Pea weevil (*Bruchus pisorum* L.) Resistance and Genetic Diversity in Field Pea (*Pisum sativum* L.)

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Cover: *Top*_from left to right: A pea weevil on a young green pod; neoplasm formed on a green pod; and frequency and distribution of rare and private EST-SSR alleles in field peas. *Bottom*_from left to the right: two main flower types of field pea varieties, scanning electron micrograph of neoplasm on field pea pod; and Rogers genetic distance-based clustering pattern of field pea accessions from Ethiopia.
(Photo: Abel Teshome, Kerstin Brismar)

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Abstract

Field pea (*Pisum sativum* L.) is an important source of protein in developing countries like Ethiopia. However, the production of field pea is hampered by invasive pests like the pea weevil (*Bruchus pisorum* L.). In this PhD project, more than a quarter of the field pea accessions available at the Ethiopian Biodiversity Institute (EBI) have been screened for resistance against pea weevils, both under field and greenhouse conditions. The screenings led to the identification of a few relatively less susceptible accessions/genotypes. Repeated field and greenhouse trials revealed that locally released, high yielding varieties are highly susceptible to pea weevil infestation. The lack of strict quarantine systems in Ethiopia to prevent the spread of infested seeds is worsening the pest distribution within the country. Hence, there is an urgent need to develop locally adapted pea weevil resistance varieties. Developing such varieties is the most effective and cheapest approach which can be supported by other integrated pest management techniques like intercropping to minimize or possibly halt economic loss due to this pest.

In the present study, we have also developed 15 new EST-SSR markers that could be used for various applications including genetic diversity assessments and genetic mapping as well as for marker assisted selection in *P. sativum*. Most of these markers are transferable to other taxa of the genus *Pisum* and related genera. Twelve of these newly developed EST-SSR markers were used to assay the genetic diversity among 46 accessions of *P. sativum*. This study revealed high genetic differentiation among the accessions which could be valuable for field pea breeding in the future. In addition, the findings could be used as an input for *in-situ* and *ex-situ* conservation strategies of the *P. sativum* and guide future collection missions.

Keywords: Bruchus pisorum, EST-SSR, field pea, genetic diversity, intercropping, *Pisum sativum*

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Dedication

To my family

Be yourself; the world worships the original.
Ingrid Bergman

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Teshome, A., Mendesil, E., Geleta, M., Andargie, D., Anderson, P., Rämert, B., Seyoum, E., Hillbur, Y., Dagne, K. and Bryngelsson, T. (2015b). Screening the primary gene pool of field pea (*Pisum sativum* L.) in Ethiopia for resistance against pea weevil (*Bruchus pisorum* L.). Genetic Resources and Crop Evolution 62:525-538.

II Teshome, A., Mendesil, E., Bryngelsson, T. and Geleta, M. Enhancing neoplasm expression in field pea (*Pisum sativum* L.) via intercropping and its significance in pea weevil (*Bruchus pisorum* L.) management (submitted).

III Teshome, A., Bryngelsson, T. Dagne, K. and Geleta, M. (2015a). Assessment of genetic diversity in Ethiopian field pea (*Pisum sativum* L.) accessions with newly developed EST-SSR markers. BMC Genetics 16:102 DOI 10.1186/s12863-015-0261-5.

Paper I is reproduced with kind permission of the publisher.

The contribution of Abel Teshome to the papers included in this thesis was as follows:

I Planned field and greenhouse experiments, analysed data and wrote the manuscript with input from co-authors

II Planned greenhouse experiments, analysed data and wrote the manuscript with inputs from co-authors

III Planned the experiment, ran the laboratory analyses, carried out the data analysis and wrote the manuscript with inputs from co-authors

Abbreviations

CSA	Central Statistics Agency
CTAB	Cetyl Trimethyl Ammonium Bromide
EBI	Ethiopian Biodiversity Institute
EST	Expressed Sequence Tags
HPR	Host Plant Resistance
ICARDA	International Centre for Agricultural Research in the Dry Areas
IPM	Integrated Pest Management
FAO	Food and Agriculture Organization of United Nations
NCBI	National Centre for Biotechnology Information
PSD	Percent Seed Damage
Sida	Swedish International Development Agency
SSR	Simple Sequence Repeats

1 General Introduction

Field pea (*Pisum sativum* L.) is amongst the prominent ancient crops which contributed to the culmination of the hunting and gathering era of mankind and the upsurge of civilization (Martin-Sanz *et al.*, 2011; Upadhyaya *et al.*, 2011; McPhee, 2003; Swain, 1979). The ability of this crop to adapt to various environmental conditions worldwide made it successful (Smartt, 1990). The spread of field pea is also attributed to reduced toxicity levels obtained during domestication which made it safe both as a food and forage crop (Harlan, 1992; Liener, 1982). Field pea cultivation in recent times has been favoured by the ability of the crop to fix atmospheric nitrogen which is both an economic and an ecological advantage (Smýkal *et al.*, 2012; Stenvovic *et al.*, 2005; Hardie, 1992). Currently, field pea is a major protein source for humans in the developing world and a key fodder crop in developed countries (Kapila *et al.*, 2012; Fraser *et al.*, 2004; Khan & Croser, 2004).

The main field pea producing countries include Canada, Russia, USA, India and France. Ethiopia holds the number one spot in Africa and the sixth spot in the world in field pea production (FAOSTAT, 2015). Field pea is traditionally an important staple food in Ethiopia and has recently become an export commodity. Despite the recent rise in field pea production in the country due to more acreage devoted to this crop (FAOSTAT, 2015), the progress is hindered by invasive pest species like the pea weevil (*Bruchus pisorum* L.) (Teshome *et al.*, 2015b; Seyoum *et al.*, 2010).

In recent years, the pea weevil has been the major setback in field pea production, especially in the northern and central part of Ethiopia (Teshome *et al.*, 2015b; Seyoum *et al.*, 2010; Tesfaye *et al.*, 2002). This pest may cause up to 60% reduction in yield annually (Teka, 2002; Assayehegne, 2002). Pea weevils begin their attack at the field and continue to do so during storage. Two to three months after harvest, infested seeds are of no use either for human consumption or as animal feed (McDonald, 1995). As a result, local landraces are becoming less profitable for subsistence farmers as they are forced to spend more resources on insecticides and/or after harvest treatments like fumigation. Currently, the most effective approach worldwide for pea weevil management is the use of chemical insecticides (Seidenglanz *et al.*, 2011; Horne & Bailey, 1991). However, such an approach is too expensive in small scale farming systems and environmentally unfriendly (Byrne *et al.*, 2008). In addition to the aforementioned pest problem, soil degradation and unpredictable climatic conditions are further downgrading the success of local landraces.

To circumvent further economic losses due to this pest and other biotic and abiotic factors, screening and characterization of the available genepool is a primary step. Such characterization can lead to the identification and development of resilient varieties armed with genes of agronomic interests. There are more than 2000 accessions of *Pisum* species at the Ethiopian Biodiversity Institute (EBI) which could contribute to this cause. Screening these accessions for possible host plant resistance (HPR) against pea weevil is a way forward to develop resistant varieties that can be used in tandem with other pest management techniques. In parallel, quantifying the genetic diversity of these collections could contribute to breeding programs via development of molecular markers associated with traits of interest.

2 Literature review

2.1 *Pisum* biosystematics

The Fabaceae (Leguminosae) family commonly, known as the pea or bean family, consists of 700 genera and more than 20,000 species (Upadhyaya *et al.*, 2011). This family is the third largest family among flowering plants with a distinct fruit architecture from which the family name arises from (Upadhyaya *et al.*, 2011; Doyle & Luckow, 2003). It consists of herbs, shrubs trees and aquatic species that are distributed in all parts of the world (Kosterin & Bogdanova, 2015; Doyle & Luckow, 2003). With 41 domesticated species, Fabaceae is the hub for most domesticated crops in comparison to all other families (Harlan, 1992). The Fabaceae family consists of three sub-families: Mimosoideae, Caesalpinioideae and Faboideae (Papilionoideae). The subfamily Faboideae comprises most of the important edible legumes like broad beans (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L.), pea nuts (*Arachis hypogaea* L.) and soybean (*Glycine max* L.) (Smartt, 1980).

The genus *Pisum* falls within the Faboideae sub-family and tribe Viceae. There are several taxonomic levels and nomenclatures given to the species in the genus *Pisum*. For example, Linnaeus (1753) classified the genus into four species: *P. sativum* (garden pea), *P. arvense* (field pea), *P. ochrus* and *P. maritinum*. On the other hand, Boissier (1872) only recognized a single species of *Pisum i.e. P. sativum* L. with three wild relatives (*P. elatius* Beib., *P. humile* Boiss. and Noe and *P. fulvum* Sibth and Sm.). Davis (1970) proposed only two species in this genus, *P. sativum* and *P. fulvum* and placed the other at the subspecies level under *P. sativum*. Despite the ongoing debate on the taxonomic status of the species in this genus, *P. sativum*, *P. fulvum* and *P. abyssinicum* are the three major species recognized in recent times (Kosterin & Bogdanova, 2015; Maxted & Ambrose, 2001). Nonetheless, all *Pisum* taxa are diploid with 2n=14 chromosomes (Ben-Ze'ev & Zohary, 1973).

