation phase (Desroches *et al.,* 1995). Nwanze and Horber (1976) stated that seed coat serves no nutritional purposes, since it always remains even after severe infestation. Lale and Kolo (1998) suggested that presence of elevated levels of certain chemicals or a reduced level of oviposition stimulants on seed coat, irrespective of its texture, are likely factors responsible for reduced egg deposition on resistant samples. Oviposition preference on seeds with intact seed coats by C. *maculatus* in this study suggests that resistance may not be due to seed coat deterring oviposition. A significant correlation between level of secondary compounds contained in legume seeds and their susceptibility to bruchid attack was described by Singh and Singh (1990).

From this study, it may be revealed that seed coat occasionally acted as a physical barrier for C. *maculatus* emergence since few resistant RIL seeds registered bruchid emergence, mostly in larval form [Plate. 22(b)]. However, factors responsible for resistance were present in cotyledon. This may be explained from the present examination that no sign of bruchid presence or development was observed when uninfested resistant RIL seeds, with or without seed coats, were cut open. This again reconfirms the resistance status of the selected RILs.

5.11. Host ovipositional preference by normal and antennae ablated *Callosobruchus maculatus* females

One of the major aspects to understand the ecology and evolution of interactions between insects and plants is to study the behaviour of insect oviposition and its deterrence (Kumari and Kaushik, 2016). According to Thompson and Pellmyr (1991), oviposition behaviour is when a number of potential hosts are available; a female insect will lay maximum eggs on the most

preferred host species, lesser number on the next, and so on. Insect females follow certain sequence of events leading to oviposition, which includes searching, orientation, encounter, landing, surface evaluation and acceptance (Singer, 1993). All these sequences of host finding depend on a wide variety of sensory cues and most studies in this area have been focused on visual factors such as shape, size and colour (Kumari and Kaushik, 2016). During the present study, variation in host preference for oviposition by normal (with antennae) and antennae ablated (without antennae) bruchid females were noticed in some of the samples evaluated. However, it was not significant between their means (p>0.05). Similar non-significant results were also recorded for other bruchid parameters. This may indicate that antennae have little or no role in host preference by *C. maculatus*. These results are in good accordance with Szentesi (1976), who suggested that maxillary palpi played a significant role in recognition of oviposition site while studying the effect of amputation of head appendages on *Acanthoscelides obtectus* oviposition. In bruchid beetles, detection of chemical for host finding is thought to be mediated by receptors located in the labial palpi and maxillary palpi (Messina and Renwick, 1985c). Moreover, Messina *et al*. (1987a) did not observe any direct involvement of *C. chinensis* and *C. maculatus* antennae in egg distribution on seeds, which corroborates the present finding. Kumari and Kaushik (2016) reported that sensory receptors involved in oviposition may be present on different body parts of insects such as tarsi, antennae, proboscis and ovipositor. Based on the earlier reports and results obtained in this study, it clearly indicates that antennae do not play any role in host preference for oviposition in bruchids. Moreover, SEM analysis of *C. maculatus* antennae and mouthparts supported the present findings, discussed in the later section. Nonetheless, Mbata *et al*. (1997) reported that antennae play a role in mate finding through pheromone reception in bruchid beetles.

Egg-laden seeds are effective in deterring ovipositing females and scraping off eggs from seeds caused the deterrent effect of oviposition marker to dissipate after 4 weeks (Ramaswamy et al, 1995). They also reported that females are able to distinguish between egg-laden and pristine seeds under various regimes of temperature, humidity, and light. Furthermore, age of females did not affect their ability to distinguish between egg-laden and pristine seeds. It is speculated that both contact and olfactory cues are necessary for the avoidance of egg-laden seeds. Females of this bruchid species mark the oviposition substrate after egg deposition with an epideictic pheromone, which promotes resource partitioning (Giga and Smith, 1985; Messina *et al*., 1987b;

Credland and Wright, 1988). However, the chemical identity of such oviposition-marker pheromones is unknown (Ramaswamy *et al.,* 1995).

5.12. Effects of indigenous plant extracts on the bionomics of *Callosobruchus maculatus*

Inadequate literature is available on the effects of indigenous plant extracts on C. *maculatus* in Tamil Nadu. Moreover, besides neem, work on other botanical pesticides needs to be strengthened considerably. Hence, this study was undertaken to find any possible effect(s) on *C. maculatus* development and to assess and validate the efficacy of selected indigenous plants. Aqueous extracts of notchi, cashew and *Neerium* at 10 % concentration were effective in killing *C. maculatus* adults as evident from high adult mortality released for oviposition. This suggests that indigenous plants may be useful in controlling bruchids to some extent, if applied timely (before egg laying). Similar findings were observed by Ahad *et al*. (2013) who reported high adult mortality in *C. chinensis* when mungbean seeds were treated with ethanol extracts of *Vitex negundo* (notchi) and *Nerium oleander* (korobi) at 3% concentration. Present results were in also agreement with the reports of Rahman and Talukder (2006) on black gram seeds.

According to Elhag (2000), a treatment is considered effective if it killed emerged bruchids as this would reduce the re-infestation and few adult bruchids would emerge in the next generation which would result in reduced damage. This may be explained from the assumption that when eggs are laid on treated seeds, the toxic substance (active ingredient) present in plant extract might have entered into the egg through chorion and suppressed further embryonic development (Raja *et al*., 2001). Considering this point, in this study, notchi extract @ 10 % concentration significantly prevented emergence of F1 adults of *C. maculatus* from treated seeds, which is in corroboration with the results of Ahad *et al*. (2013) that showed oviposition deterrent activity, percentage reduction in adult emergence and reduced seed damage when seeds were treated with ethanol extract of notchi at three different concentrations (1 %, 2 % and 3 % respectively).

