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Morphological, Physiological and Genetic Variation of Wild and Cultivated Pea (*Pisum sativum*)

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For all….

Thank you

Do'a

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Abstract

In this study the morphological, physiological and genetic variations of pea (*Pisum sativum)* accessions were addressed. Accessions included two different wild populations from Bani-Noaim and Dura, local cultivated genotypes from three different sources (Union of Agricultural Work Committees (UAWC), Dura, Al-Dahria), and other two commercial varieties imported from Fito company (Spain), and (Canada).

Investigated morphological characteristics were plant height, flower color, number of branches, number of pods, number of seeds in pod, and number of stomata of the lower leaf epidermis. Total chlorophyll content was also analyzed. In addition, plant weight and average weight of seeds were also recorded.

The genetic variation within and between populations were assessed using Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) molecular markers, and the Amplification of the Internal Transcribed Spacer (ITS) sequencing.

Results showed separate clustering between wild, local and commercial accessions at all levels of investigation. The two wild accessions have no overlap between them, which indicate that there is no gene flow; they represent a good source for cultivated pea improvement and should be protected. In contrast local accessions were different in their diversity for example local Dahria has low diversity between their accessions.

Using morphological and physiological measurements beside genetic diversity clear and complete view of the relatedness between and within accessions was reveled. Our results suggested that fingerprinting techniques should be utilized characterization of the local and wild plant species mainly by those institutes working on conservation of these plants.

Introduction

Plants have evolved to survive in almost every climatic and environmental niche available. To achieve that, plants evolved sophisticated and complex mechanisms and structures to enable them to survive environmental changes in light, temperature, atmosphere composition, water and nutrients and salinity (Ferguson, 2004). Accordingly, genotype – environment interaction is the source of plant diversity and variation at all. This is necessary because of the sessile nature of plants (Ferguson et al. 1998). As the world population, currently is more than six billions, it is expected to almost double by the middle of 21st century (Kang, 2002), the key of doubling agricultural production is to increase the efficient utilization of resources; this includes better understanding for genotype- environment interactions and ways of exploiting it well.

The variation between wild and cultivated plants is a result of the interactions between genotype and environment; beside that the variation among genotypes in phenotype sensitivity to environment may be necessary to enhance development of locally adapted varieties (Flconer, 1952). Although wild plants are better adapted to survive stressful conditions than commercial genotypes, they still have lower productivity rates.

The low genetic diversity in most of the varieties developed may encourage breeders to include exotic materials in their breeding programs, which will broaden the genetic base and maximize genetic gains from selection, as favorable alleles may be accumulated. In tomato, for example, and despite their apparent inferior phenotypes, exotic germplasm and wild genotype contain desirable alleles for various economically important characters (de Vicente and Tanksley 1993). However to date, there has been limited use of

exotic germplasm or wild accessions as sources of genetic variation in pea improvement (Tar'an, 2005), despite the extensive attempts to collect and develop a diverse germplasm base. A greater effort to introgression diverse germplasm into locally adapted cultivars that do not carry a yield penalty may offer greater rewards in crop improvement and reduced genetic vulnerability (Sorrells and Wilson 1997). In pea, for example, wild accessions were considered, to provide alleles to attain resistance to *Mycosphaerella* blight, for which no resistance was found in the cultivated species (Priliouk et al. 1999). To minimize the negative effects of distantly related, non-adapted germplasm, Sorrells and Wilson (1997) suggested the concept of parent building, the gradual incorporation of a few traits through some form of backcrossing or marker-assisted selection to make use of allelic variation. Previous genetic diversity studies in pea focused on genetic relatedness within the *Pisum* genus using morphological characters and/or molecular techniques (Ellis et al.1998; Hoey et al. 1996; Pearce et al. 2000), or comparison of different marker types to assess genetic relatedness among a limited number of accessions or specific groups of pea genotypes (Lu et al. 1996; Samec et al. 1998; Simioniuc et al. 2002). In this respect, morphological, physiological, and genetic measurements are conducted to recognize and assess plants for the aim of developing more adapted cultivars to our requirements that combine the advantages of wild and cultivated plants.

Knowing a plant in ecological, morphological, physiological and genetic ways make studies geared towards developing it easier (Bozoglu, 2006).

Objectives

The aims of this study are:

1- To generate molecular fingerprint to discriminate between different peas accessions (wild, local and commercial).

2- To evaluate the relatedness between wild, local and commercial pea accessions using the morphological, physiological and molecular character of these accessions.

3- To measure the influence of the environmental factors on growth and development of wild pea plants.

Chapter One

Literature Review

1.1 Pea (*Pisum sativum*)

Pea (*Pisum sativum*) is one of the most important food legumes in the world not only for its very old history of domestication (more than 8000 years ago) (Lazor, 2006), but also for its versatile use as vegetables, pulses and feed (Choudhury, 2007). Pea production ranks second among the cool season pulses in the world (Tar'an, 2005), and has the third largest sowing area in the world after beans and chickpeas (Bozoglu, 2006). Additionally, pea is a significant contributor to agricultural sustainability through N –fixation (Rowland et al. 1994), and as a rotation crop allowing the diversification of agricultural production systems (Gen, 2005). Moreover, dry pea seed is a rich source of protein (19–27%), and is relatively free of anti-nutritional substances (Petterson et al. 1997).

Pea (*Pisum sativum*) is one of the most studied plants from genetics point of view and a source of immense variation (Marx, 1977; Choudhury, 2007). Botanically pea (*Pisum sativum* L.) belongs to family Fabaceae, and comprises only two species *Pisum sativum* and *Pisum fulfum* (Smart, 1990). The wild pea's flowers have five sepals, five petals (two fused keel petals, two wing petals and a standard petal), ten anthers (nine fused into a filament tube and one partially free) and a single central carpel (Yaxley, 2001; Tucker, 1989; Ferrandiz et al., 1999). Further, pea is a self pollinated, annual herb, with weak stem, alternate leaves, leaflets ovate or elliptic and terminal branched tendrils (Duke, 1981; Ghafoor, 2005).

Depending on the variety and the weather conditions, a mature pea seed needs 25 to 35 days after pollination to be formed (Irzykowska, 2002).

In respect to the growing condition, pea is grown in cool climates with an optimum daily temperature of 17°C, a minimum of 10°C and a maximum of 23°C. The normal growing period is 65 to 100 days for fresh pea, with an additional 20 days for dry peas, although the growing period is extended under cool conditions. Under irrigation, pea yields 2 to 3 ton/ha shelled fresh pea (70% to 80% moisture) and 0.6 and 0.8 ton/ha dry pea (12% moisture) (Tzitzikas, 2005). The major component of pea seed is starch, which accounts for up to 50% of the seed dry matter (DM) (Borowska et al. 1996; Wang et al. 1998). Protein and total dietary fiber account for about 24% and 20% DM, respectively, where as lipids are present in lower amounts (2.5% DM) (Black et al. 1998). However high variation in starch and protein contents were observed frequently, whereas the variations in the other components are usually lower (Borowska et al. 1996).

1.2 Effect of environmental factors on plant characteristics

The impact of environmental factors on plants may appear in the form of acclimatization or adaptation to severe climates, or the evolution of morphological and/or physiological characteristics to maintain alive.

Under natural conditions, plants frequently encounter combinations of stress factors (Bazzaz, 1996; Sultan et al. 1998). Consequently, the individual ability to tolerate multiple stresses through morphological adjustments is a major feature that determines species survival and colonization, and hence the ecological breadth of the species (Chapin et al., 1987; Bazzaz, 1996; Sultan et al., 1998).

According to Larcher (1987) stress contains both destructive and constructive elements and is a selection factor as well as a driving force improving resistance and adaptive evolution. Rivero (2007) found that annual plants in arid areas combine a relatively short life cycle with a high growth rate during the wet season to avoid drought. These plants adjust also sink/source allocation by increasing root growth and by decreasing leaf area (Karatassiou, 2009).

Plants have evolved diverse mechanisms to respond to environmental conditions, both in terms of growth and development, and tolerance mechanisms (Ferguson, 2004). Among these are structural traits which are indexes of biological characteristics of different plant species, and reflect the adaptation strategies of plants to the environment. These traits, among others, include leaf area, leaf dry weight, specific leaf area and chlorophyll content. The high- specific leaf area leaves are productive (Vander, 1998; poorter, 1998) but are necessarily also short lived and vulnerable to herbivory (Grime, 1996; Pandey, 2009), where as, low- specific leaf area leaves perform better in resources-poor environments (Wilson, 1999). Leaf area plays an important role in light interception, water and nutrient use, growth and yield potential (Aase, 1978; Williams, 1987). It is well known that leaf area adjustment is the major response of adapted plant to decreasing moisture and/or nutrient availability (Hamann, 1979; Fonseca, 2000). Lower specific leaf area, due to thicker and/or denser leaves, contributes to long leaf survival, nutrient retention, and protection from desiccation (Pandey, 2009), whereas small leaf size reduces boundary layer resistance, and helps maintain favorable leaf temperatures and higher photosynthetic water-use efficiency under the combination of high solar radiation and low water availability (Parkhurst, 1972; Evans, 1989).