2.1.1 Domestication of *P. sativum* L.

Domestication that involves intentional or unintentional selection has resulted in modification of traits like yield, seed size, seed dormancy, maturation and other traits that mankind needs (Weeden, 2007). The achievement of plant domestication and breeding has benefited some crops to remain valuable for thousands of generations. The earliest archaeological evidences on cultivated pea are as old as 8000 BC from the Fertile Crescent (Messiaen *et al.*, 2006). Between the year 7000 and 6000 BC, pea appeared in the Nilotic farmlands but there is no conclusive evidence that pea was cultivated by then (Ben-Ze'ev & Zohary, 1973). According to Oelke *et al.* (2003), *P. sativum* is the earliest species to be embraced as a crop, though there are other theories which dispute this claim. Nevertheless, cultivation of pea is well documented in the writings of Greek and Roman literature which shows the importance of this crop during ancient times (De Candolle, 2011).

Despite the fact that the progenitors of *P. sativum* so far are unknown, Ethiopia, Western and Central Asia and the Mediterranean region are proposed as possible centres of origin because of the high pea genetic diversity sampled in these regions (Messiaen *et al.*, 2006; Muehlbauer & Tullu, 1997). For centuries, pea has been an important legume crop in the Mediterranean region as an annual crop (Smartt, 1984). This crop might have reached North America after the visit of Christopher Columbus (Muehlbauer & Tullu, 1997). Pea spread to China in the first century AD and by the Middle Ages, pea were grown in UK but no specific variety was in cultivation until the 16th century (Davies, 1995).

2.1.2 Botanical description of *P. sativum*

P. sativum is a herbaceous legume that is either climbing or bushy type which can grow from 0.6 to 1.2 m (Oelke *et al.*, 2003). It has self-pollinating hermaphrodite flowers with less than 5% cross pollination (Lazaro & Aguinagalde, 2006). In most varieties, the flowers are either reddish-purple or white (Fig. 1) but there are also cultivars with pink, lavender and blue flowers (Muehlbauer & Tullu, 1997). The pod length can vary between 2 to 6 cm (Hardie & Clement, 2001) and each pod can have one to nine seeds (Oelke *et al.*, 2003). The seed coat can have different colours, *e.g.* green, brown, variegated green, variegated brown, creamy and rarely violet colours (Teshome *et al.*, 2015b).

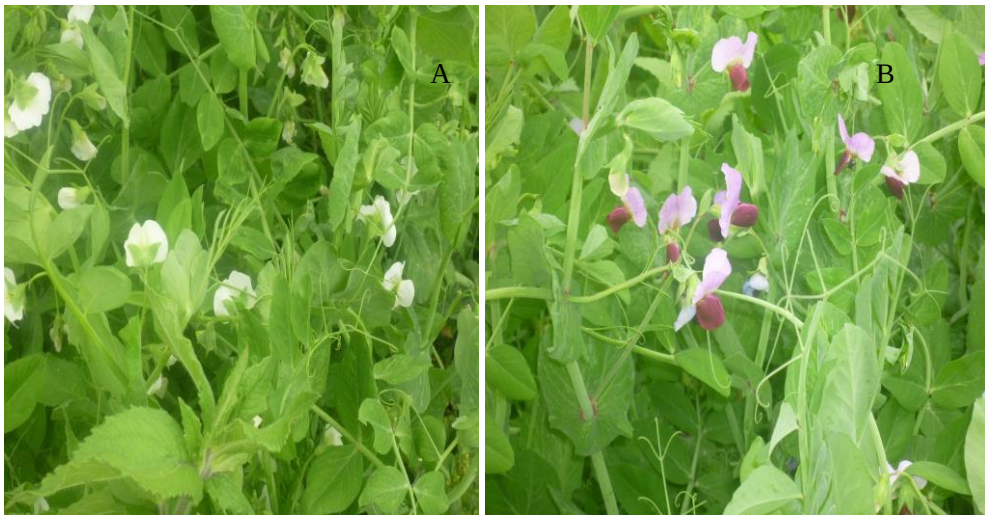

Figure 1. Field pea varieties with white (A) and purple flowers (B).

2.1.3 Use of field pea and its nutritional quality

As a result of the processes of domestication, there are now two major types of field pea; green pea for vegetable use and dry pea for feed and fodder (Santalla *et al.*, 2001). Field pea is known to have essential amino acids such as lysine and tryptophan that are scarce in cereals (Schatz & Endres, 2009; McPhee, 2003). The nutrient content of dry pea seeds can vary from variety to variety but in general, they contain 18-30% protein, 35-50% starch and 4-7% crude fibre (McPhee, 2003). Because of the low level of trypsin activity, the amino acids composition and the high digestibility, dry peas are suitable as fodder for meat production (Schatz & Endres, 2009; Hickling, 2003). Field pea is also an excellent source of protein in developing countries where dairy products are scarce or limited.

2.1.4 Field pea production in the world and in Ethiopia

According to Schatz and Enders (2009), about 25 million acres of land is covered by field pea every year. Canada, USA, Europe and Australia are the major producers of peas and China and India take the lions share in Asia (Fig. 2) (FAOSTAT, 2015). Field pea production in Ethiopia has shown steady increase during the last decade (Fig. 3). Unfortunately the increase in production is not due to increase in yield/hectare but rather due to an increase in acreage (Fig. 3) (FAOSTAT, 2015). The crop is popular among farmers due to its high return value even when grown in degraded soil, and it requires less management input than cereals to give good yield. Its nitrogen fixing capacity makes the crop popular among famers as a break crop in between two to three years of cereal cropping (Personal comm. with farmers around experimental sites).

Figure 2. Top ten field pea (dry pea) producers in 2013. (FAOSTAT, 2015).

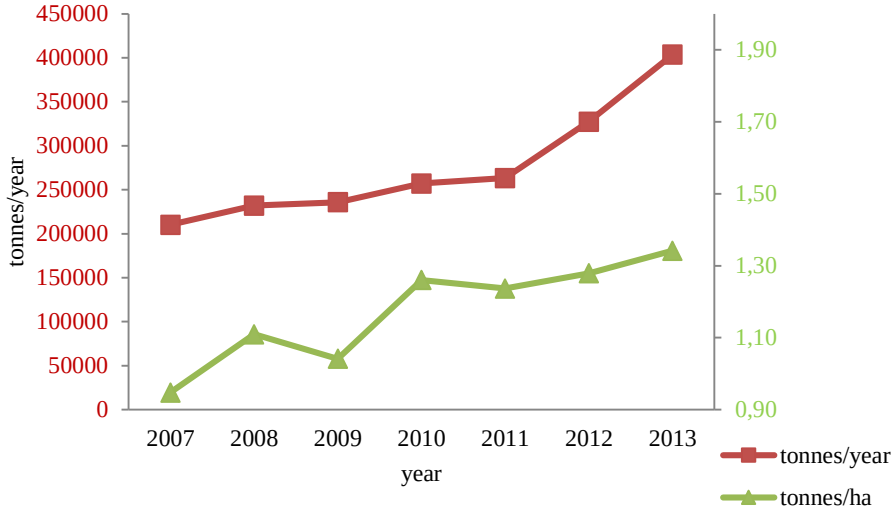

Figure 3. Field pea production (tonnes/year) and seed yield (tonnes/ha) in Ethiopia from 2007 to 2013 (modified from FAOSTAT, 2015).

Field pea is grown twice per year in Ethiopia during the short rainy season (March-July) and the main rainy season (July-October) (Getachew, 2000). In recent times, the production of local landraces has been hampered by diseases like powdery mildew and downy mildew as well as by lodging (Fikere *et al.*, 2010; Keneni *et al.*, 2003; Getachew, 2000), but the major setback in field pea production is the pea weevil (Teka, 2002; Teshome *et al.*, 2015b; Seyoum *et al.*, 2010). The economic loss due to this pest is getting worse and it is forcing farmers to give up field pea production in certain parts of the country. Since most farmers are unable to afford appropriate pesticides, they rely on fumigation as post-harvest containment alternative. However most farmers, lack proper containers and technical expertise to precisely fumigate their yield. Hence, the cheapest and the most environmentally friendly approach against this pest would be the development of resistant varieties and employment of other pest management techniques to keep the pest below the economic threshold level.

2.1.5 Genetic diversity of *P. sativum* and its use in breeding

In the face of erratic climatic conditions and a degrading environment, genetic diversity is an assurance for the success of agriculture vis à vis human development. However, these resources are in danger because of the advent of modern agriculture that heavily invests on the end product like high yield at the expense of

diversity and environment. In relation to this, Harlan (1970) stated, ‘‘*the varietal wealth of the plants that feed and clothe the world is slipping away before our eyes, and the human race cannot simply afford to lose it*’’.