The first point in insect-host relationship at which the plant may show resistance is in deterring insect oviposition (Beck, 1965). Oviposition deterrents, which deter an insect from laying eggs are important and proper understanding of these deterrents shall provide necessary insight into new vistas for insect pest management. Present studies revealed that maximum oviposition deterrent activity was shown by 10 % cashew extract (T_{14}), which also showed 40 % reduction in adult emergence. This is in agreement with Adedire *et al.* (2011), thus stating

similar results, when seeds were treated with cashew kernel extracts. Raja *et al*. (2001) reported that leaf extract of *Jatropha* showed oviposition deterrence and antifeedant activity as well as reduction in adult emergence of *C. maculatus*. Boateng and Kusi (2008) also reported that *Jatropha* seed oil was highly toxic to *C. maculatus* eggs and acts as a repellent for bruchid adults. Moreover, Chudasama *et al*. (2015) recorded *C. maculatus* oviposition deterrence and reduction in adult emergence of 53.24 % and 52.01 % with 5 % aqueous leaf extract of *Jatropha* on cowpea seeds. However, these reports are in contradiction to the present findings since aqueous leaf extracts of *Jatropha* at 5 % and 10 % concentrations did not give any negative effect on bruchid development. Contradictory results may be due to differences in the plant parts and the type of formulation used in the experiments. Oviposition deterrence of 80.77 % was observed in treatment with 5 % *Lantana* extract, which is again supported by Ahad *et al*. (2013) and Chudasama *et al*. (2015).

Certain non-host plant compounds, when applied to a host plant, are likely to render the plant less attractive to a phytophagous insect and more attractive to its parasitoids (Kumari and Kaushik, 2016). Extracts of non-host plants may be effective in reducing *C. maculatus* oviposition load on susceptible hosts (Elhag, 2000; Jayakumar, 2010). Results obtained from this study also showed that aqueous extracts of TNAU Red (bruchid resistant rice bean parent) cotyledon (T24) and one resistant rice bean landrace, LR(M)4 cotyledon (T30), caused mortality of *C. maculatus* adults with oviposition deterrence of 51.28 %, 64.10 % respectively and percentage reduction in adult emergence of 50 %, 40 %, respectively. It may be assumed that constituents present in these bruchid resistant rice bean cotyledons are responsible for these adverse effects on *C. maculatus* bionomics. Report on the effect of seed cotyledon extract on bruchid development is not available. However, this can be supported from the results of Somta *et al*. (2006a) who reported presence of antibiotic factor when they studied bruchid resistance evaluation on artificial seeds made of resistant rice bean cotyledon.

VRM(Gg)1 seeds treated with notchi extracts had the most profound effect of all the extracts evaluated on *C. maculatus*, followed by cashew. Other plant extracts such as *Tecoma*, *Datura*, *Calotropis*, manjanathi, *Dodonea* did not show any effective results and hence these indigenous plant species may not be recommended for bruchid management. Obembe and Kayode (2013) also reported effective protection of cowpea seeds against *C. maculatus* with aqueous extracts from tropical trees. It may be assumed that the negative effect of these plant

extracts on bruchid might be as a result of contact toxicity since most insects breathe by means of tracheas, which usually open at the body surface through spiracles. The extracts that were mixed with seeds might have blocked these spiracles thereby leading to suffocation and death. (Rahman and Talukder, 2006). Oviposition and percentage adult emergence of *C. maculatus* were significantly lower in notchi treated mungbean seeds compared to control. The extracts might have possibly inhibited locomotion, thereby affecting mating activities and fecundity. Similar results were reported and fairly well documented by Boeke *et al*. (2004a). Additionally, these effects could be linked with respiratory impairment, thus probably affecting metabolism and consequently other systems of the bruchid beetle (Rahman and Talukder, 2006). Failure of the eggs to stick to treated seeds due to presence of extracts may also reduce survival after adult emergence (Obembe and Kayode, 2013).

5.13. Identification of biochemical compounds through Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS analysis of the methanolic and hexane extracts of seed cotyledons and seed coat of both resistant and susceptible samples were conducted in order to identify the possible chemical constituents, which may be responsible for conferring resistance to bruchids. Till date, no reports exist on GC-MS analysis of the underutilized *Vigna* crop, *V. umbellata*. Bioactive compounds present in the methanolic extracts of seed cotyledon and seed coat of *V. umbellata* were identified by GC-MS analysis [Fig. 14(b)]. The identified major compounds possess some important biological potential for future pharmaceutical studies.

Recent studies show that several plant products, including polyphenolic substances (e.g., flavonoids and tannins) and various herbal extracts, have antioxidant and anti-inflammatory activities. From the present GC-MS analysis of seed contents, it is revealed that the extract contains various bioactive functional groups, *viz*. alcoholic, ester, aldehydic etc., and hence it can confirm that the plant accumulated with diverse bioactive molecules. Most of these compounds have not been reported from this underutilized pulse crop (*V. umbellata*) so far. While GC-MS analysis of *V. radiata* (mungbean) has been reported by many workers, there may be variation in the chemical composition based on topography (Kusolwa, 2008).

Malic acid was detected in the hexane extract of resistant rice bean landrace. This finding is in trend with Gupta and Bhat (2016) who reported the presence of malate and oxalate as the main components of chickpea exudates. Rembold *et al.* (1990) suggested that varieties with

highest amount of malic acid had the highest resistance to *H. armigera*. Malic acid acts as deterrents to *H. armigera* larvae and resistant lines have more amount of malic acid than the susceptible lines (Bhagwat *et al*., 1995). However, there are no reports on the role of malic acid in resistance to storage pests.

Flavonoids and tannins are considered to be the most promising polyphenolic compounds among plant secondary metabolites (Tomczyk *et al*., 2010). Though many studies conducted elsewhere have reported the presence of total phenolics in hexane extract (Ifeson *et al*., 2013), in the present investigation, no phenolic compounds were detected in hexane extracts of mungbean and rice bean cotyledons and seed coats. Stanisavljevic *et al*. (2015) reported that there are significant differences in both phenolic content and antioxidant activity among seed coat extracts from different pea genotypes. They stated that pea seed coats from their study contained certain amounts of rosmarinic acid, rutin, galangin, morin, naringin, hesperetin and pinocembrin, as well as ten flavonol glycoside, which is in accordance with the present findings on seed coat compounds. Moreover, Temmink *et al*. (1989) reported simple phenols, hydrolysable tannins and condensed tannins found to be present in pulse seed coat, that confer defense properties towards attack by microbes and insects. According to Monteiro *et al*. (2005), tannins have the function of inhibiting herbivorous organisms, since at high concentrations they become unpalatable to phytophages, especially insects.