Leaf movement is another common adaptive response to drought stress in plants (Begg, 1980; Ehleringer and Forseth, 1980). Many species can reduce the quantity of radiation that they intercept when suffering from drought stress either by leaf folding and paraheliotropism or by leaf rolling. In fact, leaf rolling, as a dehydration avoidance mechanism, is an effective protective mechanism from the effects of high light levels in agricultural fields and protects leaves of non-irrigated plants from photo damage (Corlett et al., 1994).

Saatchi et al. (2007) found that environmental variables such as topography, geomorphology, soil types, solar radiation, wind, temperature, and rainfall are important factors affecting the plant, its diversity, structure, density, and productivity. Plants exhibit a variety of responses to abiotic stresses that enable them to tolerate and survive adverse conditions (Knight and Knight, 2001).

Concerning the biochemical changes, which considered also as important adaptations to stress (Goodwin and Mercer, 1986), the accumulation of proline under low-water stress conditions is the most obvious change (Sivaramakrishnan et al., 1988). In addition, changes in the activities of various enzymes are important in inducing tolerance to stress (Rensburg & Kruger, 1994). Moreover, the physiological changes are another major determinant of survival and productivity of plants (Kosakivska, 2008). In this respect, abscisic acid (ABA) is considered as the first-response mediator to sense environmental changes leading to certain adaptive metabolic adjustments. Among these adjustments, the structural closure is the most obvious adaptative response. In addition, ABA upregulate the gene expression of certain genes to induce new polypeptide synthesis which increases the hardiness of plants (Allagulova et al., 2003; Rodgriguez et al., 2005).

Plants reactions to environmental factors lead also to hardening of plants by establishing a new physiological standard, which is an optimum stage of physiology under the changed environmental conditions (Yordanov, 2003). Accordingly, plant response is complex, as it reflects over space and time the integration of stress effects and responses at all underlying levels of organization (Blum, 1996). Under field conditions these responses can be synergistically or antagonistically modified by the superimposition of other stresses (Yordanov, 2003).

1.3 Plant Morphology

A lot of agricultural studies have examined plant responses to stresses, and their impact on crop yield. However, studies deals with morphogenic effects (Nobel, 1988, 1996; Larcher, 1995) are limited, although variation in plant morphology may be the key to understand mechanism behind local adaptation (Knight, 2004). Individuals ability to tolerate multiple stresses through morphological adjustments is a major feature that determines species survival and colonization, and hence the ecological breadth of the species (Bazzaz, 1996; Sultan et al., 1998). As an example, trichomes in plant are considered as morphological trait that can impose resistance to herbivore insects (Fernandes 1994; Wagner et al. 2004; Medeiros, 2007). Furthermore, in standing water, plants tend to reduce their total dry mass and leaf number to cope with increasing nutrient stress (Zhang, 1996; Crossley et al., 2002), by an increased allocation to root and stem and a decreased allocation to leaves (Madsen and Cedergreen, 2002), reduce the water content of their organs (Craine et al., 2001), reduce leaflet number, and reduce specific leaf area (Li et al., 1999). These trait values correspond to adaptations observed, also in nutrient-poor

habitats. In addition to that morphological adaptations reduce aimed to damage risk have also been identified in plants exposed to mechanical stress. A reduced size and allocation to leaves exposed to flow, together with an increased allocation of below-ground organs (Idestam-Almquist and Kautsky, 1995; Bagger and Madsen, 2004) would result in weak forces and a greater anchorage efficiency, thus reducing the uprooting risk (Puijalon et al., 2005). Moreover a reduced leaf number and leaflet number per leaf (Puijalon and Bornette, 2006) could make the reconfiguration into a more streamlined shape easier (Pratt and Johnson, 2002). Also, the reduced water content of organs and a lower specific leaf area (Retuerto and Woodward, 1992; Boeger and Poulson, 2003), revealing thicker leaves and denser tissues, probably provide greater strength against breakage (Niklas, 1999). The above-mentioned morphological traits were also used in order to identify species, genera, to evaluate systematic relationships, and to discriminate between varieties. In practice, distinctness is assessed by comparing the variety (or variety mean) of the candidate with the means of varieties of a reference collection (Smykal, 2008).

1.3.1 Number of Stomata

A wide variety of anatomical and morphological adaptations enable plants to survive and grow under dry or wet conditions. Among the most important adaptations are variations in stomatal numbers, arrangement, size and behavior, which vary widely among plants. Stomata are the pathway for gas exchange (transpiration), where most of the water leaves the plant (Gurevitch, 2002). Stomatal features are used in taxonomic differentiation (Nabin, 2000) and also give clues about plant culture; when and how plants are affected by environmental factors.

Stomata numbers can change according to species and cultivars (Kliewer, 1985; Caglar, 1999). Yang-Chuan (1998) stated that stomata are stably inherited and can be used to distinguish cultivars and lines of apples. In this respect, a comparative study was conducted on the foliar epidermis and venation pattern of *Aquilaria khasiana* and 3 phenotypic variants of *A. malaccensis* in North-East India. Some quantitative values such as stomata density, stomatal size, stomatal index, and size of aeroles were found to be very significant in differentiating the taxa at species and interspecies level (Bozoglu, 2006). In some resources, it has been reported that stomatal density change with changes in water supply (drought), net photosynthesis production (Bierhuizen, 1984), vegetative development phases (Caglar, 1999) and altitude (Caglar, 2004). In another study with seedless grape the stomatal density changed in relation to the years and cultivars, and there was no correlation between stomata numbers and leaf characteristics (Kara, 1999). Moreover, it was established that stomatal density is an important factor in resistance against diseases. It is proposed that stomatal density and conductance of lower leaves on tabacco plant be used as a selection marker for disease-resistance breeding programs (Yang, 2004). Further more, other results showed that the resistance of downy mildew in cucumber was negatively correlated to stomatal density (Gu, 2004).

The acquisition of stomata and an impervious leaf cuticle are considered to be key elements in the evolution of advanced terrestrial plants, allowing the plant to inhabit a range of different, and often fluctuating environments but still control water content (Hetherington, 2003). Even experiments examining effects of elevated CO_2 seem to study few anatomical responses other than changes in stomatal density (Raschi et al., 1997; Arnold, 1999). Carbon

dioxide (CO_2) enrichment changes the stomatal density of different species and different accessions (ecotypes) of *Arabidopsis thaliana* (Woodward, 2002). With stomatal densities ranging from 45 to 720 mm the mean response is an 11% reduction in density with the doubling of the CO_2 concentration, in a way that is insensitive to the basal stomatal density. The reduction in stomatal density with CO_2 enrichment leads generally to a decrease in maximum stomatal conductance but an increase in the maximum rate of photosynthesis (Beerling, 1997; Woodward, 1998).

Royer (2001) suggested that this increased frequency of response was due to the transition from a variable plastic response by species in short-term experiments to a genetic response, as a result of selection on longer time scales. Supports the suggestion that genetic change can alter the stomatal density–size relationship, whereas environmental changes seem primarily to move the position of a species along the stomatal size–density curve.

1.4 Fresh weight

Rapcan et al. (2006) found that pea biomass production is influenced of environmental conditions. According to Marcos-Filho and McDonald (1998) the seed quality is defined by its genetic, physical, physiological and sanitary characteristics, which influences the agro-ecological conditions of production (McDonald, 1998; Siddique and Wright, 2004; Nemeskeri, 2004; Fallon et al., 2006.; Fowler et al., 2006), seed processing (Schaffer and Vanderlip, 1999), and storage conditions (Vieira et al., 2001; Gordana, 2007). Ayodele (1990) found that fresh and dry weights were significantly reduced by drought stress at many reproductive stages. Another study done by Okcu et al. (2005) found that NaCl reduced the seedling fresh weight of pea plant.

1.5 Physiological aspects

The major environmental factors; light intensity, air, soil temperature and humidity, have profound influence on physiological processes. Physiological mechanisms for rapid and long-distance communication between plant tissues and organs, particularly in response to such external stimuli, are poorly understood (Kang, 2002).