There is huge collection of *Pisum* germplasm in different parts of the world. To mention some: Vavilov Institute (Russia), John Innes Institute (UK), NordGen (Scandinavia), ICARDA (Lebanon), USDA (USA) and EBI (Ethiopia). Some of these collections have been characterized with morphological and molecular markers but still *P. sativum* lags behind in terms of characterization of its gene pool as compared to cereals. Nonetheless, there are some notable efforts to characterize these valuable germplasm collections. For instance, the core collection of USDA have been characterized with phenotypic markers like flower colour and related traits and with molecular markers (Kwon *et al.,* 2012). The fact that these collections were characterized with both morphological and molecular markers makes them suitable for breeding programs.

On the contrary, despite the large collection of *Pisum* accessions at EBI, efforts to characterize them are limited due to various reasons. Furthermore, the findings of the national screening programs have not been properly documented. There are some notable characterizations of Ethiopian field pea germplasm collections with morphological and molecular markers (Fikere *et al.*, 2010; Keneni *et al.*, 2005; Keneni *et al.*, 2003). These studies have only considered a limited number of accessions and do not fully represent the existing diversity. Therefore, similar studies that quantify genetic diversity needs to be carried out on more *Pisum* germplasm collections at EBI.

2.1.6 Morphological and molecular markers for assessing genetic diversity in *P. sativum*

Similar to many other crops, the genetic variation of *P. sativum* populations has been studied using morphological, biochemical and DNA markers. In morphological diversity studies, traits like days to emergence, days to 50% flowering, plant height, number of pods/plant, green pod length, grain filling period (days to maturity minus days to flowering), number of podding nodes/plant, number of pods/podding nodes, number of seeds/pod, 100-seed weight (fresh), 100-seed weight (dry), grain yield/plot (g) have been studied (Azmat *et al.*, 2011; Smýkal *et al.*, 2011; Smýkal *et al.*, 2008; Keneni *et al.*, 2005). Although these traits are vital in quantifying genetic diversity, they are prone to the influence of the environment and hence quantification based on these traits is influenced by biotic and abiotic factors in play at the time of characterization.

Using DNA based markers like RAPD, RFLP, AFLP, ISSR, SSR and SNP could give more precise quantification of genetic diversity as they are not influenced by the environment. There are many such studies on *P. sativum* including the use of SSR markers (Sarikami *et al.*, 2010; Nasiri *et al.*, 2009; Smýkal *et al.*, 2008; Zong *et al.*, 2008), ISSR markers (Tar'an *et al.*, 2005), and RAPD markers (Samec & Našinec, 1995). Some of these DNA based genetic diversity studies were conducted together with morphological markers which would make the quantification comprehensive and vital in breeding programs. However, the genome of *P. sativum* is yet to be sequenced. Therefore, expressed sequence tags (EST) are the most vital tools for marker development in relation to functional genes. EST based SSR markers have been developed for this species (De Caire *et al.*, 2012; Xu *et al.*, 2012; Gong *et al.*, 2010; Teshome *et al*., 2015a) but are very few in comparison to other crops like wheat and barley.

2.2 Pea weevil, *Bruchus pisorum* (Coleoptera: bruchidae)

Bruchus pisorum L. (Coleoptera: Bruchidae) commonly known as pea weevil is a univoltine bruchid first described by Linnaues (1758) as *Dermestes pisorum*. However, Linnaues, later created the *Bruchus* genus which included pea weevil. Pea weevil is a monophagous cosmopolitan pest and field pea is its main target (Clement *et al.*, 2000; Pesho *et al.*, 1977). The pest was first mentioned in the early 18th century by Swedish traveller Kalm (Larson *et al.*, 1938). However, its first record as a pest was in South Africa in the Cape Province (Skaife *et al*., 1918). In 1931, it was established as insect pest in Australia (Newman, 1932). It is proposed that infested seeds are the cause of expansion of this pest to Europe, America, Africa and the Australian sub-continent (Clement *et al.*, 2000). Pea weevil’s ability to withstand extended periods of dry conditions has contributed to its successful expansion (Hardie, 1992). At the moment, pea weevil is a major menace in most field pea growing regions in the world (Fig. 4) (CABI, 2015).

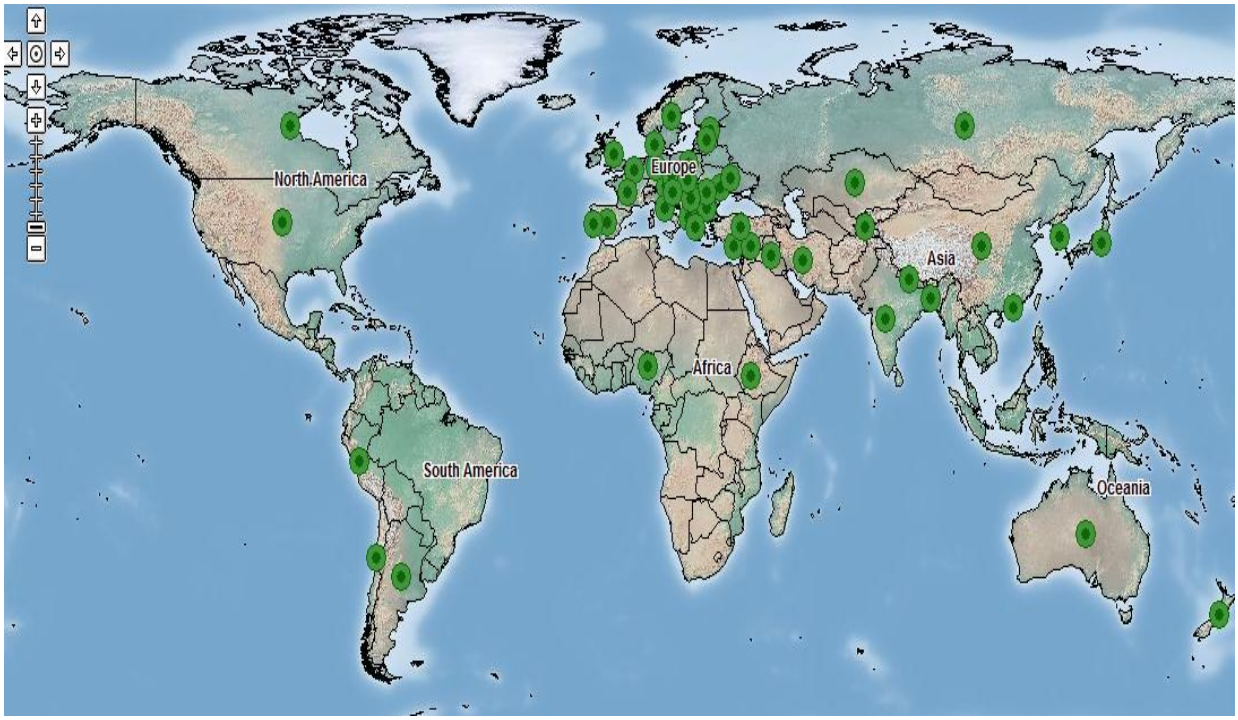

Figure 4. Distribution of pea weevil (*Bruchus pisorum* L.) in the world (CABI, 2015)
Source:http://www.plantwise.org/KnowledgeBank/Map/Global/Bruchus_pisorum/.

2.2.1 Pea weevil description

The adult pea weevil is on average 5 mm long and 2.5 mm wide (McDonald, 1995; Larson *et al.*, 1938). The antennae of the pea weevil is as long as one third of its whole body length (Hardie, 1992). The eggs are bright yellow in colour and about 1.5 mm in length (McDonald, 1995). The larva of the pea weevil is creamy in colour, limbless and curled and they can grow as long as 5 mm (Baker, 1998). The female weevils are slightly bigger in size than the male counterparts. The male can be distinguished from the females by their tiny spine located on the distal end of the tibia of the middle leg (Larson *et al.*, 1938). In general, pea weevils have a brownish colour with grey or white or black patches. Despite having the title of weevil, pea weevils do not possess snout which is typical for most true weevils (Newman, 1932).

2.2.2 Life cycle of pea weevil

Adult weevils invade pea fields at the time of flowering (Baker, 1998). For example, they invade pea fields by mid-August or late September depending on the weather of the locality in Ethiopia. Unlike their male counterparts, female weevils are sexually immature at the time of arrival (Baker, 1998; Pesho & Van Houten, 1982). Therefore, female pea weevils need to feed on the pea flower and its parts to mature sexually which can take two to three weeks after arrival (Armstrong & Matthews, 2005; Pesho & Van Houten, 1982). After mating, the females oviposit eggs on young green pods (Fig. 5) (Hardie & Clement, 2001). The eggs are usually oviposited in couples and glued to the pod by a white transparent substance that keeps the eggs from falling off the pod (Seidenglanz *et al.*, 2011; Skaife *et al.*, 1918). The pea weevil has four larval stages before turning to pupae (Schroeder *et al.*, 1995). Two to three weeks after oviposition, the first larval instar hatches and attempts to drill through the pod wall and the seed coat and, if successful, embed itself inside the young seed (Baker, 1998). Multiple larvae attempt to penetrate a single seed in case of heavily infested pods but only one adult per seed will complete its lifecycle (Smith *et al.*, 1982).