Flavone, isoflavone, flavonoids, and isoflavonoids are the important metabolites found in mungbean. Flavonoids are involved in stress protection including protection from insect and mammalian herbivores. Wang *et al*. (2008) identified twelve phenolic acids from mungbean seeds and sprouts. This report is in agreement with the present findings on compounds identified in seed extracts of few RIL seeds and rice bean landraces. Pulse crops are excellent sources of protein, dietary fibre, micronutrients and phytochemicals (Messina, 1999). In addition to nutrients, it can also supply many bioactive substances in small quantities which have significant metabolic and/or physiological effects. The effects of some fractions of the phytochemicals from resistant seed cotyledons might be the responsible factor(s) conferring resistance to *C. maculatus*, which may have attributed to antibiosis mechanism. Styrene occurs naturally in small quantities in some plants and foods (cinnamon, coffee beans, and peanuts). Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds like tannins. According to Marathe *et al*. (2011), phenolic content of legumes varies in the range of

0.325-6.378 mgGAE (gallic acid equivalent)/g. The phenolic acids are a large family of secondary metabolites having either derivatives of benzoic acid (e.g. gallic, syringic and vanillic acid) or of cinnamic acid (e.g.caffeic, ferulic, sinapic and ρ-coumaric acid), which are commonly found as esters of caffeic and quinic acids (Kawsar *et al.*, 2008). Some of these acid derivatives were identified in few samples in the present study. For example, cinnamic acid (naringinin acid) was detected in methanolic extract of resistant RIL 158 cotyledon, which might be responsible for conferring bruchid resistance, supported by Kashiwaba and Tomooka (2002).

The above mentioned isolated compounds from methanol extract of *V. umbellata* seed seem to posses some biological activity and further study of these phytoconstituents may prove the insecticidal action in the near future. The information regarding phytochemical compounds are not only supportive for discovery of therapeutic potential, but also have an active contribution towards discovery of new semi-synthetic and synthetic compounds which can be used in the manufacture of new insecticides (Sermakkani and Thangapandian, 2012). More compound identification from seed extracts of hexane compared to that of methanol may probably be due to the polar nature of the solvent, which might have interfered in the solvent flow chamber inside GC-MS apparatus, which is supported by Reena and Sinha (2012) suggesting similar reasons.

5.14. Anatomical structures for resistance through scanning electron microscope (SEM)

5.14.1. SEM analysis of *Vigna* seeds

Scanning electron micrographs of seed coat and cotyledon cross sections of different seed samples were taken and differences were recorded among them. This study was undertaken to examine and compare seed microstructures of mungbean, rice bean and some of their RILs (inter-specific populations) in relation to bruchid resistance. Randomly distributed pits with surface deposits were observed on the seed coat of mungbean [Fig. 16A(d)]). Similar pits were reported to be observed in red gram (Joseph *et al.*, 1993) and soybeans (Wolf *et al.*, 1981). Microstructure of RIL (158, 116 and 125) seeds exhibited distinct structural features such as pits with surface deposits, prominent ridges and pillar cells in the sub-epidermal layer of seed coat [Fig. 16C(a, e); Plate. 16D(a)], which are distinctly different from that of rice bean [Fig. 16B(c)]. In case of mungbean seed coat, these pits were not noticed [Fig. 16A(c)]). The absence of pits is also a characteristic of seed coats of mature common beans *(Phaseolus vulgaris* L.) (Hughes and

Swanson, 1985) and adzuki beans (*Vigna angularis* cv. Express) (Chilukuri and Swanson, 1991) which supported these findings.

Starch granules from different seed samples were also evaluated with respect to their morphological characteristics, using SEM images. In VRM(Gg)1 and TNAU Red, starch granules were basically oval shaped, which is in accordance with Joseph *et al*. (1993). Starch granules presented sizes in the range of 15 to 25μm and elliptical or oval in shape, with RIL 158 starch granules being the smallest. Legumes contain 30-70 % of starch on dry basis (Guilbot and Mercier, 1985). Samples examined in the present study were typical of legume starch granules. Data on the starch content of rice bean are limited. Chavan *et al*. (2009) reported 56.58 % starch content in rice bean seeds. In legume seeds, the occurrence of starch granules in cotyledons depends on the species and the cotyledons store protein, lipids and starch, although the proportions vary for different species. For example, in broad bean (*Vicia faba*) the ratio of protein:lipid:starch is 23:1:56 (Torres *et al*., 2009). Mungbeans have much greater carbohydrate content (50%–60%) and starch is the predominant carbohydrate in the legume (Tang *et al*., 2014), which was evident in RIL 158 cotyledon microstructure [Fig. 16C(b)]). Most legume seeds are starch rich but are also rich in protein as compared to cereals. Scanning electron microscope (SEM) has been employed to study microstructure of food grains. Microstructural variations can be related to textural, chemical and physical variations in bean cultivars (Sefa-Dedeh and Stanley, 1979) and identification of different cultivars of soybean (Wolf *et al*., 1981), adzuki bean (Enquist and Swanson, 1992).

In general, observations on the microstructure of cotyledons of legumes are available in literature due to the interest in studying changes during maturation, storage, soaking and processing (Hughes and Swanson, 1985; Sefa-Dedeh and Stanley, 1979). In the present study, SEM examination of cotyledon cells of mungbean, rice bean and some of their inter-specific lines illustrated spherical starch granules embedded in a protein matrix. Protein bodies were differentiated and more visible in the protein matrix of mungbean than in rice bean cotyledon cells [Fig. 16B(E)]. In rice bean seeds, the collapse of the protein matrix on starch granules may probably be due to partial digestion. Microstructure information for the under-utilized *Vigna* crop, *V. umbellata* is not available in any literature till date. According to Lott (1981), seeds of angiosperms store most of their reserve proteins in storage vacuoles known as protein bodies, which encloses globoid crystals. Seed storage proteins accumulate in sub-cellular organelles

called protein bodies. These are membrane bound spherical organelles, a few microns in diameter, filled with proteins and phytates. Scanning electron microscopic (SEM) study of these protein bodies have been undertaken in many legume species (Mosse and Pernollet, 1982). The membranes of the protein bodies are generally smooth; though in some cases have granular appearance (Barker *et al*., 1976). These reports are in accordance with the present study. Radley (1976) also affirmed that bean starch granules showed a diameter between 30 and 50μm and a rounded or oval shape. According to Lindeboom *et al*. (2004), granule size, size distribution and shapes are amongst the most important morphologically distinguishing factors of starch from different origins. They also cited that granule size influences the physicochemical properties of starch, its composition, gelatinization and pasting properties, enzyme susceptibility, crystallinity, swelling and solubility. Enzyme susceptibility might have played a minute role with regard to starch granules in some resistant samples evaluated during this study.