Water stress results usually in stomatal closure and reduced transpiration rates. In addition to that a decrease in the water potential of plant tissues, decrease in photosynthesis and growth inhibition, accumulation of abscisic acid (ABA), proline, mannitol, sorbitol, formation of radical scavenging compounds (ascorbate, glutathione, α-tocopherol etc.), and synthesis of new proteins and mRNAs are the normal responses. Besides these physiological responses, plants also undergo morphological changes. One of the largest changes is the adaptation of plants and chloroplasts to high light (sun) and low light (shade exposure) (Yordanov, 2003).

1.5.1 Total chlorophyll content

Chlorophyll content analysis has become one of the most powerful and widely used techniques available to plant physiologists and ecophysiologists (Maxwell, 2000). Most studies on photosynthesis have focused mainly on species of economic importance, while similar studies on wild species are relatively rare (Goldstein et al., 1991; Filho, 2004). The measurement of chlorophyll content has become established as a sensitive method for assessing photosynthetically the efficiency of PSII and its role in environmental perturbations of photosynthesis (Baker 1991; Bolhàr-Nordenkampf and Öquist 1993; Figueroa, 1997).

The Mediterranean-type climate offers contrasting challenges for plant growth that oscillate between hot, arid summers with high irradiance and cool winters with irregular rainfall and episodic frosts. Plants growing in such an environment should, in principle, represent a good test of the ecological utility of field measurements of chlorophyll content. Year to- year variations in the abundance of many Mediterranean- grassland species can be correlated with similar variations in the accumulated rainfall over the growing season (Figueroa and Davy 1991).

1.6 Genetic diversity

Genetic engineering is the most revolutionary tool to impact agricultural research in recent years (Grover, 2001). Determination of genetic diversity in a gene pool is the key to crop improvement (Choudhury, 2007); the analysis of genetic diversity and relatedness among different individuals, species or populations is the central topic in genetics (Smykal, 2006). For estimation of genetic diversity and establishment of varietals identity, various types of marker systems are being used by plant breeders (Choudhury, 2007).

The last two decades have seen an exponential increase in the number of plant sequences in databases and the explosion of investigations on the molecular functions and physiological roles of these genes (Burstin, 2007). The functions of thousands of genes have been identified (Østergaard and Yanovsky, 2004). These tools can now be used to address the question of phenotypic plasticity—which genes control plant functioning in which environments—and to provide some clues about which forces shaped natural variation and the strategies that should be used to breed more adapted

cultivars (Paran and Zamir, 2003; Reymond et al., 2003; Koornneef et al., 2004; Mitchell-Olds and Schmitt, 2006).

1.6.1 Pea

Pea genetics has been an object of study for a long time (Knight, 1799; Mendel, 1866), but surprisingly it is only relatively recently that a coherent picture has emerged of the overall structure of the genome, both in terms of karyotype and the organization of the genetic linkage groups. At present, pea is unpopular as a model for genetic analysis because plants are large and the genome is complex (Ellis, 2002). However, the large genome provides an opportunity to study genome architecture, and the large size of the plants and their suitability for physiological and biochemical analysis may yet prove an advantage in some contexts. While there are current technical and financial obstacles to the pursuit of pea genetics and genomics, this organism has many interesting mutants available for study (Hofer and Ellis, 1998; Ellis, 2002). The genetic map of pea has been developed gradually over time, from the initial version given by Lamprecht in 1948 up to the latest, published in 1998 by the *Pisum* Mapping Committee (Irzykowska, 2002).

In Palestine the pea varieties available to growers are either locally developed from crossing and selection for improved yield, adaptation, and disease resistance or introduced as new varieties developed through breeding programs. However, the relatively narrow gene pool (Heath and Hebblethwaite, 1985) and the heavy use of a small number of parents by competing breeding programs have led to low genetic diversity among pea varieties (Baranger et al. 2004; Simioniuc et al. 2002). Like all major crop species, cultivated peas have a condensed gene pool relative to its wild relatives (in this case *P. fulvum* and *P. elatius*), and the relationships within

the genus *Pisum* have generated substantial debate (Jing, 2007). Studies have indicated that *P. fulvum* can reasonably be considered as a distinct species, with *P. sativum* forming a subset of *P. elatius* (Vershinin et al. 2003; Baranger et al. 2004; Tar'an al. 2005; jing, 2007). Recent researchers also isolate *Pisum abyssinicum* as bona species, represented by cultivated and some wild forms from South Arabia and Ethiopia which differ from *Pisum sativum* by chromosome rearrangements as well as some morphological traits. Ellis et al. (1998) suggested that *Pisum* genus includes three main groups *P. fulvum, P. abyssinicum*, a distinctive Ethiopian form domesticated independently from *P. sativum* and all the other *Pisum* subspecies. Including two wild groups, i.e. *P. humile and P. elatius*, both considered as the basis for domestication of cultivars and *P. sativum* subspecies. Sativum, which comprises wild and cultivated forms, among which morphologically distinct types, dedicated to different end-uses, have been, selected (Smart, 1990). Samec and Nasinec (1996) classified the peas using Random Amplification of Polymorphic DNA (RAPD) technique in the subspecies *P. arvense, P. humile, P. sativum and P. abyssinicum*. It is worth to mention that between the different species there was extensive genetic variability, for example, in plant height, leaf type, number of branches and pods.

Main germplasm collections have accumulated a great number of wild pea accessions, but many of those are doubled under different designations, and some are contaminated by genes of other wild and cultivated forms in the course of reproduction that poses difficulties for classification and phylogenetic analysis (Oleg, 2008). Such information is critical for the design of optimal breeding strategies for continued progress in pea improvement. In a plant breeding program, estimates of genetic relations among parental lines

may be useful for determining which material should be combined in crosses to maximize genetic gain. Diverse genetic background among parental lines provides an ample supply of allelic variation that can be used to create new favorable gene combinations (Tar'an, 2005). Based on quantitative genetic theory, the probability of producing unique genotypes increases in proportion to the number of genes by which parents differ. In soybean (*Glycine max* L. Merr.), for example genetic variance for yield was positively associated with parental genetic distance, and genetic variance declined to near zero when the coefficient of parentage was above 0.27 (Manjarrez-Sandoval et al. 1997). In studies with oat (*Avena sativa* L.), genetic variance was also positively associated with genetic distance (Cowen and Frey 1987). Kisha et al. (1997) made a general observation that the more distantly related soybean parents generated larger genetic variances than closely related parents, although they concluded that genetic distance did not accurately predict the genetic variance for any given cross. In studies with wheat (*Triticum aestivum* L.), Souza and Sorrells (1991) and Cox and Murphy (1990) reported that the relation between genetic distance and variance varied among traits and populations.

1.6.2 Molecular markers

The development of cultivated species and the breeding of new varieties have always relied on the availability of biological diversity, issuing from the long-term evolution of species. Modern plant breeding methods focusing on wild adaptation and high crop yield and intensive selection on crop species have raised the question of the amount of genetic variation still available in breeding pools for sustainable improvement (Hodgkin, 1995). Together with a need for biodiversity conservation, the need for an assessment for crop diversity and a better understanding of the impact of breeding on this diversity has emerged. Molecular markers have allowed the study of diversity through

DNA sequence variation, thus facilitating the understanding of crop species domestication (White and Doebey, 1998), the deciphering of elite cultivar breeding history (Dubreuil and Charcosset, 1999; Narvel et al. 2000; Russell et al. 2000), the assessment of genetic diversity within germplasm and/or cultivated types for various species (Prasad et al. 2000; Liu et al. 2001; Metais et al. 2002; Steiger et al. 2002) and the identification of cultivars (Rongwen et al. 1995; Russell et al. 1997; Baranger, 2004). Recently, molecular marker have been used to examine the effect of modern plant breeding method on genetic diversity in barley (Russell et al. 2000; Koebner et al. 2003) wheat (Christiansen et al. 2002; Soleimani et al. 2002), and maize (Lu and Bernardo 2001). These studies concluded that the level of genetic variability within the cultivated plants had been maintained during modern selection, either through the differentiation of heterotic groups or through the maintenance of independent breeding programs (Baranger, 2004).

During the past few decades a wide variety of DNA based markers have been developed. These markers have the potential to provide useful information regarding genetic diversity, genotype identification and the estimation of relatedness between different genotypes. One of the major advantages of molecular markers (for e.g. RFLP, RAPD, SSRs etc.) is their independent of environmental interactions. Moreover, molecular markers do not show stage specific expression of characters and provide high degree of polymorphism and thus preferred over other marker systems (Choudhury, 2007). Accordingly, Tinker et al. (1993) suggested that diversity estimates based on molecular markers are better suited than pedigree data for parental selection.