Figure 5. Ovipositing gravid female pea weevil (left) and heavily oviposited pod (right) on the same field.

The fourth instar burrows a transparent exit from the inside without breaking the seed coat completely and the young adult awaits the right moment to break free (Brindley & Hinman, 1937) (**Fig. 2, Paper I**). Cues like shaking and elevated temperature trigger the young adults to leave the seed and hibernate. Adults hibernate over winter in holes, trees or branches until the next season of flowering (Baker, 1998). The arrival of pea weevils on pea fields usually coincides with the time of flowering otherwise the weevils remain hidden until the right cues trigger their flight to nearby pea fields (Hardie, 1992). The adults can even remain at the field within leftover seeds and survive until the next growing season (Armstrong & Matthews, 2005). When the surrounding temperature rises to ca. 20ºC, the adults fly to pea fields lured by the scent of pea flowers (Baker, 1998; Brindley & Hinman, 1938). It is estimated that adults can travel as far as 5 km to locate their host, guided by scents of pea flowers (Brindley & Hinman, 1938).

2.3 Insect resistance in crops

2.3.1 Plant insect co-evolution

In constant struggle to survive the attack by insect pests, plant species in general have mastered the ability to deter, resist and/or tolerate attack in a form of herbivory and oviposition. The struggle to have the upper hand over a myriad of insects attempting to take advantage of their hosts has been in play for millions of years and is still ongoing (Gatehouse, 2002). Resistance in host species can broadly be defined as the ability of a specific group or population to deter insect pests in one way or another and/or revive after attack has taken place as compared to a population lacking such virtues (Smith, 2006). The non-stop interface between host plants and their respective insect pests has contributed to the coevolution of both groups and brought diversity into both groups (Rausher, 2001; Gatehouse *et al.*, 1990). In the long run, individuals or populations with enhanced resistance will have the upper hand in the race that subsequently ensures their success.

2.3.2 Mechanisms of insect resistance

There are three well known mechanisms of resistance which was first proposed by Painter (1958). These are non-preference (antixenosis), antibiosis and tolerance. Non-preference is a category of plant resistance that merely influences the pest from devouring or ovipositing on the host plant via morphological traits or with a volatile release (Smith & Clement, 2012). Antibiosis is another form of resistance with which plants combat insect pests. This type of resistance is effective after the arrival of the pest and it diminishes the survival, development and reproduction success of the pest (Smith & Clement, 2012; Byrne, 2005). The insect pest that has fed upon antibiotic plant parts shows reduced growth, smaller size and weight, reduced

fecundity, a lengthy completion of life cycle, early death etc. (Byrne, 2005). The third form of resistance, tolerance, is a mechanism of defence where the plant survives despite becoming a host for the particular pest without too much loss (Smith & Clement, 2012). In this type of resistance, the insects' survival and reproduction is not affected rather the plants have the capacity to survive dire consequences invoked by the pest in comparison to non-tolerant types. Although these mechanisms of resistance differ in their mode of action, all could be involved at the same time in some plant-insect interactions (Horber, 1980).

2.3.3 Breeding for insect resistance in crops

According to Painter (1958), plant resistance is genetically controlled and measurable qualities possessed by the plant could potentially minimize injury inflicted by the insect pest. Since the 17th century, plant resistance has been implemented in agricultural practices intentionally or unintentionally (Byrne, 2005). However, most resistance breeding efforts have been against pathogens rather than insect pests due to the relative ease to deal with diseases and the success of pesticides (Lowe, 1987). Breeding for insect resistance is a huge undertaking that requires in-depth understanding of the pest's lifecycle, feeding and reproduction and also detailed knowledge of the interface between the pest and its host (Hardie, 1992). Breeding for insect pests has been successful in recent times with crops like wheat, rice, maize, field pea and beans (Sharma & Ortiz, 2002). Legumes in particular are a rich source of resistance against bruchids because of their anti-nutritional and toxic secondary metabolites stored in the seeds (Birch *et al.*, 1985).

2.3.4 Resistance against bruchids in legumes

Legumes are known to produce secondary metabolites like storage compounds, growth or metabolism regulators and some antimetabolites (Gatehouse *et al.*, 1990; Rhoades, 1979). These compounds include an array of tannins, lectins, alkaloids, cyanogenic glycosides, enzyme inhibitors etc. These secondary metabolites protect the plants from herbivory by repelling attackers or being toxic after ingestion. Many species in the Fabaceae family have lectins in their cotyledons which are anti-metabolic for many insects (Marconi *et al.*, 1997; Murdock *et al.*, 1990). For example, Gatehouse *et al*. (1990) discovered enough dosage of lectins in *P. vulgaris* with the potential to kill larvae of the cow pea seed weevil. Furthermore, Ishimoto and Kitamura (1989) identified α-amylase inhibitors in kidney bean (*Phaseulus vulgaris* L.) that prevent carbohydrate digestion and cause gradual death of the azuki bean weevil (*Callosobruchus chinensis*) and the cowpea weevil (*Callosobruchus maculatus*) larvae. Transgenic field pea lines with an α-amylase inhibitor gene were less susceptible to pea weevils in comparison to conventional varieties since the activity of α-amylase inhibitor in the mid-gut of the larvae prohibits digestion and causes gradual death due to starvation (Morton *et al.*, 2000; Schroeder *et al.*, 1995). These transgenic *P. sativum* lines also showed resistance to the azuki bean weevil and the cowpea weevil thus making them resistant to multiple bruchid pests (Shade *et al.*, 1994). However, since a genetically modified field pea has not been prioritized in breeding and cultivation, the crop is still suffering from pea weevils.

2.3.5 Integrated pest management (IPM) for pea weevil

The fact that pesticides use is yet a treadmill in most small-scale farming systems in Africa, there is still a window of opportunity for adopting IPM for managing insect pests like the pea weevil (Abate *et al.*, 2000). Pea weevil was first reported as an invasive pest in Ethiopia in the late 1970s with limited distribution (Abate, 2006). Recent reports suggest that the pest has been reported in many field pea producing regions in the country (Seyoum *et al.*, 2010). However, there might be a window of opportunity to limit its distribution and possibly contain the pest population below economic threshold levels with strict quarantine and adoption of IPM techniques tailored for this pest.

IPM techniques include HPR, cultural methods, biological control, transgenic technology and, in worst case scenario, chemical methods (Fig. 6) Cultural methods like early harvesting and after harvest grazing in Australia did not bring significant success against the pea weevil (Baker, 1998). However, such practice could potentially be effective in small-scale farming systems where most farmlands are less than a hectare. Planting and harvesting early has been a successful pest management technique in different crops in Africa (Abate *et al.*, 2000) and hence similar mechanisms can also be tailored for pea weevil management. Intercropping is also an important part of IPM with proven capacity to restrict disease and pest incidence and bring soil fertility benefits. The fact that specific genotypes of field pea could express neoplasm

formation when grown under shade (Nuttall and Lyall 1964; Doss *et al*., 1995), which can be facilitated via intercropping, is an extra impetus to adopt IPM against pea weevil (Teshome *et al, unpublished*).

The most important component of IPM is HPR. HPR has been successfully implemented against different pests in many legume crops like pigeon pea, chickpea, field pea and cow pea (Sharma *et al.*, 2004). However, their application is limited due to insufficient attention given to this method and the ease of using pesticides whenever affordable (Sharma & Ortiz, 2002). HPR augmented by biological control and proven cultural practices is a silver bullet option for subsistence farmers that requires no skillset for adoption and with no environmental costs (Sharma & Ortiz, 2002).