5.14.2. SEM analysis of South Indian *Callosobruchus maculatus* strain

Scanning electron microscope (SEM) study of *C. maculatus* egg showed a projection on one end and plain surface on its membrane. However, Thakur and Kalpna (2015) reported the presence of hexagonal plates on egg membrane of another bruchid species, *Acanthoscelides macrophthalmus* without any protrusion. This might indicate variation in egg morphology, which may be useful in bruchid species identification. During host preference study between normal and antennae ablated *C. maculatus* females, no significant role of antennae was observed statistically. Based on SEM observations, morphology of *C. maculatus* sensilla were investigated and was found to have three different types of sensilla on labial palpi and antennae. Observation of three types of sensilla on maxillary and labial palpi was also reported by Mbata *et al*. (1997). In classifying sensilla, terminology of Schneider (1964) was applied. Hu *et al*. (2009) suggested possible functions of all the sensilla with supportive studies. According to them, sensilla trichoidea1 (ST1) may have an olfactory function (smell), whereas sensilla trichoidea2 (ST2) probably function as sex pheromone receptors. Sensilla chaetica (SC) in *C. chinensis* and *C. maculatus* is believed to have a dual function of mechanoreception and contact chemoreception. Also, sensilla basiconica1 (SB1) was implicated to have an olfactory function which was verified by the use of electrophysiological recordings Hu *et al*. (2009). In the present SEM study, sensilla trichoidea (ST1), sensilla basiconica (SB) and sensilla chaetica (SC) on *C. maculatus* mouthparts were more abundant and pronounced than their antennal sensilla. This finding is in conformity

with Mbata *et al*. (1997), who reported that a greater number of sensilla were present on female maxillary palps than on male maxillary palps and labial palps of both male and female, which may suggest the involvement of these sensilla in stimulus detection, possibly food source and/or oviposition sites. Limited literature is available on the sensilla types of *C. maculatus*. Fouda *et al*. (2016) reported two sub-types of sensilla basiconica (SB1 & SB2) and one type of sensilla chaetica (SC) in mouthparts of rice storage beetles, *Sitophilus oryzae* and *S. granarius*, which supported the present findings.

Sensilla trichoidea1 (ST1) are described on the antennae of most investigated insects. Hu *et al*. (2009) reported that ST1 is the most abundant sensilla type on the whole antennae of *C. maculatus* which was evident and confirmed in the present investigation. The types of sensilla on the antennae of *C. maculatus* recorded are largely in conformity with those reported for other bruchid and beetle species; *C. chinensis* (Hu *et al*., 2009), *Acanthoscelides macrophthalmus* (Thakur and Kalpna, 2015), *Sitophilus oryzae* and *Sitophilus granarius* (Fouda *et al*., 2016). Thanjavur *C. maculatus* female ovipositor had sensilla trichoidea1 (ST1) surrounding the region which is also supported by Mbata *et al*. (1997), who stated that sensilla found on ovipositor lobes resemble trichoid sensilla on the ovipositor of several insects. Although, these sensilla are hypothesized to function in different roles based on their distributional pattern and observed morphological characteristics, the actual physiological role may be attributed only after studying their ultrastructure and with detailed electrophysiological and behavioral investigations.

5.15. Bulked segregant analysis (BSA)

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as 'signs' or 'flags' (Jones *et al*., 1997). Markers that are located in close proximity to genes (i.e. tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes (like genes) called 'loci' (singular 'locus') (Winter and Kahl, 1995).

Deviation of markers from the expected segregation ratio is a common occurrence that has been observed in inter-specific mapping populations (Paterson *et al*., 1988). Segregation distortion of markers has been noted in previous genetic mapping studies of *Vigna* species such as mungbean, cowpea and azuki bean with the level of distortion ranging from 12 to 30.8%

(Kaga *et al.*, 2004). Paterson *et al.* (1988) attributed abnormal segregations in inter-specific hybridizations as the product of linkage between markers and factor(s) that operate in the pre- and post-zygotic phases. They further suggested that if a gene that causes segregation distortion is segregating in a population, then markers close to it would tend to exhibit distorted ratios as well. All he markers evaluated for polymorphism between parents and bulked (resistant and susceptible) DNAs showed segregation distortion and were skewed towards the susceptible parent (Plate. 23, 24, 25 and 26). Similar phenomenon might have taken place in the segregating RIL populations of VRM(Gg)1 and TNAU Red analysed in the present study.

SSR markers screened for polymorphism between parent DNA and extreme bulked groups showed a skewed segregation distortion towards the susceptible parent. Fernandez and Talekar (2005) reported that the inheritance of bruchid resistance is controlled by the genotype of the maternal parent and appeared to be controlled by recessive genes which complicate the selection and breeding for bruchid resistance in pulse crops.

5.16. Marker-trait association analysis

5.16.1. Genomic regions associated with bruchid resistance

Concerted efforts have been made in mapping a *Br* gene in TC1966 (bruchid resistant wild mungbean) and other sources derived from TC1966. First, a single *Br* gene, flanked by RFLP marker, was proposed (Young *et al.*, 1992). Later, it was reported to be linked with RAPD and SSR markers on linkage group 8 (Lambrides *et al.*, 2000; Miyagi *et al.*, 2004). Another *Br1* gene was reported on chromosome 5 (previously known as LG 9), linked with SSR markers (Wang *et al.*, 2016). Nevertheless, the nature of *Br* gene(s) in TC1966 remained unclear and inconclusive. Many QTLs were also identified near this *Br* gene using different bi-parental mapping populations (Young *et al.*, 1992; Kaga and Ishimoto, 1998; Somta *et al.*, 2006b; Somta *et al.*, 2008b; Chotechung *et al.*, 2016). However, the number and effect of those QTLs associated with bruchid traits showed great variations, presumably due to poor resolution in detecting QTL with traditional linkage mapping. In addition, QTL analysis in such mapping populations require development of a high-density genetic map and validation of markers, and are limited in scope to SNPs segregating in parents (Henning *et al.*2015).