The use of molecular markers for diversity analysis can also serve as a tool to discriminate between closely related individuals from different breeding

sources (Lombard et al. 2000; Métais et al. 2000; Sun et al. 2001), and it may help to eliminate redundancy in phenotype based germplasm collections (Tar'an, 2005).

1.6.2.1 Randomly amplified polymorphic DNA (RAPD)

Williams *et al.* (1990) described a novel type of genetic markers based on DNA amplification but requires no knowledge of target DNA sequence. These RAPD markers are generated by the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Laucou, 1998); RAPD markers can be used for genetic mapping applications, cultivar identification, phylogenetic studies, and diversity studies (Rameau, 1998). Amplification with RAPD primers is extremely sensitive to single base changes in the primer target site, which makes RAPDs a highly useful technique for phylogenetic analysis of closely related individuals (Cheghamirza, 2002). Also RAPD is a multi locus marker (Karp et al. 1997) with the simplest and fastest detection technology and used for diversity analysis in several crop plants (Choudhury, 2007), and it was used to evaluate and reveal genetic polymorphism in rice (Virk et al. 1995; Choudhury et al. 2001), wheat (Cao et al. 2000), maize (Pejic et al. 1998), barley (Yu et al. 2002), leguminous crops like mungbean (Lakhanpaul et al. 2000), *Cicer* (Ahmad, 1999), adzuki bean (Yee et al. 1999) and pea (Wolko et al. 2000; Simioniuc et al. 2002; Tar'an et al. 2005; Choudhury et al. 2007). Concerning peas Tar'an et al. (2005) used 21 RAPD primers to distinguish between 65 varieties and 21 accessions from wild *Pisum* subspecies (subsp.): *abyssinicum, asiaticum, elatius, transcaucasicum*, and var. *arvense*. Their main finding was that accessions could be distingushed by comparing polymorphism in genomic fingerprints. In this respect, Nei and Li's genetic similarity (GS) estimates calculated using

the marker data showed that pair-wise comparison values among the 65 varieties ranged from 0.34 to 1.00 genetic similarity (GS). Furthermore, in Cheghamirza (2002) study, 13 RAPD primer were used for identifying and mapping new molecular markers in pea (*Pisum sativum*); RAPD analysis of various cultivars and lines of pea was carried out using 10-mer random primers. The study of individual plant DNA from the F2 populations allowed determining the genetic distances between genes and the RAPD markers linked to them. Rana and Bhat (2005) estimated the genetic diversity in 59 cotton cultivars belonging to four cultivated species of cotton. Eighteen RAPD primers produced a total of 251 amplicons, generating 97.21% polymorphism. The number of amplification products ranged from 7 to 24 for the different primers, whereas the per cent of genetic similarity for the studied primers ranged from 42 to 79%. Among the 59 cotton cultivars, 36% genetic diversity was observed. In 41 *Gossypium hirsutum* cultivars included in the study, the average genetic similarity was 74%. More genetic diversity was observed in diploid than in tetraploid cotton cultivars. Un-weighted pair group method using arithmetic averages (UPGMA) cluster analysis placed tetraploid cotton cultivars into two distinct clusters that are in agreement with the traditional taxonomic arrangement of these cultivars. Analysis of molecular variance in *G. hirsutum* cultivars revealed that most of the variance could be attributed to within breeding-centre variance. All the cultivars could be discriminated from one another based on the combined profiles for eighteen oligonucleotide primers. A negative correlation between average genetic similarity for a primer and the number of cultivars identified and observed. Genetically distinct cultivars were identified that could be potentially important sources of germplasm for further cotton improvement in India.

1.6.2.2 Simple Sequence Repeats (SSRs)

Microsatellites consist of tandem repeats of mono-, di-, tri- or tetra-nucleotide patterns (Burstin, 2001). They are also referred to as simple sequence repeats (SSRs). These sequences provide an interesting source of markers, as they are frequent in eukaryote genomes and are well distributed throughout the genome. The sequencing of the flanking regions of a microsatellite allows one to design specific primers to amplify the fragment containing this microsatellite using PCR. The length polymorphism of the amplified fragment is then visualized following agarose or acrylamide gel electrophoresis. These markers are highly polymorphic, and their length polymorphism results from a variable number of tandem repeats (VNTR), probably stemming from replication slippage and/or unequal recombination. High levels of polymorphism have already been observed with this kind of marker (Saghai-Maroof et al. 1994, Innan et al. 1997, Udupa et al. 1999; Burstin et al. 2001).

To relate the results obtained in different populations for the same or for different traits, the development of common tools for applying molecular markers becomes important. The availability of highly polymorphic, locus-specific, easily transferable and cost-effective molecular markers distributed throughout the genome is of great value (Loridon, 2005). Because microsatellite markers have all these qualities and are the choice markers for many applications, significant effort has been dedicated to their development in various species during the last decade. They are now widely used in plants for genetic mapping, genetic diversity assessment, population genetics and marker assisted selection (de Vienne 1998, Gupta and Varshney 2000) and for investigating genetic diversity among cultivars. SSR maps now exist for a large range of species of maize (Sharopova et al. 2002); sunflower (Yu et al.

2003); wheat (Somers et al. 2004); bean (Blair et al. 2003); peanut (De Carvalho Moretzsohn et al. 2004); strawberry (Sargent et al. 2004); lytchee (Viruel et al. 2004); kiwi (Fraser et al. 2004); cacao (Pugh et al. 2004); soybean (Song et al. 2004); grape (Adam- Blondon et al. 2004); Eucalyptus (Brondani et al. 2002); melon (Ritschel et al. 2004); cassava (Mba et al. 2001); cherry (Dirlewanger et al. 2004); and Rose (Yan et al. 2005).

For pea (*Pisum sativum* L.), genetic maps have been also developed using different types of markers (isozymes, RFLPs, RAPDs, AFLPs), and trait loci (Ellis and Poyser 2002; McPhee 2005), and a comprehensive consensus linkage map was developed integrating linkage relationships from multiple maps and linkage studies (Weeden et al. 1998; Ellis and Poyser 2002). However, few studies have used microsatellite markers in pea for either mapping studies (Weeden et al. 1998; Pilet-Nayel et al. 2002; Prioul et al. 2004) or diversity assessment (Burstin et al. 2001; Baranger et al. 2004; Tar'an et al. 2005; loridon et al. 2005). In their study, Tar'an et al. (2005) used 25 SSR primers to distinguish between 65 pea varieties (*Pisum sativum* L.) and 21 accessions from wild *Pisum* subspecies (subsp.): *abyssinicum*, *asiaticum*, *elatius*, *transcaucasicum*, and var. *arvense*. They concluded that accessions could be distingushed by comparing polymorphism in genomic fingerprints.

In another study done by Choudhury (2006), 24 popular and widely adapted varieties of pea (*Pisum sativum*) were subjected to RAPD analysis to find out the genetic relatedness among them using 60 decamer primers. Primers used were found to be polymorphic and seven of them showed 100% polymorphism. Among their finding, it was obvious that the 1053 genotypes can be unambiguously distinguished by employing 60 RAPD primers.

Moreover, Burstin (2001) worked in microsatellite polymorphism in *Pisum sativum*, using 43 microsatellite markers, which were generated from these sequences and used to assess the genetic variability among 12 pea genotypes: 31 of these marker were polymorphic and the average number of variants per marker was 3.6. Overall, the number of variants for a given SSR marker was correlated with the length of the SSR but some 12-bp long SSRs showed the same degree of polymorphism as longer ones. The groupings resulted from the SSR genotyping among the 12 genotypes gave an interesting insight into the possible origin of one recent cultivar.

1.6.2.3 Why more than one primer?

Different marker methods can give different views of diversity, depending upon the evolutionary parameters of the underlying DNA sequence variation. Rapidly evolving DNAs, such as simple sequence repeats (SSRs), give high resolution views of relatedness (Jing, 2007).

Furthermore, the genomic compartment in which the markers reside might affect the diversity pattern seen, and it is possible that markers residing mainly in junk DNA might produce different results from markers based upon genes, which are predominantly euchromatic (Jing, 2007). For these reasons there is a need to compare the diversity patterns obtained using different molecular approaches for diversity assessment.

1.6.2.4 Comparison between RAPD and SSR

Hussein et al. (2002) used 49 RAPD, 14 ISSR, 8 SSR primers and 6 AFLP primer combinations to estimate the genetic variability among 12 Egyptian cotton varieties (*G. barbadense*) and one *G. hirsutum*. The level of

polymorphism among all genotypes as revealed by RAPD, ISSR, SSR and AFLP was 30.4%, 53%, 68%, and 56.3%, respectively, although the variability levels among the 12 Egyptian barbadense genotypes were 24.9%, 44.4%, 58.9% and 43.1%, respectively.