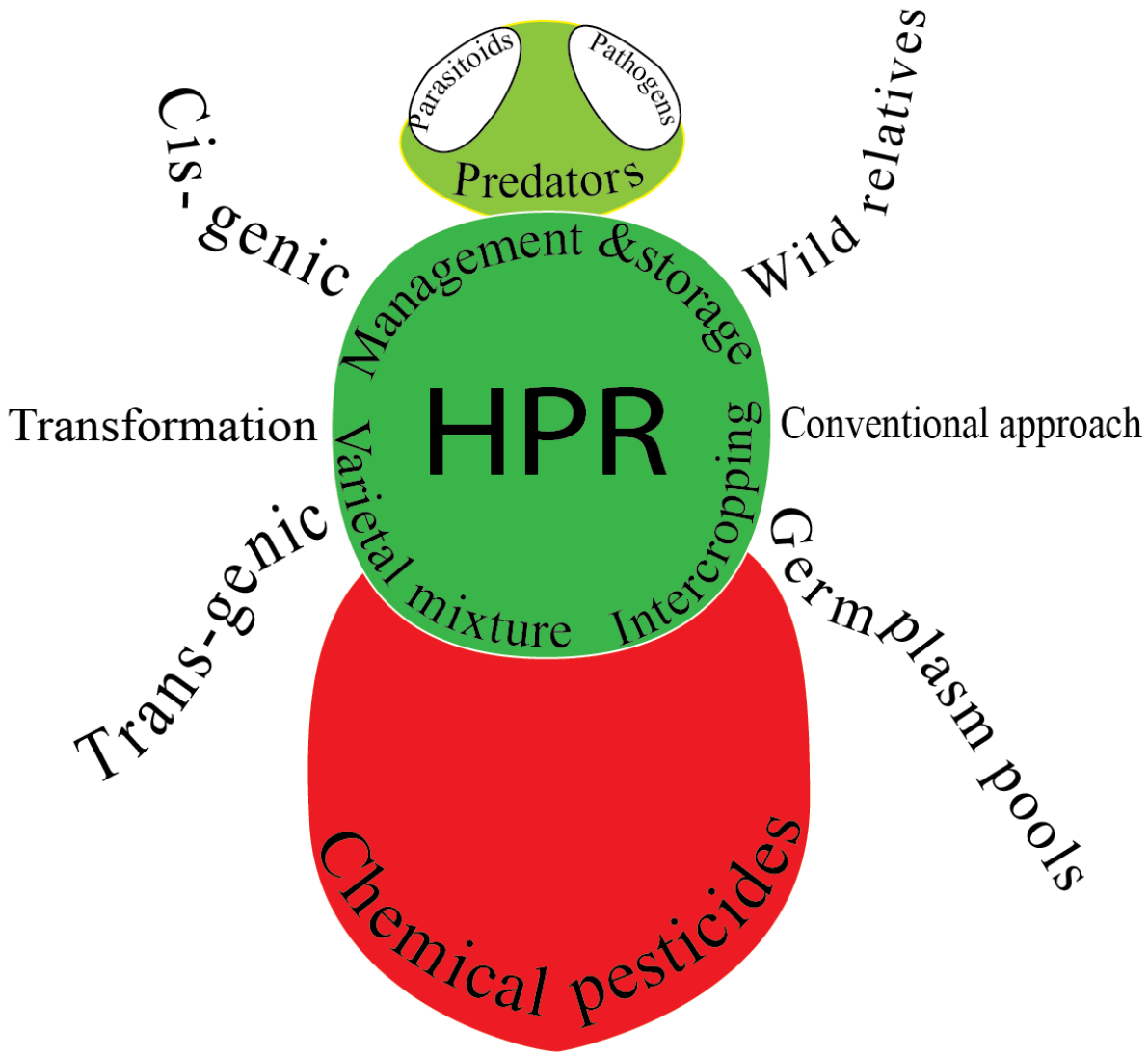

Figure 6. Different components of IPM technique.

The primary step for identifying HPR is screening the primary and secondary gene pool of crops for enhanced resistance and/or reduced susceptibility. Screening germplasm for resistance against *Helicoverpa armigera* in chick pea and pigeon pea has given moderate level of resistance (Sharma & Ortiz, 2002). Likewise, there have been efforts to find HPR against pea weevil in the field pea genepool (Byrne, 2005; Clement *et al.*, 2002; Hardie *et al.*, 1995). In all these studies, enhanced resistance was mainly identified in *P. fulvum* which is the wild relative of *P. sativum*. Aryamanesh *et al.* (2012) was able to introgress the antibiosis and antixenosis capacities of *P. fulvum* into *P. sativum* lines. However, the negative consequence of linkage drag on those lines is yet to be quantified. Recently, Teshome *et al.* (2015b) has identified

enhanced pea weevil resistance in germplasms collected within Ethiopia. These accessions are now under further investigation both at the greenhouse and field levels.

There is another form of host plant resistance against pea weevil that prevents larval entry into pods. In this type of resistance, specific genotypes grow neoplastic tissue on the site of oviposition (Doss *et al.*, 2000; Berdnikov *et al.*, 1992). Such a response on the surface of pods will force the larva to move around looking for a clean site for penetration which in turn exposes the larva for desiccation, parasitoids and risk of detachment from the pod (Doss *et al.*, 2000). However, this trait is attenuated under field conditions because the expression of the *Np* gene controlling this trait is prevented by UV rays (Burgess & Fleming, 1973; Nuttall & Lyall, 1964). Therefore, in order to allow the expression of this trait under field conditions, intercropping is being investigated to mimic greenhouse conditions (**Paper II**).

In conclusion, in order to effectively adopt IPM techniques against pea weevil, starting with genotypes possessing host plant resistance is a crucial step that is economically wise, environmentally safe and easily applicable. The HPR can further be strengthened with effective aforementioned cultural methods. In addition, generalist predators could potentially contribute to the successful containment of the pest. It is also possible to use intercropping as one parcel of the IPM for enhanced neoplasm formation. As a last resort, chemical pesticides can be applied before and after harvest.

3 General objectives

The main aim of the present study is to identify relatively resistant *P. sativum* genotypes against pea weevil in the primary genepool of Ethiopian field pea collections and characterize the genetic diversity and population structure of selected field pea accessions.

3.1 Specific objectives

- Mass screening of the primary gene pool of *P. sativum* for enhanced host plant resistance against pea weevil.
- Evaluating neoplasm expression among Ethiopian field pea collections and its significance in pea weevil resistance.
- Evaluating the possibility of adopting intercropping for enhanced neoplasm formation.
- Developing new EST-SSR markers for *P. sativum* and related genera.
- Assessing the genetic diversity among selected accessions of *P. sativum* with newly developed microsatellite markers.

4 Material and methods

4.1 Field trials

Field trials were conducted for three consecutive years (from 2011 to 2013) in Ethiopia from June to October during each year. In these field trials, selected accessions were exposed to natural pea weevil infestation. Sites used for the trials were predetermined based on a survey conducted ahead of the experiment and records of pest incidence in those sites in earlier years obtained from the local bureau of agriculture. In all trials, pesticides were not applied and trials were conducted under natural rain fed conditions. Land preparation, planting, weeding and harvesting were all carried out manually.

In the first field trial (FT1), 602 accessions were studied at three sites (Ebinat, Liben and Sekota). These sites represent different agro-ecological zones but all share high pea weevil incidence (**Paper I, Fig 1**).

For the second field trial (FT2), two sites within the Liben district were chosen. One of these sites was used for FT1 and the second was 15 km away from site-one. In FT2, 100 accessions were selected for planting based on the results from FT1. At site-one, about 23 of the 100 accessions were highly susceptible. At site-two, only the least infested accessions and a single highly susceptible check were tested.

The third field trial (FT3), the same 100 accessions used during FT2 were tested. FT3 was conducted in the Liben district in a manner similar to that of FT2 although new plots of land were used for this trial. The two sites were different in that at site-one finger millet and at site-two field pea was grown during the previous growing season.

4.2 Greenhouse experiments

The best and the worst performing genotypes in terms of their resistance against pea weevil during FT1 and FT2 were further studied under greenhouse conditions at SLU Alnarp. The selected genotypes were planted in two litre plastic pots in a greenhouse chamber at 18-22ºC and a minimum of 12 h light. Ahead of flowering, the plants were transferred to insect rearing cages (60 cm x 60 cm x 120 cm) (MegaView Science Co Ltd, Taiwan) for artificial infestation (Fig. 7). Different mixes of genotypes were tested in subsequent generations of screenings.

Freshly emerged pea weevil adults were used for infestation in the greenhouse experiment. The sex of the weevils was determined as described in Larson *et al.* (1938) and afterwards the weevils were kept at 4ºC until time of release. Damage analysis was conducted three months after harvest.

Figure 7. Field pea genotypes in cages during greenhouse experiment conducted for screening potentially resistance genotypes against pea weevil.

4.2.1 UV light experiment on neoplasm producing genotypes

Genotypes known to produce neoplasm under greenhouse conditions were selected for this experiment. These genotypes were investigated for neoplasm formation under a single UV lamp, double UV lamps and a control chamber (no UV lamp). Furthermore, a replica of these genotypes was tested at the field under intercropping conditions. In this experiment, non-neoplastic genotypes were also included as a control.

In addition, F1 hybrids of neoplastic pollen donor and non-neoplastic pollen recipients were subjected to double UV lamp and control chamber test with the aim of observing the inheritance of neoplastic formation at the F1 generation.

4.3 Development of field pea EST-Simple Sequence Repeat markers

Field pea EST sequences from National Centre for Biotechnology (NCBI) were assessed for SSR motifs using Msatcommander-0.8.1 (http://code.google.com/p/msatcommander/) (Faircloth, 2008). SSRs with two to six repeat motifs were targeted. Primer3 primer designing program (Rozen & Skaletsky, 1999) was used for designing the primers for SSR containing unique sequences. Of the total 37 designed primers, 15 were consistently amplifying target loci across various accessions. Across-taxa transferability of these newly developed SSRs was also evaluated on other *Pisum* species and sub-species and related genera. Twelve of these markers were further used for a genetic diversity study on selected accessions of *P. sativum*.

4.4 DNA isolation and PCR amplification

For the genetic diversity and population structure study, 46 accessions were chosen. 43 of these accessions were landraces obtained from EBI and three were varieties obtained from NordGen. The EBI accessions were chosen based on their place of collection in a way that they represent most of the field pea growing areas in the country. The three NordGen varieties were included to determine the extent of genetic differentiation between the landraces from Ethiopia and the varieties in the northern hemisphere.