Alternatively, several methods are available other than this classical mapping, which includes selective genotyping, association mapping and nested association mapping. Among

these, association mapping offers high resolution with either prior information on candidate genes or a genome scan with very high marker coverage (Boopathi, 2013); searches for functional variation in a much broader context and offers three advantages, *viz.* strong statistical power, greater allele number, and reduced research time (Yu and Buckler, 2006). Association studies are also important in assessing correlations between specific genetic variants and trait differences on a population level (Risch, 2000). Marker-trait association study was recommended due to poor resolution in constructing linkage map and identifying QTLs for bruchid resistance in rice bean (Pavithravani *et al.*, 2015). With this background, association mapping was carried out in 108 RILs derived from the cross of VRM (Gg) 1 (*V. radiata*, highly bruchid susceptible) and TNAU Red (*V. umbellata*, completely bruchid resistant), using a set of 1,310 SNP markers and five bruchid resistance related traits (adult emergence, seed damage, adult survival, mean developmental period and index of suitability), to identify genomic regions associated with bruchid resistance.

In this study, general linear model (GLM; TASSEL 4.0 software) was used to determine significant marker-trait associations and it was found that 17 SNPs on chromosome 2, 5, 6 and 8 showed strong and highly significant associations at probability level of $p \leq 1 \times 10^{-3}$ (Table. 61). Henning *et al.* (2015) suggested *p*-values ($p \leq 10^{-3}$) for strong and significant associations between marker and phenotype without sacrificing conventional estimates of threshold significance.

A recent study by Chotechung *et al.* (2016) reported a possible candidate gene for bruchid resistance around position 5,598,000 bp of chromosome 5 (DMB_SSR-158 marker linked to *Br* gene), which codes for a polygalacturonase-inhibiting protein (PGIP) useful in plant defense against many diseases. This SSR marker was reported to co-segregate perfectly with the *Br* locus and they delimited this locus to a genomic region of 38 Kb on chromosome 5. One highly significant SNP (QTL) was also mapped to chromosome 5 at position 5,178,332 to 5,179,402 bp, reported to be associated with bruchid resistance in mungbean (Schafleitner *et al.*, 2016). In both the reports, the mapping populations used were derived from crosses between the resistant mungbean (TC1966, wildtype) and cultivated mungbean accessions (intra-specific), which explains the detection of nearby genomic regions, around 5.17 to 5.59 Mb.

In the present study, highly significant and more SNPs were identified on chromosome 5 and 8 compared to chromosome 2 and 6. However, none of the SNPs was close to the already

reported *Br* locus on chromosome 5. Given that mapping populations used in this study is an inter-specific cross between mungbean and rice bean, chromosomal rearrangement due to recombination events and their divergence at marker level may attribute to why nearby *Br* regions were not detected. The extent of genomic similarity between two species determines the extent to which markers can be transferred from one species to the other (Pavithravani *et al.*, 2015). This further elucidated the identification of SNPs at position 18,756,676 bp on chromosome 5 [Table. 61; Fig.16(b)], which is approximately 13.1 Mb apart from the QTL reported by Schafleitner *et al.* (2016), which is at position 5,178,332 to 5,179,402 bp on chromosome 5.

5.16.2. Possible candidate genes for bruchid resistance

In association mapping, alleles at a few selected candidate genes may be tested for association with a phenotype, or the whole genome may be scanned to identify regions that are associated with a particular phenotype (Remington *et al.*, 2001). Since none of the identified SNPs co-localized with the previously reported QTLs in mungbean, candidate genes were annotated from the mungbean genome database (*http://plantgenomics.snu.ac.kr*).

No genes containing the associated SNPs (C2_SNP-15 and C2_SNP-15) were located on chromosome 2. But, a gene (*Vradi02g02990*) at the nearby region encodes a squalene synthase enzyme, which is involved in terpenoid synthesis. Plant-derived terpenoids act as deterrent towards insects. Terpenoids also exhibit direct mode of defense mechanism through accumulation of phytochemicals that have antibiotic activities against insects, whereas indirect mode includes defense against herbivores by enhancing the effectiveness of their natural enemies (Singh and Sharma, 2015) (Table.62).

On chromosome 5, a gene (*Vradi05g14370*) encodes a ribosome biogenesis protein (pre-rRNA protein family), which contains two significant SNPs (C5_SNP-89 and C5_SNP-90). Recent studies in other plant species have demonstrated that this protein has an important role in the synthesis of defense related proteins (Chen *et al.*, 2007) and provides defense at the cellular level. It is also reported to associate with MAPK (Mitogen-activated protein kinases) signaling and play a critical role in regulating the herbivore-induced dynamics of phytohormones. Furthermore, they are required for transcriptional activation of herbivore defense-related genes and accumulation of defensive metabolites, detrimental to insects (Hettenhausen *et al.*, 2015). Another significant SNP (C5_SNP-109), on chromosome 5, is in a gene (*Vradi05g17250*)

encoding biotic stress response protein, a phosphate kinase. This protein has been reported to regulate indirect responses to different types of biotic stress, including insect and pathogen attacks (Bajsa *et al.*, 2011) and play a very important role in signal transduction of jasmonic acid, the hormone which regulates defense genes against herbivores.

A gene, *Vradi05g10400* on chromosome 5 containing three associated SNPs (C5_SNP-60, C5_SNP-60 and C5_SNP-62), encodes an uncharacterized protein with unknown functions. Further studies are required to characterize this protein since more SNPs are found in this gene. However, a gene (*Vradi05g10410*) adjacent to the 3 significant SNPs mentioned above, on chromosome 5, encodes a cellulose synthase enzyme, which functions as defense-related proteins. These proteins regulate the synthesis of three major components of secondary cell wall (lignin, cellulose and hemicellulose) in plants with fortified cell wall, which offers resistance to insect attack.