Agrama and Tuinstra (2003) applied two DNA-based fingerprinting techniques; SSR and RAPD analyses, in sorghum germplasm analysis to compare suitability for quantifying genetic diversity. The 22 sorghum genotypes, representing an array of germplasm sources with important agronomic traits, were assayed for polymorphism using 32 RAPD primers and 28 sets of SSR primers. The results indicated that SSR markers were highly polymorphic with an average of 4.5 alleles per primer. Where as the RAPD primers were less polymorphic with nearly 40% of the fragments being monomorphic. An analysis of genetic diversity among sorghum lines indicated that the genetic distances calculated from SSR data were highly correlated with the distances based on the geographic origin and race classifications. Based on the results of these studies, SSR markers appear to be particularly useful for the estimation of genetic similarity among diverse genotypes of sorghum. On the other hand, Hussein *et al.* (2006) assayed 21 cotton accessions using 28 RAPD and 12 ISSR primers, in addition to 24 SSR specific primer pairs. The total number of amplicons detected by RAPD, ISSR and SSR were 323, 125 and 62, respectively. Where as the number of polymorphic amplicons were 191, 62 and 39, respectively. Thus, the level of polymorphism as revealed by RAPD, ISSR and SSR were 59.1%, 48.8% and 53.7%, respectively. However, all dendrograms clearly clustered accessions belonging to *G. hirsutum* in one group and those of *G. barbadense*, expect Pima Early American, in another group. Furthermore, the five SSR alleles were able to discriminate between the accessions belonging to the species *G.*

barbadense and those of *G. hirsutum*. In another study Meszaros *et al.* (2007) determined the genetic relationships between 38 barley genotypes using of 36 RAPD, 54 STS and 26 SSR markers. The dendrogram groups showed high coincidence with growth habit and ear type. There were significant correlations between the Jaccard coefficients obtained using the matrices of each single marker type and their combined matrix. When the varieties were grouped using markers with above-average Polymorphic Information Content (PIC) values, the same groups were obtained as when using all markers, outlining their usefulness for estimating diversity between the varieties. Based on Meszaros *et al.* study, three RAPD or four SSR primers are sufficient to distinguish all the barley varieties from each other, although the applicability of the various types of primers differed. The STS markers could best be used for estimating relationships between the varieties and the SSR markers for distinguishing genotypes from each other, while RAPD markers could be employed both for estimating the relationships between varieties and for variety identification.

1.6.2.5 Amplification of the Internal Transcribed Spacer (ITS):

For more than a decade, sequencing of ribosomal DNA (rDNA) has become a commonly used procedure and one of the most widely applied molecular markers for reconstructing plant phylogenies at the intrafamily level (intra- and intergeneric, and interspecific levels) (Soltis et al. 1998; Baldwin et al. 1995). Nuclear ribosomal DNA is classified as a multigene family and is arranged in the Nucleolar Organizing Region (NOR) in arrays of hundreds to thousands of copies. Each unit within this array is composed of specified segments (Figure 1). The two internal spacers, ITS1 and ITS2 are located between genes encoding 18S (small subunit), 5.8S and 26S (large subunit)

nuclear ribosomal RNA. Both spacers ITS1, ITS2 and 5.8S are referred to as the ITS region (Polans, 2002).

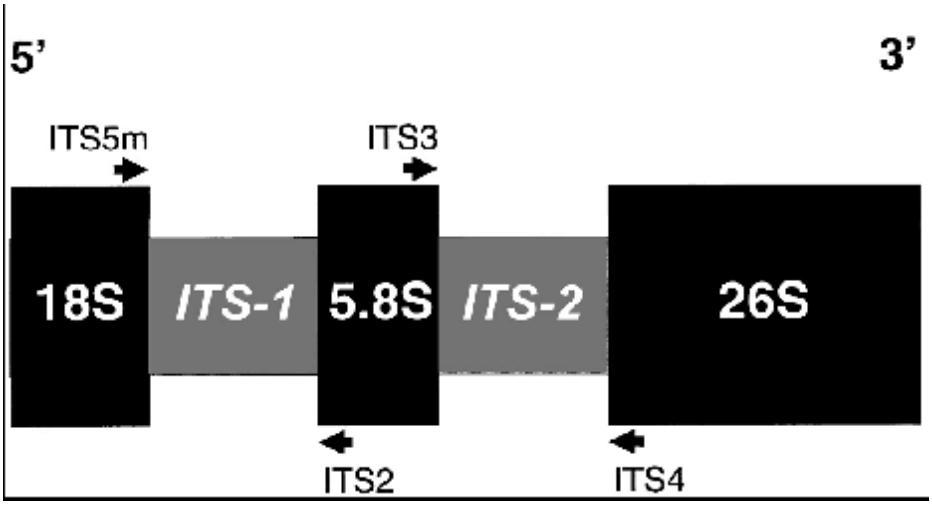


Figure (1) the three coding and two internal transcribed spacer regions of the nuclear ribosomal DNA repeat unit of a typical angiosperm (not drawn to scale).

Accordingly some criteria make the ITS region (18S-5.8S-26S) a powerful marker in inferring phylogenetic relationships. Hong (2003) worked on analysis of nucleolar pre-rRNA processing sites in pea (*Pisum sativum*) using hybridization with ITS1 probe in pea (*Pisum sativum*). The results showed that rRNA processing sites were in dense fibrillar components (DFCs) and granular components (GCs), but not in fibrillar centers (FCs). Polans (2002) also studied the ITS sequence variation in wild species and cultivars of pea. He revealed a number of base differences separating *P. fulvum* from 52 *P. sativum* accessions ranges from 11 to 17, with 10 of these sites being unique to *P. fulvum*.

Moreover Saar (2000) examined pea ITS-1 and ITS-2 regions, his study contain 298 and 349 alignable base pairs (bp), respectively, totaling 647 bp for each of the plants analyzed. Researchers conclude that ITS sequence variation for the selected taxa of his study suggests very close genetic affinities throughout the genus *Pisum*, with *P. fulvum* exhibiting the greatest degree of divergence.

Chapter Two

Materials and Methods

2.1 Plant sampling

Seven pea accessions were used in this investigation, two commercial from Canada and from Fito company Spain , three local accessions two of them are collected from farmers in Dahria and Dura towns and the third one is from the Union of Agricultural Work Committees (UAWC), and two wild accessions collected from two different sites (Bani-Noaim and Dura). Each accession was comprised of ten pea plants (replicates), (Tables 1, 2 and Figure. 3)

Table (1) Sources of the seven pea accessions

	Sources	**Location**
Comm. Canada	Canada	-
Comm. Spain	Fito Co.	-
Local UWAC	UAWC	Farmer in Al-klab valley
Local Dahria	Dahria	Mohammed Naif
Local Dura	Dura/Bet-alrosh	Ismael Abu-sharar
Wild Bani-noaim	Wild	Bani-Noaim
Wild Dura	Wild	Dura

Table (2) the geographical position of the wild pea accessions

Location of wild	Latitude N^o	Longitude E^o	Altitude (m)
Bani-Noaim	51.89	50.76	677
Dura	22.40	45.23	460

2.1.1 Plant material

For wild pea accessions sampling, ten samples of fully mature pea plant from each location were collected during March through April, 2008. Samples were placed in a paper bag and taken to Hebron University Agricultural Lab and stored at $4C^{o}$ until the assessment.

Seeds of local and commercial accessions were planted in Al-Arub Agricultural Station in 18-3-2008 in a completely randomized design with 40 plants for each accession (Fig. 2). The planting distance was 50 cm between plants and 80 cm between rows. All agricultural practices were conducted according to standard procedures (drip irrigation, add fertilizers, weed control). Plants were checked at weekly intervals.

Figure (2) Local and commercial pea plant grown in Al-Aruob station

At full mature stage (31-5-2008), 10 plants (replicates) from each accession were selected randomly and taken for further assessments.