Isolation of DNA was conducted based on modified CTAB protocol (Geleta *et al.*, 2012). The PCR was carried using 96-well plates in GeneAMP PCR 9700 thermo cycler (Applied Biosystems Inc. USA). A combination of touchdown and constant annealing temperature was used for amplification. The PCR products were stored at 4ºC until electrophoresis.

After multiplexing of the PCR products, capillary gel electrophoresis was conducted at the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. The capillary gel electrophoresis was conducted with ABI Prism 3730 DNA Analyser (Applied Biosystems).

4.5 Data analysis

4.5.1 Field and Greenhouse data

The field data was analysed using R version 2.15.3 (R Core Team, 2013). The percent seed damage (PSD) of accessions was first arcsine transformed to obtain homogenous variances and normal distribution before analysis of variance was conducted. In FT2, ANOVA was conducted on PSD of accessions. Finally, Bonferroni adjustment was used to compare PSD of each accession with that of a susceptible check, cv *Adet*.

In case of greenhouse experiments, the PSD of each genotype in each cage was calculated as the ratio of infested seeds and the total number of seeds produced by the plant. These data was then compared with other cohabiting genotypes' PSD and with the average damage within the cage. In addition, data from different cages and generations were averaged for particular genotypes.

4.5.2 Genetic diversity analysis

Peak identification and fragment sizing was conducted with GeneMarker® V2.2.0 software (SoftwareGenetics, LLS, Sate College, Pennsylvania). Default settings with 200 threshold intensity in the Genemarker software were used for identification but the final acceptance of the peaks was made after checking each band's bin sharpness.

Genetic diversity parameters were calculated using POPGENE 1.31 software (Yeh *et al.*, 1999). Molecular variance (AMOVA) was calculated according to Excoffier *et al.* (2005) with Arlequin software. Dendrogram was generated with Free-Tree Freeware program (Pavlicek *et al.*, 1999), and viewed using TreeView (Win32) 1.6.6 program (PAGE, 1996). The 46 populations were classified into probable clusters based on 12 polymorphic loci with STRUCURE software (Pritchard *et al.*, 2000). The most likely population number (*K* value) was calculated according to Earl and von Holdt (2012) using STRUCTUREHARVESTER software. The output of the STRUCTUREHARVESTER was viewed using CLUMPP and DISTRUCT softwares (Jakobsson & Rosenberg, 2007; Rosenberg, 2004).

5 Results and discussion

5.1 Field and greenhouse experiments

In the first field trial, a few of the 602 accessions studied had a relatively low percent seed damage (PSD) across the three sites. Accessions like 213192, 32294, 32362, Nc-03, 213965, Nc-18, 244802 and 32409 scored less than 20% PSD across the three sites. On the contrary, varieties like Milky and Wolmera and accession 227143 scored the highest PSD values which was above 90%. However, ANOVA of mean PSDs revealed no significant variation among accessions. This is partly due to the fact that the replications were across locations and each site was influenced by different factors other than the high pest pressure.

In FT2, the released variety cv *Adet* scored the highest PSD. An interesting similarity among seeds from the locally released varieties was that they were all round in shape with creamy seed coat colour and relatively larger cotyledon size in comparison to the landraces. Among different seed colour groups, creamy coloured seeds were the most susceptible, which suggest the presence of a different chemical signal produced by genotypes with creamy seed colour that strongly attract this pest. Such trend of higher susceptibility in creamy coloured seeds was also the case when different coloured seeds exist within an accession. For example, when PSD of green and creamy seeds within the same accessions were compared, the green seeds were unattacked in most accessions while creamy seeds scored a minimum of 45% mean PSD (Fig. 8). A possible explanation for high susceptibility in these groups (creamy coloured seeds) could be a thinner seed coat (testa) and/or a larger cotyledon which can play role in the fecundity of the larvae. For example, seeds of wild relatives of cow pea have smaller size and a darker seed coat colour than cultivated varieties which is correlated with higher tannin content (Chang *et al.*, 1994). Tannins are secondary metabolites that are directly or indirectly involved in the deterrence of herbivory by making protein digestion hard to attain. Previous studies reported antibiosis and antixenosis capacities of pods of *P. fulvum* against pea weevil (Byrne *et al.*, 2008). Interestingly, seeds of *P. fulvum* are small in size with a relatively stark dark seed coat colour, which makes them different from most cultivated varieties of *P. sativum*.

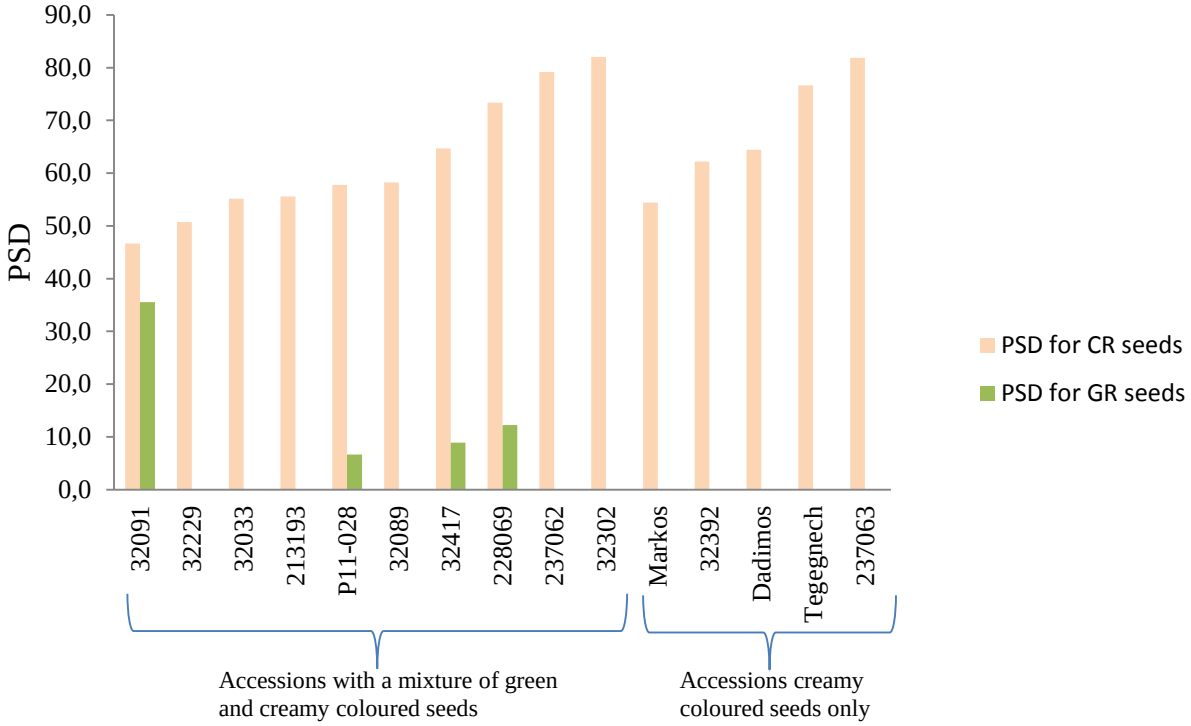

Figure 8. Comparison of PSD of creamy (CR) and green (GR) coloured seeds in FT1.

Another interesting observation related to pea weevil infestation was the gradual change in seed coat colour of infested seeds. In most infested seeds, the green and variegated-green seed coat colour gradually turns into brown and variegated brown, respectively, upon infestation (Fig. 9). Such alteration was not observed in creamy coloured seeds which remained the same before and after infestation. When the mean PSD of green and brown coloured seeds within accessions was compared, it was easy to see the high PSD score in the brown coloured seeds (Fig. 10). The fact that pea weevil infestation causes a change in seed coat colour and that specific colours of seeds are more susceptible, imply the importance of seed coat colour in relation to pea weevil susceptibility or resistance. According to Ceballos *et al.* (2002), seeds of *Sesbania drummondii* undergo changes in seed coat colour and physiology following attack by the nymph of *Hyalymenus tarsatus*. Our findings indicate the need to consider seed coat colour and seed coat thickness in search for pea weevil resistance in field pea.

Figure 9. Seed colour of healthy (upper row) and infested (lower row) seeds from the same plant, showing change in seed coat colour due to infestation by pea weevil.