A protein with pepsin-like aspartic protease encoded by a gene (*Vradi05g17240*) is also located nearby another significant SNP (C5_SNP-109) on chromosome 5. Aspartic protease in plants is involved in storage protein degradation, stress responses, programmed cell death, and antimicrobial defenses (Darabi and Seddigh, 2015). It is reported to display dose-dependent antimicrobial activity and induced response to infection by the fungus, *Phytophthora infestans* (Guevara *et al.,* 2004). Plants have inhibitors for all four classes of proteinases, which can delay larval development without directly causing mortality. They are supposed to inhibit the proteolytic activity of midgut enzymes and thereby decrease the availability of amino acids. This in turn leads to reduction in amino acid synthesis needed for growth, development and reproduction in insects (Wolfson and Murdock, 1995). These inhibitors are often found in places where insect attack is most likely to occur, i.e. in seeds, bulbs, leaves, etc. For aspartic protease from leaves, a role in defense responses of potato plants against pathogens or insects has been suggested (Guevara *et al.,* 2004). This finding may particularly be noted since similar enzymes such as trypsin and chymotrypsin inhibitors belonging to this protein family (proteinase inhibitors) were found in higher concentration in resistant rice bean seeds (Srinivasan and Durairaj (2007) and other pulses (Ignacimuthu *et al.*, 2000), which are reported to confer resistance against bruchid insects.

On chromosome 6, a gene (*Vradi06g04670*) contains two significant SNPs (C6_SNP-38 and C6_SNP-39) associated with MDP, which codes for another uncharacterized protein. One

more gene (*Vradi06g04680*) was found near these two SNPs and another significant SNP (C6_SNP-40), which encodes a glycosyl transferase protein. This protein is reported to be involved in the biosynthesis of phenolics in plants and is responsible for providing defense mechanism against insect attack (xxxxxx).

In addition, genes involved in different phytohormone signaling pathways were also identified. On chromosome 8, a gene (*Vradi08g01680*) containing two significant SNPs (C8_SNP-13 and C8_SNP-14) encodes a zinc finger protein domain. Recent work has highlighted this protein domain to be associated with ubiquitin-proteasome system (UPS) (containing stress-associated proteins) and its enzyme, ubiquitin ligases, which functions as regulators of plant defense response. Also, it is reported that these proteins play an important role in biotic stress resistance through rapid induction of salicylic acid, jasmonic acid and ethylene signaling (Takai *et al.*, 2002).

Another gene (*Vradi08g12980*), which contains a significant SNP (C8_SNP-94) on chromosome 8, encodes an ethylene responsive protein kinase. It is reported when jasmonic acid (JA) pathway is induced in response to wounding and tissue-damage from insect feeding, ethylene (ET) is released after the attack. This leads to increased production of volatiles and secondary metabolites, which are toxic to insects. Hence, ethylene often regulates defense signaling pathways, including those by salicylic acid (SA). Moreover, hormonal signal integration with ethylene steer the defense signaling network to activate specific defenses that can have direct effects on the attackers (Broekgaarden *et al.*, 2015). This same gene (*Vradi08g12980*) also encodes a serine/threonine kinase protein. It is well known that kinase proteins play a vital role in plant hormone signaling during pathogen recognition, herbivore wound-mediated defense response and maintenance of plant cell wall integrity (Afzal *et al.*, 2008).

A gene (*Vradi080g13050*) encoding a retrovirus-related pol polyprotein was also located on chromosome 8, which contains one significant SNP (C8_SNP-96). No previous evidence about the involvement of this particular protein family in imparting resistance to insects was found. However, it is predicted that these are one of the defense-related protein families, which are involved in biosynthesis of defensive proteins when attacked by micro-organisms (Chen *et al.*, 2007). Khan *et al.* (2003) reported that defense-related proteins may play a role in maintaining bruchid resistance in resistant mungbean.

It is interesting to note that a gene (*Vradi08g19320*) nearby two significant SNP (C8_SNP-140 and C8_SNP-140) on chromosome 8, encodes a naringenin-chalcone synthase, a defense-related enzyme, which is involved in biosynthesis and pathway induction of the flavonoid naringenin (Mierziak *et al.*, 2014). Naringenin is also reported to have growth inhibitory functions towards insects (Goławska *et al.*, 2014) and fungal pathogens (Mierziak *et al.*, 2014). Earlier, Kashiwaba and Tomooka (2002) (US Patent 6,770,630B2) postulated that the biochemicals responsible for bruchid resistance (*C. maculatus* and *C. chinensis*) in "Menaga" (Japanese *V. umbellata* cv.) were derivatives of the flavonoid naringenin and quercetin, which were isolated and identified from seed cotyledon. The SNP adjacent to this gene is associated with three bruchid traits, *viz*., adult emergence, seed damage and index of suitability.

Identification of genes containing or physically close to the significant SNPs and the consistent association of these SNPs with adult emergence and seed damage, on chromosome 5 and 8, gives a plausible explanation for involvement of diverse genes present on these two chromosomes, which may be responsible for conferring bruchid (*C. maculatus*) resistance in rice bean. Therefore, these genes may be proposed as potential candidate genes for bruchid resistance, which is also supported by their involvement in defense-related mechanisms, signal transduction during wound response (role of secondary metabolites) and presence of enzyme inhibitors (anti-nutritional factors in seeds).

Also, the present results suggest that bruchid resistance in rice bean is primarily due to polygenic background and contradicts the previous reports of monogenic control in mungbean (Dongre *et al.*, 1996; Somta *et al.*, 2007) and that it is controlled by a single *Br* gene (Kitamura *et al.*, 1988; Fujii *et al.*, 1989; Somta *et al.*, 2007). Polygenic control of resistance in rice bean was also reported by Pavithravani *et al.* (2015). To date, no SNPs or QTLs have been reported in bi-parental mapping populations of mungbean and rice bean in genomic regions where the present associated SNPs are located. Therefore, apart from the earlier reported linked markers, association mapping carried out in the present 108 RILs uncovered additional genomic regions on chromosome 5 and 8, associated with bruchid resistance in rice bean and RIL populations.