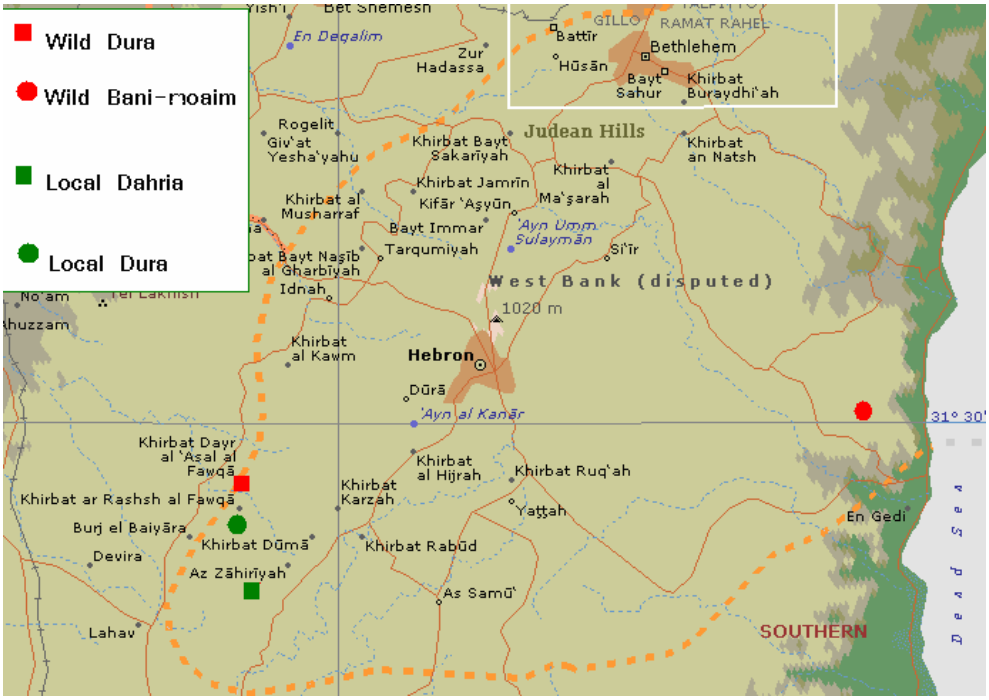


Figure (3) Map for location of local and wild pea accessions

2.2 Morphological Measurements

The distance between the base and the highest point of the longest branch of the plant was recorded as main stem length. Ten replicates for each plant group were measured, and the number of branches was counted for each plant from the base. The color of petals was also recorded.

The following morphological measurements were recorded:

a) Number of pods per plant and number of seeds per pod.

b) The weight of ten seeds taken randomly from each plant group.

c) Plant fresh weight (of shoot system).

d) Stomatal density: impressions were taken from the abaxial surface of fully mature leaves using nail polish, and examined subsequently under compound microscope.

For each accession, ten leaves were taken randomly, and each leaf was used to obtain five impressions. Stomatal number from every slide had been determined from three different microscopic fields under a magnification of 40x. Photos have been used to get stomata number per field.

2.3 Physiological Measurement

2.3.1 Total chlorophyll content

The chlorophyll content was evaluated using 10 replicates according to Arnon (1949) 0.2 g pieces of fresh leaves was added to 10 ml acetone (80%), incubated for 30 minutes in ultrasonic bath, followed by over night incubation at room temperature. Second incubation was for 30 minutes in ultrasonic bath, second addition of 10 ml acetone 80%, for the third time the solution was incubated for 30 minutes in ultrasonic bath and finally incubation for 4 hours, followed by 30 minutes in ultrasonic bath.
The volume of the supernatants was completed with acetone (80%) to 50 ml (Fig. 4). Detection was carried out at 645 nm and 663 nm using spectrophotometer.
Recorded numbers were applied on the following equation:

$$\text{mg chlorophyll /0.2gm fresh weight} = 20.2A_{645nm} + 8.02A_{663nm}$$

Then the values were calculated for 1 gm fresh weight by dividing them on 0.2.

Figure (4) Flasks ready for measuring chlorophyll content by spectrophotometer for pea accessions

2.4 Genetic Measurement

2.4.1 Plant Material

Samples of the wild peas were used for morphology and physiology measurement and also used for DNA extraction. The local and commercial plants (five group accessions) were planted in 11-5-2008 in greenhouse (Fig. 5). Thirty plants from each accession group were planted, and then 10 samples (replicates) were chosen randomly for DNA extraction.

Figure (5) Local and commercial accessions seedling grown in the greenhouse for DNA extraction

Pea accessions with ten replicates of young fresh leaves for each accession were used for genetic measurements.

2.4.2 DNA extraction, purification, and quantification

2.4.2.1 DNA extraction and purification

DNA was isolated using a modified version of the protocol described in Fulton (1995). Approximately 4-8 new leaves, up to 1.5 cm long from a 1-3 week old pea seedling were loosely placed in the bottom of mortar, prepare fresh microprep buffer (2.5 parts DNA Extraction Buffer, (0.35 M Sorbitol, 0.1 M Tris-base, 5 mM EDTA; pH 7.5), 2.5 parts Nuclei Lysis Buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, 2% CTAB), 1.0 part 5%

Sarkosyl). Sodium Bisulfite (0.3-0.5 g) was added then kept at room temperature. Buffer 200µl were added and grind tissue with pestle, another 550µl were added of buffer and either vortex lightly or shake entire rack by hand. Then samples were incubated at 65^{o}C water bath for 30-120 minutes.

After that chloroform/isoamyl (24:1) was added and the mixture was shaked 50-100 times, subjected farther to centrifugation at 13,200 rpm for 10 minutes, and the aqueous phase was taken. To precipitate DNA, two volumes of cold isopropanol were added, and tubes were farther centrifuged at 13,200 rpm for 10 minutes. Pellet was washed with 70% ethanol, and dried at room temperature for one hour.

DNA was resuspended in 50 µl of TE at 65 C^{o} for 15 minutes, and finally stored in -20 C^{o} until future use, upon use; DNA was purified from RNA by adding 5 µl of RNAse (1mg/ml) Sigma, incubated over night.

2.4.2.2 Estimation of DNA quantification

DNA quality and quantity was tested on 0.8% agarose gel electrophoresis using Lambda DNA as a standard. Other measurements done in Palestine Polytechnic University (PPU), Biotechnology Research Unit for DNA concentration and purity using spectrophotometer. Final concentration of DNA was adjusted to 25ng/µl.

2.4.3 RAPD and SSR primer sequence

Twenty five RAPD primers were used according to Tar'an (2005), and Cheghamirza Kianoosh (2002). Moreover, another 16 SSR primers were used according to Tar'an (2005), and Burstin (2001). All reactions were conducted using a thermocycler in PCR (PTC-200) to obtain the polymorphic primers.

The six polymorphic primers (tables 3 and 4) were used for the genetic fingerprinting.

Table (3) Sequences of the six RAPD primers used to detect polymorphism in pea accessions

No.	Primers	Sequences (5'-3')
1	BC261	ctggcgtgac
2	BC305	gctggtaccc
3	BC318	cggagagcga
4	BC329	gcgaacctcc
5	G10	agggccgtct
6	R11	gtagccgtct

Table (4) Sequences of the six SSR primers used to detect polymorphism in pea accessions

No.	Primers	Forward sequences	Reverse sequences
1	PSMPD21	tattctcctccaaaatttcctt	gtcaaaattagccaaattcctc
2	PSMPD23	atggttgtcccaggatagataa	gaaaacattggagagtggagta
3	PSMPSAD135	tggcattagattctccagcaca	tgaggaggtgaacgtaaaagca
4	PSMPSAD141	aatttgaaagaggcggatgtg	acttctctccaacatccaacga
5	PSMPSAD148	gaaacatcattgtgtcttcttg	ttccatcacttgattgataaac
6	Y14273	aattcggcacgaggagaga	tgcagccttgagctggttat

2.4.4 Randomly Amplified Polymorphic DNA (RAPD) PCR reaction mixture and program

DNA amplification was done in a PTC-200 Peltier thermal cycler.

Amplification was carried out in 20 µl volumes, containing 25 ng template DNA, 1 µl 50 mM $MgCl_2$, 2 µl 10X (10 mM Tris-HCl, pH 8.8 and 50mM KCl), 0.2 µl of a 2 mM dNTPs solution in equimolar ratio, 0.8 µl of primer at 10 pmol/µl, and one unit taq polymerase. PCR program was as following: 3 min at 94^{o}C, followed by 40 cycles of 30 s at 36 ^{o}C, 1 min at 7 2 ^{o}C and 20 s at 94^{o}C, and a final elongation step 72 ^{o}C for 8min. After the run, samples were stored at 4 ^{o}C until analysis (Arafeh et al, 2002).

2.4.5 Simple Sequence Repeats (SSRs) PCR mixture and program

Amplification was carried out in 20µl volumes, containing 50ng template DNA, 1µl 50mM MgCl2, 2µl 10X (10mM Tris-HCl, pH 8.8 and 50mM KCl), 0.2µl of a 20mM dNTPs solution in equimolar ratio, 2µl primer at 10 pmol/µl, and one unit taq polymerase. PCR program was as following: initial step of 3min at 94C^{o}, 40 cycles of 30s at 50C^{o}, 1min at 71C^{o} and 20s at 94 C^{o}, and final elongation step at 72 C^{o}, for 8 min. Samples after run were kept at 4 C^{o} until analysis (Tar'an, 2005).