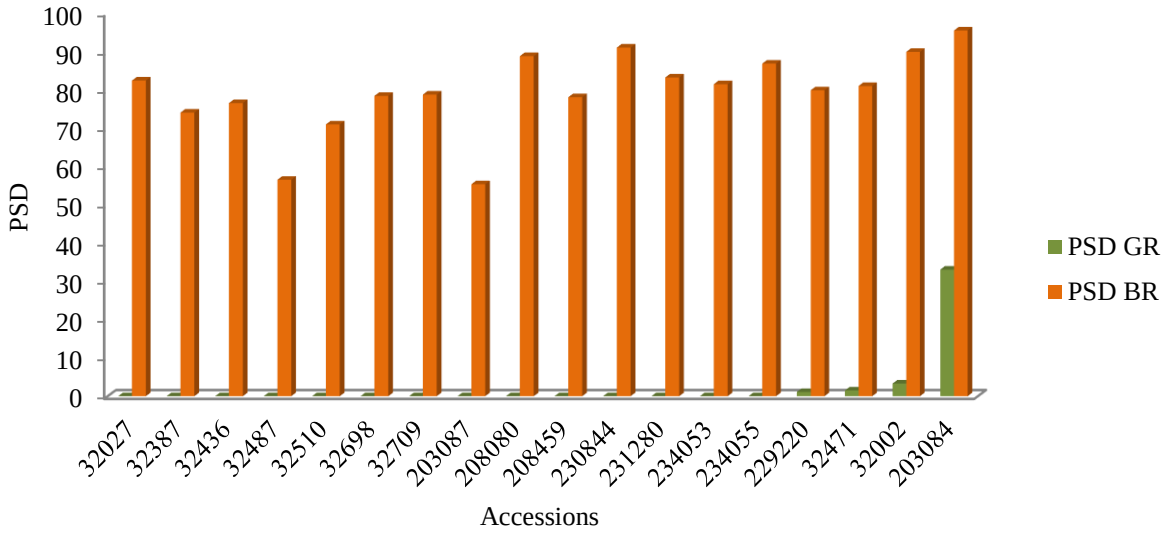

Figure 10. Comparison of PSDs of green (GR) and brown (BR) seeds for selected accessions.

In FT2, only accessions which scored less than 30% or above 70% mean PSD during FT1 were studied. It was interesting to observe that there was a significant difference in performance of the accessions between the two sites used during FT2. As can be seen from Fig. 11, the average damage (PSD) at site-one (B1 & B2) was above 80% while the average damage at site-two (B3 & B4) was below 20%. It should be noted that only relatively resistant accessions (according to the results from the FT1) with a single susceptible check (cv *Adet*) were planted at site-two whereas at site-one about 23% of the 100 accessions were highly susceptible to pea weevil. Hence, when only a single susceptible accession is present within a field (site-two), the PSD of most accessions, including the most susceptible accession, was significantly low. On the contrary, where there are a number of susceptible accessions (site-one), the average PSD was high for all. Previous studies on cherry-oat aphid (*Rhopalosiphum padi* L.) on barley (*Hordeum vulgare* L.) have shown that resistance could be invoked on susceptible checks if both resistant and susceptible types are planted in close proximity under greenhouse conditions (Ninkovic & Åhman, 2009). Similar allelopathic patterns have also been observed on some *Hordeum* genotypes in accepting bird cheery-oat aphid under field conditions (Ninkovic *et al.*, 2002). Hence, the low PSD observed for most accessions at site-two could be due to varietal mixtures with some possessing relative resistance and such resistance having positive influence on the performance of all accessions, including the susceptible check cv *Adet*. Varietal mixtures is one of the strategies advocated and applied for management of pests in other crops but with limited application due to various reasons (Ratnadass *et al.*, 2012). In general, consistent results were obtained for some accessions like 32454 and 235002 where the mean PSD was less than 40% in both FT1 and FT2.

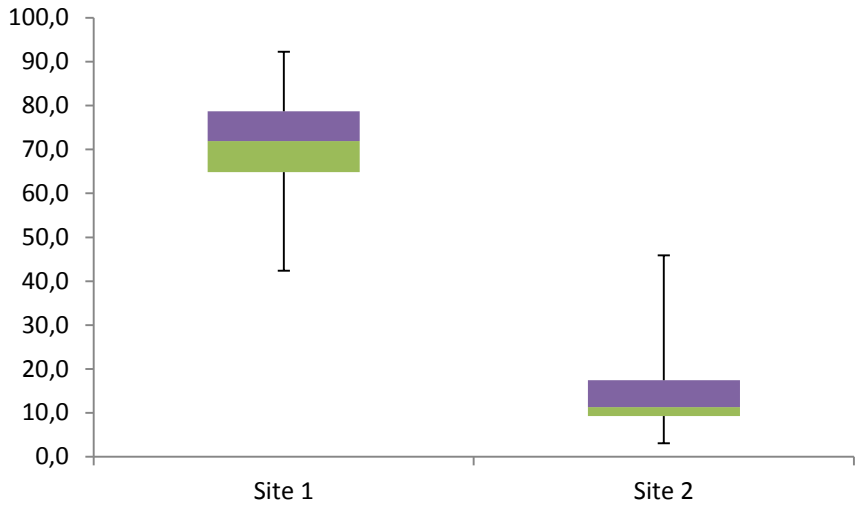

Figure 11. Comparison of average percent seed damage at the two sites during the second field trial.

In the greenhouse experiments, some genotypes consistently showed lower PSD despite high average seed damage on other plants in the same cage. Genotypes in the left group of Fig. 12 consistently scored less PSD while genotypes on the right group scored high PSD, in most cases higher than the average seed damage of the plants in the same cage. Furthermore, some admixtures of genotypes performed very well against pea weevil in repeated trials (data not shown). It is possible that the admixture of genotypes contributed to the enhanced resistance recorded in plants within a cage rather than the resistance of individual genotypes. A similar trend was observed in the second field trial where the mean PSD of the susceptible check was low when planted together with relatively resistant genotypes (Fig 11). Further studies are underway to prove the consistency of these results.

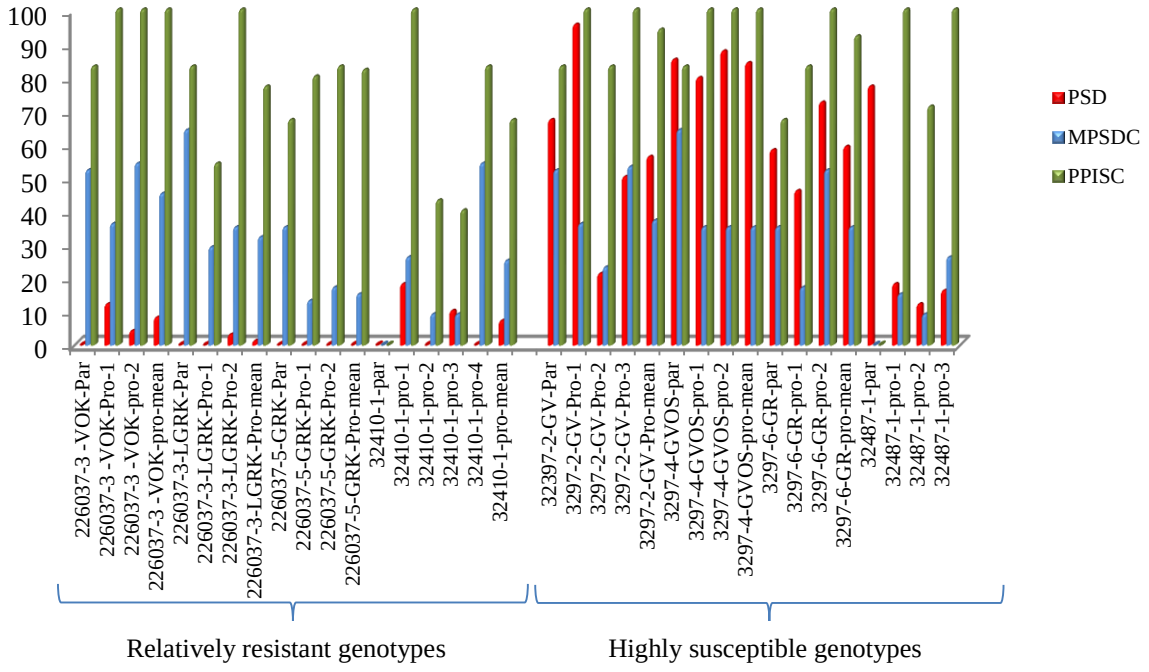

Figure 12. Performance of selected genotypes under greenhouse conditions. PSD=percent seed damage, MPSDC=mean percent seed damage per cage, PPISC=percent of plants with infested seeds per cage.

Some of the best performing genotypes under greenhouse conditions were those that produce neoplastic pods (**Paper I, Fig. 6**). Neoplastic forming genotypes are known to have less susceptibility to pea weevil (Doss *et al.*, 2000; Doss *et al.*, 1995). In this case, genotypes forming neoplastic pods were studied in consecutive experiments against pea weevil attack under greenhouse conditions. As can be seen from Fig. 13, seeds from neoplastic pods were usually free of pea weevil infestation. Genotypes with this trait scored lower PSD in comparison to other genotypes (**paper II, Table** 2). Furthermore, neoplastic genotypes were less preferred for oviposition by gravid female pea weevils in choice experiment (Mendesil *et al*., *unpublished*). Interestingly, accession 226037 that contains neoplasm forming genotypes in the greenhouse also scored relatively low mean PSD values in three consecutive field trials.