In summary, the possible candidate genes associated with bruchid resistance were identified. However, the current study does not have functional evidence to support the resistance conferred by the gene. And so, the function of these resistant genes needs to be validated through functional genomics approaches, such as VIGS or genetic transformation in

future. The pinpointing or nearby SNPs in gene can be assayed in some cost effective marker platform such as CAPS (cleaved amplified polymorphic sequences) or KASP Assay for use in the marker assisted selection (MAS) in breeding programs for resistance to bruchids. Molecular breeding using such genes will help in the development of superior lines with enhanced resistance to bruchid that will eventually enhance crop production and protection of mungbean.

Summary and Conclusions

CHAPTER V
SUMMARY AND CONCLUSIONS

Results of the various experiments conducted on phenotyping of bruchid resistance and mapping in inter-specific populations of *Vigna radiata* and *Vigna umbellata* are summarized hereunder.

- Damage potential evaluated for *Callosobruchus maculatus* and *C. chinensis* indicated *C. maculatus* as the dominant and more destructive species since it caused significantly higher seed damage of 91.33 ± 3.06 % than caused by *C. chinensis* (70.67 ± 3.06 %) on susceptible mungbean and was superior in all developmental aspects, which corroborates their dominant status in Southern India, especially Tamil Nadu.

- *Callosobruchus maculatus* female fecundity was highest (113.6 eggs/50mungbeanseeds) at 30°C (controlled) in Thanjavur (T) population, whereas lowest oviposition (29.6 eggs/50 black gram seeds) was observed for Coimbatore (C) population under uncontrolled (ambient or laboratory) conditions. T-population registered highest seed damage (91.2 % on mungbean) compared to 72.4 % on black gram under controlled (favourable) conditions. Based on these results, Thanjavur *C. maculatus* population was selected for resistance evaluation and resistance mechanism studies. With regard to mass-culturing and long-term maintenance of bruchid generations, mungbean was found to be a more suitable host for *C. maculatus* than black gram. Individual effects of the three factors *viz.* insect population source, rearing conditions and rearing host, studied on some bruchid parameters were more profound than their interaction effects.

- Fecundity, survival and development studies of *C. maculatus* (Thanjavur population) for 25 generations showed that female oviposition potential reached to a peak within 2 days after its commencement and then declined with time. Longevity study indicated that *C. maculatus* females have shorter lifespan than males. Sex ratio (emergence) was in favour of females in all the subsequent generations. Maximum adult emergence (mean of 8.60 bruchid adults out of 10) was observed on second day from their first emergence per generation studied.

- New sources of bruchid resistance were identified in Manipur rice bean landraces, which includes LR(M)-3, LR(M)-4, and TNAU Red procured from ARS, Virinjipuram. However, mungbean varieties collected from different research stations in Tamil Nadu showed high to

moderate degree of susceptibility towards *C. maculatus* infestation, thus indicating that bruchid resistance source(s) are limited in mungbean germplasm collection.

- Bruchid infestation had negative influence on mungbean seed qualities as evident from high seed weight loss, reduced seed protein content and reduction in germination percentage.

- Regression and correlation studies revealed that quantitative seed characters such as seed size, hardness and seed coat thickness did not play any significant (p>0.05) role in conferring resistance to bruchids in rice bean and mungbean. Although *C. maculatus* is a polyphagous insect of legumes, mostly in the *Vigna* spp., its capacity for development in rice bean seeds was generally restricted, mainly due to resistance factor(s) present in the seed.

- VRM(Gg)1 (bruchid susceptible parent) received 67.33 eggs/50 seeds whereas 189.00 eggs/50 seeds was observed in TNAU Red (bruchid resistant parent). VRM(Gg)1 and TNAU Red showed 94.00% and 0% seed damage, confirming high susceptibility and complete resistance reaction to bruchid.

- 187 inter-specific populations (RILs) of VRM(Gg)1 and TNAU Red were subjected to *C. maculatus* infestation under free-choice test in the newly designed "Innovative model" for free-choice insect resistance screening. From this result, nearly half (47.62%) of the total 187 RILs were considered susceptible (>50% damage) in first screening and thus the rest of the total RILs (about 52.38 % of 187 RILs with ≤ 50% seed damage) were subjected to "no-choice" test thus optimizing seed usage and time.

- Results of free and no-choice tests showed varied differences in the reaction of RILs to *C. maculatus* (Thanjavur populations) and suggested different degree of resistance or susceptibility. Among 187 RILs, RIL 158, 165 and 169 showed consistently high and moderate levels of resistance when artificially infested with *C. maculatus* in both the tests.

- Seed damage percentage was considered as the most reliable parameter for bruchid resistance determination, other than the widely used index of suitability (IS). Calculation of IS is dependent on survival and mean developmental period (MDP), where MDP considers only 50 per cent of adult emergence. On the other hand, damage percentage considers complete bruchid emergence at the end of the experiment, from all seed samples evaluated, which is accurate, reliable and provide effective results.

- Results on antixenotic mechanism (relationship studies) on rice bean landraces, mungbean varieties and RIL seeds showed that although oviposition responses varied on these different

samples, seed qualitative traits such as seed colour, seed lustre, seed shape, seed texture and quantitative characters such as seed size (100 seed weight), seed coat thickness and seed hardness were not correlated with bruchid parameters and hence, did not influence resistance or susceptibility towards *C. maculatus*

- The three lines (RIL 158, RIL 165 and RIL 169) which showed resistance also exhibited antibiosis mechanism when infested with *C. maculatus*. Presence of antibiosis factor(s) were confirmed from the results of several experiments conducted such as reduction in adult size, decreased longevity and reduced or no fecundity of adults emerged from resistant RILs compared to normal bruchid biological parameters on susceptible host. Results also suggest that antibiosis factor(s), present in the resistant seed cotyledons, may alone be responsible for such bruchid behaviour.

- *C. maculatus* females preferred seeds with intact seed coat for oviposition than decorticated seeds. From the role of seed coat study in resistance evaluation, it may be revealed that seed coat occasionally acted as a physical barrier for *C. maculatus* emergence since few resistant RIL seeds registered bruchid emergence, mostly in larval form. There was an evidence of seed coat being partly responsible for *C. maculatus* emergence in resistant RILs.

- From antennal ablation study, it was observed that antennae did not play any significant role in *C. maculatus* oviposition preference since their removal did not cause any loss in sensitivity and did not show significant (p>0.05) difference from oviposition preference studied using bruchid adults with antennae.