2.4.6 RAPD and SSR gel processing, evaluation and generating the binary data matrix

PCR products were electrophoretically separated on a 1.4% agarose gel containing ethidium bromide using 1X TE buffer. The amplified products were visualized and photographed under UV light source.

DNA bands were scored (1) for presence and (0) for absence for each primer- genotype combination. Only reliable and clear bands were scored. Only unambiguous bands were scored for the estimation of genetic similarity.

2.4.7 RAPD and SSR data analysis

Data matrix was utilized to generate genetic similarity data among genotype. Jaccard's similarity coefficient was used:

$$S_{ij,Jaccard} = \frac{n_{11}}{n_{11} + n_{01} + n_{10}}$$

Where n_{xy} is the number of characters that have state x in individual i and state y in individual j. Un-weighted pair group method using arithmetic averages (UPGMA) phenogram was then calculated from the Jaccard's similarity using fingerprint analysis with missing data (FAMD) software version 1.108 beta. Tree view software (Win32) version 1.6.6 was used to visualize the resulted trees. All trees presented in this study were midpointed.

Furthermore, analysis of principal coordinates (PCO) was carried out using a squared Euclidean distances matrix between all pairs of individuals and projected in three dimensions using FAMD software version 1.108 beta. The genetic structure (partition of genetic variation among groups "AMOVA") and FST values (Wright, 1951) were calculated using Arlequin software v.2.

2.4.8 Amplification of the Internal Transcribed Spacer (ITS):

2.4.8.1 Primer sequences and references which were used in the ITS amplification are shown in table (5).

Table (5) ITS Primers sequences and references

Primer codes	Primer sequences	References
ITS-4	5'-TCC TCC GCT TAT TGA TAT GC-3'	White et al. (1990)
ITS-A	5'-GGA AGG AGA AGT CGT AAC AAG G-3'	Blattner (1999)

2.4.8.2 Amplification of the Internal Transcribed Spacer

The reaction was performed in 25 µl volumes with 10% 10X buffer, 5% 50 mM $MgCl_2$, 2% of 20 mM dNTPs mix, 2% from each primer at 25 pmol/µl conc., 1.0 unit (0.2 µl) BioThermTM polymerase (GeneCraft), and 1.0 µl of template DNA (30-60ng/µl). The rest of the volume was filled up to 25.0 µl with DEPC-H2O. The PCR mixture was preheated for 1 min. at 94 ^{o}C, and then subjected to 35 cycles of 94 ^{o}C for 0.3 min., 55 ^{o}C for 0.5 min, and 72 ^{o}C for 1 min., and two final incubations of 1.3 min. at 55 ^{o}C followed by 8 min. at 72 ^{o}C. Amplification was checked by ethidium bromide staining using 0.7% agarose in 1xTBE buffer and documented.

2.4.8.3 Purification of PCR product and Sequencing reaction:

The PCR amplified products were purified using three cleaning kits:

The PCR sequences kit: big Dye terminator v1.1 cycle sequencing kit (Applied Biosystem), for sequencing PCR cleaning: gel filtration cartridges (EdgeBio), and cleaning of PCR product: wizard SV gel and PCR clean-up system (Promega). All kits were used according to manufacture instructions.

To check product recovery after purification, 5.0 µl of the purified product was loaded on 1.4% agarose gel.

2.4.8.4 Sequence editing and alignment:

Sequence data of the ITS region (ITS1-5.8S-ITS2) were manually edited and automatically aligned using Sequencher v.4.1.4 software. Boundaries of the ITS sequence were determined by comparison with representatives of Compositae subtribe Madiinae (Baldwin, 1992). Gaps of single nucleotides were treated as single-site or new state. For gaps longer than one nucleotide, the first site in the gap was recoded as a new state and all other sites were coded as 'missing data'.

Chapter Three

Results

3.1 Morphology:

3.1.1 Growth characteristics

Results show that stem length for pea accessions ranges between 30 cm to 65 cm (Fig. 6).

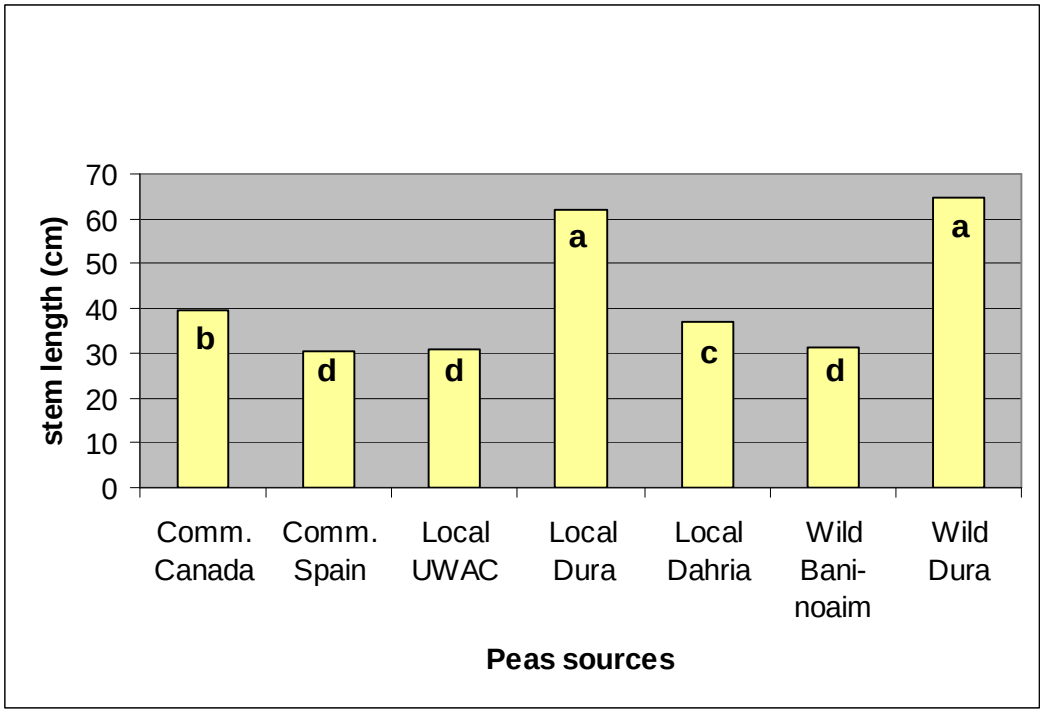


Figure (6) Average stem lengths (cm) of investigated accessions (LSD, $p \leq 0.05$).

Wild and local Dura pea accessions have significantly ($p \leq 0.05$) the highest stem length compared with other pea accessions (Fig. 6). The commercial Spain, local UWAC and wild Bani-Noaim stem length were significantly the shortest.

Data shows that local accessions and commercial Canada have significantly ($p \leq 0.05$) the highest fresh weight (Fig. 7), (local Dura= 42.5g); in versa the wild accessions have the lowest fresh weight (4g).

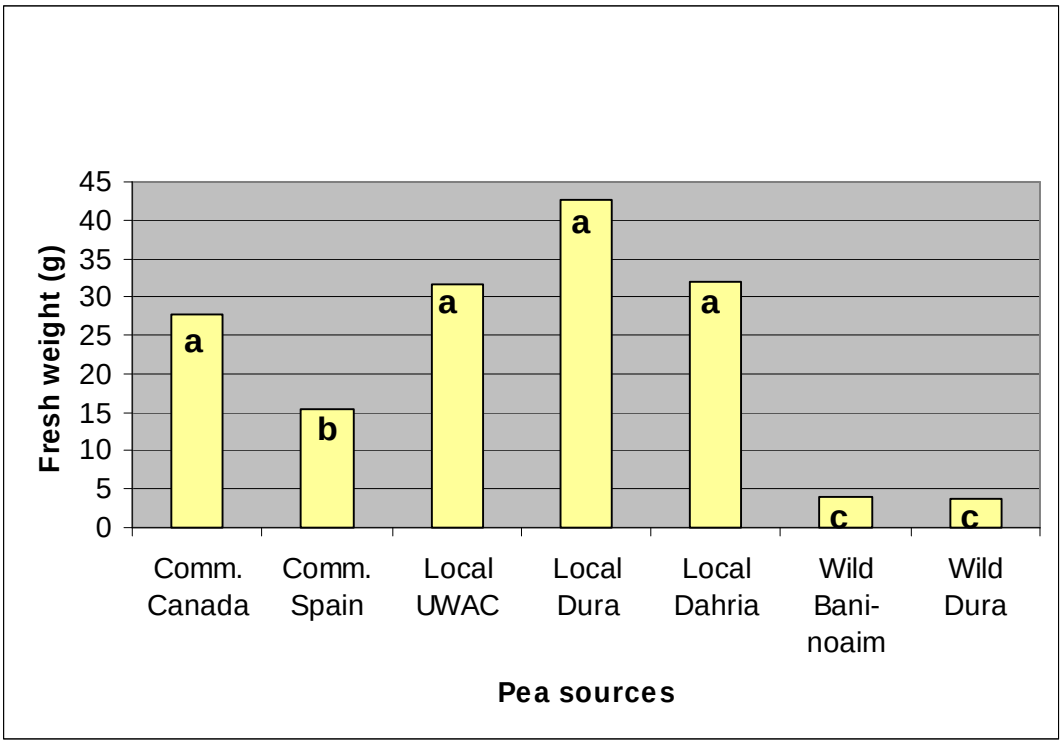


Figure (7) Average fresh weight (g) plant of the investigated accessions (LSD, $p \leq 0.05$).