It has long been known that neoplastic pod formation is controlled by the *Np* gene which is inhibited in the presence of UV light which in turn has prevented its use in pea weevil resistance breeding (Nuttall & Lyall, 1964). In the present study, neoplastic forming genotypes were intercropped with sorghum (*Sorghum bicolor* L.) accessions under field conditions to abate the negative impact of UV rays on the expression of the *Np* gene. As hypothesized, the shade provided by the canopy of sorghum has facilitated neoplasm formation in these genotypes (**Paper II, Table 3**). However, the enhanced neoplasm formation via intercropping is yet to be tested under natural infestation for resistance against pea weevil. Nonetheless, previous studies on neoplastic genotypes have shown less pea weevil susceptibility under field conditions (Doss *et al.*, 2000).

Neoplasm formation in field pea is a dominantly inherited trait (Berdnikov *et al.*, 1992; Nuttall & Lyall, 1964). The present study has also demonstrated the expression of this trait in F_1 hybrids derived from non-neoplasm mother plants and neoplasm pollen donors (**Paper II, Table 4**). In addition, one of the F_1 hybrids tested for pea weevil resistance has shown reduced PSD (**Paper II, Table 2**). However, as it was observed in the parental generation, the percentage of neoplastic pods of F1 hybrids was significantly reduced in the UV lamp chamber (**Paper II, Table 4**).

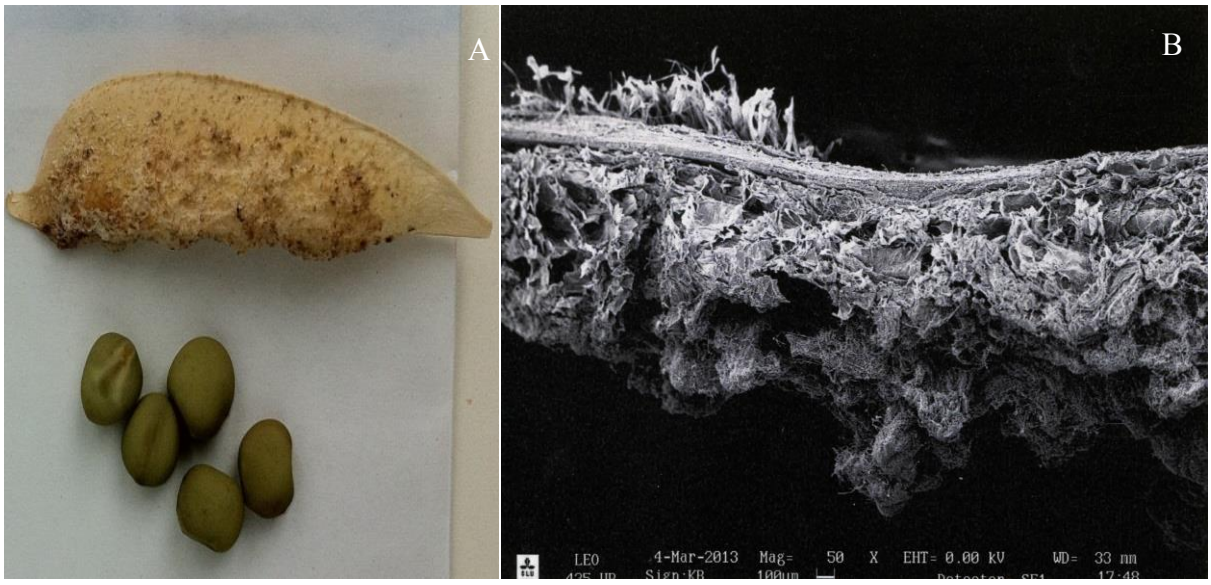

Figure 13. (A) Neoplastic pod after harvest and its seeds. (B) Scanning electron micrograph view of neoplasm on pea pods.

Since enhanced pea weevil resistance was also observed in non-neoplastic genotypes, there is possibility to cross neoplastic genotypes with other genotypes with enhanced pea weevil resistance and possibly pyramide genes of resistance into a single breeding line. For example, neoplastic genotypes from accession 226037 and the non-neoplastic genotypes from accession 32410 have scored low PSDs in field and greenhouse trials. Therefore, the intraspecific hybrids of these two genotypes may be superior in terms of resistance to pea weevil due to pyramiding of resistance genes. There is also a possibility for interspecific hybridization of these genotypes with *P. fulvum*, which is the wild relative of *P. sativum* and known to possess enhanced resistance against pea weevil (Hardie *et al.*, 1995). The evaluation of interspecific hybrids of low PSD accessions with *P. fulvum* genotypes and their progenies is currently underway. However, for

the sake of minimizing costs and time needed for screening potential genotypes under greenhouse or field conditions, the development of molecular markers related to resistant genes is the best way forward.

5.2 Microsatellite marker development and genetic diversity

Fifteen new EST-SSR markers for *P. sativum* were developed in this study. The majority of these newly developed microsatellites are trinucleotides as has been observed in previous studies on *P. sativum* and cereals (Choudhary *et al.*, 2012; Mishra *et al.*, 2012). All but two microsatellites amplified expected bands in *P. fulvum*. It is interesting that these markers have worked in *P. fulvum* as this species is known to have enhanced resistance against pea weevil (Hardie *et al.*, 1995). Polymorphic markers that differentiate the two species are candidates for genetic linkage and QTL mapping for this trait based on interspecific hybrid mapping population; and markers linked to resistance against pea weevil could play an important role in the transfer of pea weevil resistance from *P. fulvum* to cultivated *P. sativum* lines.

These microsatellites markers were also amplified in wild subspecies of *Pisum* as well as in *Vicia faba* and *Lens culinaris*. EST-SSR markers are known to be transferable to related genera (Gupta *et al.*, 2003; Burstin *et al.*, 2001). The fact that the markers were effectively transferable to species and subspecies of the genus *Pisum* is important in resolving the taxonomic status of some of the disputed taxa. For example, *P. abyssinicum* is placed at the subspecies level by some taxonomists but given a species status by other (Kosterin & Bogdanova, 2015; Maxted & Ambrose, 2001). The controversy is also valid for *P. sativum* var. arvense and *P. sativum* var. elatius which different authors rank at higher or lower taxonomic levels. In general, comparing the allelic profile of these disputed taxa using the newly developed microsatellite markers could potentially contribute to the improvement of taxonomic classification of the genus *Pisum*. These markers could also be used in genetic diversity studies of *P. sativum* and related genera.

The genetic diversity study of 548 individuals of *P. sativum* individuals representing 46 accessions with 12 polymorphic EST-SSR loci resulted in the amplification of 37 alleles. Overall, an average of 3.1 alleles was detected across the 12 loci studied. Among the loci, PS 10 was the most polymorphic with six alleles. Previous studies with genomic and EST-SSRs on *P. sativum* have also reported similar levels of polymorphism and number of alleles per locus (Mishra *et al.*, 2012; Loridon *et al.*, 2005; Burstin *et al.*, 2001).

Despite that all markers were polymorphic among all accessions; the level of heterozygosity was low in general. The highest heterozygosity was recorded for PS 13 which was 0.05. Such reduced heterozygosity at each locus show that some of the alleles are at very low frequency in comparison to the others. In addition, a number of private alleles were recorded for particular accessions at different loci. For example, accession 32048 has recorded private alleles for five of the 12 loci. Such exclusive alleles could be important both from a breeding point of view and for employing *in-situ* and *ex-situ* conservation strategies in Ethiopia.

Overall, a high level of genetic diversity was revealed among Ethiopian landraces through the use of these microsatellites, which is in line with previous studies that reported a great extent of diversity in the Ethiopian field pea gene pool. This diversity could be a resource of genes for various desirable traits in field pea breeding. The findings of this study are valuable inputs for future collection missions and setting up core collections.

6 Conclusion and recommendations

The present study has revealed enhanced host plant resistance against the pea weevil in a few accessions and/or genotypes among a large number of field pea accessions screened. Furthermore, intercropping trials have shown promising results to improve neoplasm formation under field conditions which can add extra protection against pea weevils. In parallel, the newly developed microsatellites could play a key role in marker assisted selection for pea weevil resistance so that less time is spent in the actual breeding program. The genetic diversity study revealed the existing potential within field pea gene pool in Ethiopia that could contribute to the development of pest and disease resistant varieties in the future. It is vital to make use of molecular markers for speeding up the screening phase so that less time is needed in the actual breeding program. Based on the findings of the present study, we recommend the following for developing pea weevil resistant varieties in the foreseeable future:

- Screening EBI gene bank accessions that were not included in the present study. There are around 1400 untested accessions at EBI, and their field and greenhouse screening may open up opportunity to identify more effective HPR against this pest.

- Use accessions and genotypes with enhanced resistance against pea weevil that were identified in the present study in the field pea breeding program.

- Intraspecific hybridization between identified genotypes with less susceptibility for pea weevil and interspecific hybridization with wild relatives. Such crossings can potentially lead to pyramiding of resistance genes and broaden the HPR.

- Intercropping trials of neoplastic field pea genotypes with sorghum or maize under natural pea weevil infestation conditions. Such trials help evaluate the effectiveness of the neoplasm formation in deterring pea weevil infestation.

- Develop molecular markers associated with resistance for application of marker assisted selection for pea weevil resistance.

- Bio-assay study of the mechanism of resistance in the identified relatively resistant genotypes against pea weevil.

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