- Studies on the effectiveness of indigenous plant and seed extracts revealed high efficacy of notchi, cashew @ 10 % concentration and TNAU Red (rice bean) cotyledon extracts on bruchid bionomics as evidenced from high adult mortality, maximum percentage of oviposition deterrence (POD) and highest per cent reduction in adult emergence (PRA).

- Rice bean landraces recorded comparatively lower protein content ranging from 14.62 % [LR(M)3] to 16.72 % [LR(M)1 and LR(M)6] compared to mungbean varieties and RILs. Highest protein content (25.83 %) was recorded in the susceptible RIL 116 followed by susceptible RIL 60 (24.42 %). Relationship analysis performed between protein content (%) and bruchid biological characteristics on susceptible and resistant samples revealed non-significant correlations, which suggest that protein content in seeds, had no influence on susceptibility or resistance to *C. maculatus*.

- GC-MS results from the present study provided preliminary referential information of the two pulses used and also serve as an investigative tool for appropriate identification of compounds present in the seeds. Identification of various chemical constituents suggests that both mungbean and rice bean exhibit rich phytopharmaceutical importance. Detection and presence of phenols in cotyledon extracts of resistant rice bean landraces [LR(M)3 and LR(M)4] and RIL 158 may indicate its secondary role in imparting resistance towards bruchid (*C. maculatus*).

- SEM examination of cotyledon cells of mungbean, rice bean and some of their inter-specific lines illustrated spherical starch granules, with varying sizes ranging from 7.73 μm (dia.), 15.49 μm (length) in VRM(Gg)1 to 29.75 μm (dia.), 45.97 μm (length) in TNAU Red and embedded in a protein matrix. Protein bodies were more differentiated and visible in protein matrix of mungbean than in rice bean cotyledon cells. Crude protein estimation was carried out for both the resistant and susceptible RILs, which showed varied results. No significant correlation was observed between bruchid resistance parameters (seed damage and MDP) and the size of starch granules in resistant and susceptible samples.

- SEM study on South Indian *C. maculatus* strain revealed the presence of different types of sensilla such as sensilla trichoidea (ST), sensilla basiconica (SB) and sensilla chaetica (SC) on mouthparts and antennal regions, which have different functions like mechnoreception, chemoreception. However, these sensilla were more abundant and pronounced in bruchid mouthparts than their antennae. This study may give supportive elucidation as to why no significant variations were recorded during ovipositional preference studies conducted with antennae ablated (without antennae) and normal (with antennae) bruchids.

- Bulked segregant analysis (BSA) carried out with 42 polymorphic SSRs (belonging to azuki bean genome), to identify markers linked with bruchid resistance, showed skewed segregation distortion towards the susceptible parent, VRM(Gg)1. However, polymorphism was detected at different base pair lengths in resistant parent as well as resistant bulks when surveyed with one each of ISSR (UBC810) and DAMD (M-13) primers and two RAPD primers (OPB08 and OPX04).

- Marker-trait association analysis carried out for identification of SNPs associated with bruchid resistance in selected 108 RIL populations of VRM(Gg)1 x TNAU Red, using a set of high quality 1310 SNPs, revealed 17 SNPs strongly and significantly associated ($p \leq 10^{-3}$) with 5

bruchid resistance related traits (mainly adult emergence and seed damage). Among these, 7 SNPs were significantly associated with adult emergence and seed damage, 2 SNPs with adult survival per cent, 5 SNPs with mean developmental period, and 3 SNPs with index of suitability, on chromosome 2, 5, 6 and 8.

- Seven genes harbouring 12 significant SNPs containing or physically close to these associated SNPs are proposed as candidate genes for bruchid resistance, which is supported by the identification of candidate genes mostly involved in the signaling pathways and defense-related protein synthesis. However, three genes in particular *viz. Vradi05g17240, Vradi06g04680* and *Vradi08g19320* (nearby associated SNPs) may be taken into account owing to their encoding enzymes with antibiotic / antinutritive effect against insects (proteinase inhibitory enzymes), phenol biosynthesis (glycosyltransferase) and naringenin biosynthesis-pathway induction (naringenin-chalcone synthase), which has been earlier reported to be responsible for conferring bruchid resistance in rice bean.

- In conclusion, LR(M)-3, LR(M)-4 and TNAU Red among the rice bean landraces and RILs 158, 165 and 169 among the inter-specific mapping populations were identified as bruchid resistant and could act as resistant parents (donors) in development of bruchid resistant mungbeanlines/varieties. Given the adequate genotypic variation among rice bean landraces and RILs on their resistance to *C. maculatus*, development of durable resistant varieties *via* conventional breeding and advanced breeding techniques may be recommended. Considering the immense bioactive potentials of *Vigna umbellata* seeds from GC-MS results, attempts can be made to scientifically validate and chemically standardize insecticidal formulations for insect pest management strategy. Proximate composition analysis and compound identification of the effective plant extracts should be carried out in order to unravel the active ingredient(s) responsible for the toxic effects on insect. SEM studies on microstructure of seed coat surfaces and cotyledons will be useful to study cultivars, storage conditions, maturity and relationships between structural and functional properties. Further studies on functional morphology of sensilla using transmission electron microscopy (TEM) coupled with electrophysiological recordings will confirm the functions of different sensilla observed in this study.

The potential of association mapping was successfully applied in this study to identify SNP markers (genomic regions) associated with five bruchid traits, commonly used in relation to bruchid resistance evaluation. From this study, possible candidate genes associated with bruchid

resistance were identified. However, the current study does not have functional evidence to support the resistance conferred by the gene. And hence, the function of these resistant candidate genes needs to be validated through functional genomics approaches, such as virus-induced gene silencing (VIGS) or genetic transformation in future. The pinpointing or nearby SNPs in gene can be assayed in some cost effective marker platform such as CAPS (cleaved amplified polymorphic sequences) or Kompetitive Allele Specific PCR (KASP) Assay for use in the marker assisted selection (MAS) in breeding programs for resistance to bruchids. Molecular breeding using such genes will help in the development of superior lines with enhanced resistance to bruchid that will eventually enhance mungbean productivity.