Data show that the commercial accessions tend to have lower number of branches (Fig. 8).

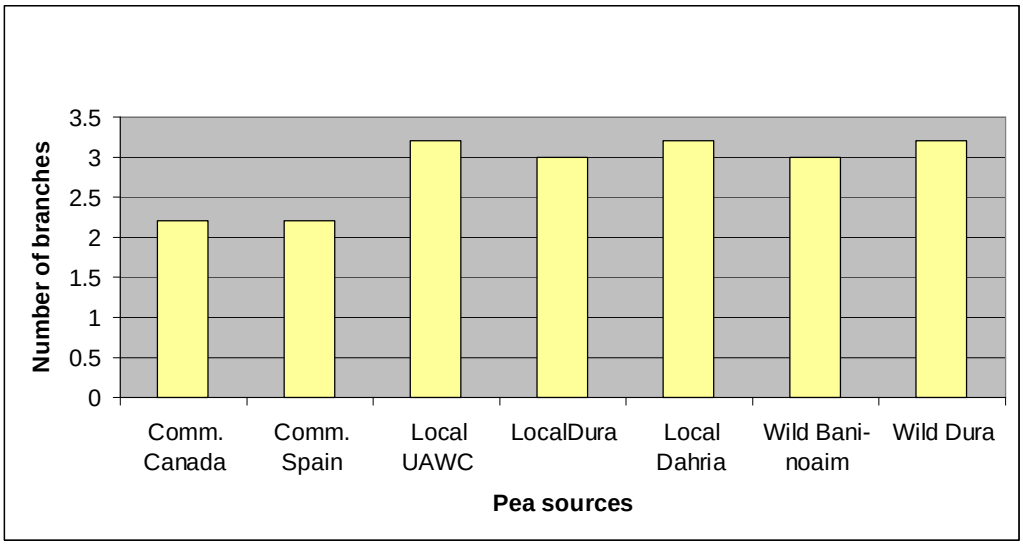


Figure (8) Average number of branch plant of investigated accessions

3.1.2 Fruit and seed production

Commercial variety from Canada and local pea accessions showed higher number of pods (Table 6), in comparison with wild accessions, and the commercial variety from Spain.

Table (6) Average number of pods, average number of seeds in pod; and average weight of ten seed of investigated accessions

	Commercial		Local			Wild	
	Canada	Spain	UAWC	Dura	Dahria	Bani-Noaim	Dura
No. of Pods	6.4 **ab**	3.2 **c**	8.4 **a**	5 **bc**	6.8 **a**	0.5 **d**	3.2 **c**
No. of seeds in pod	6.6 **a**	5.4 **ab**	6.2 **a**	5.8 **a**	7 **a**	1.6 **c**	3.7 **b**
Weight of 10 seed (g)	2.26 **b**	3.08 **a**	1.5 **d**	1.3 **e**	2.12 **c**	-	0.44 **f**

Means followed by the same letter in the same raw are not significantly different, according to Fisher LSD test at $p \leq 0.05$.

Wild Bani-Noaim and Dura accessions have significantly ($p \leq 0.05$) the lowest number of seed per pod (1.6 and 3.7), respectively, compared with commercial and local accessions (Table 6). Significant differences appear also between and within commercial, local, and wild accessions (Table 6). Commercial Spain and Canada accessions have the highest weight (3.0 g and 2.2 g), where as the wild accession (Dura) has the lowest weight (0.44 g).

3.1.3 Stomata density

Results show that commercial Canada and local UAWC have significantly higher stomatal density than other accessions, except local Dahria (Fig. 9, 10); wild accessions have the lowest stomatal density

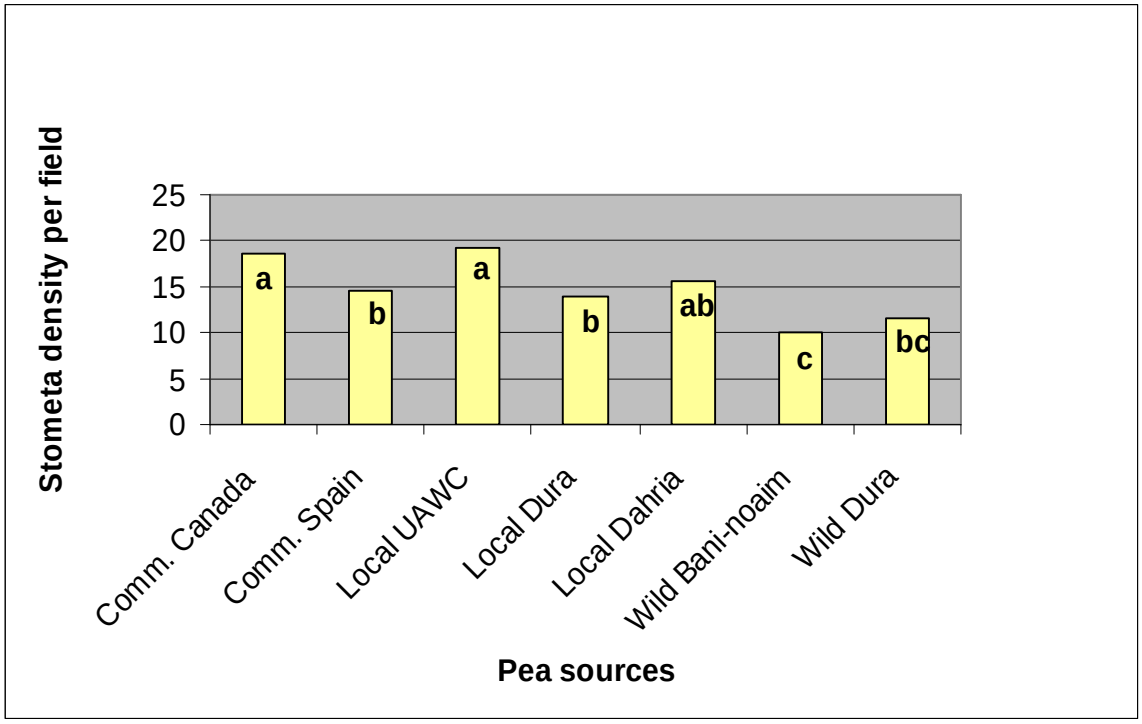
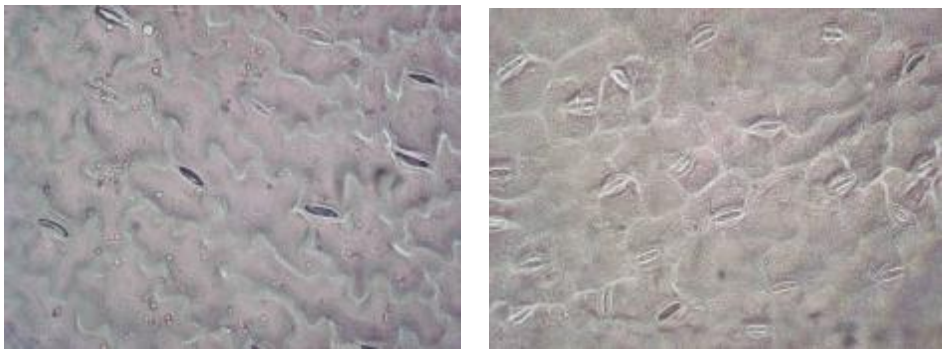


Figure (9) Average stomata density of investigated accessions



Wild Bani-Noaim Local UAWC

Figure (10) Microscopic photo of stomata for wild Bani-Noaim and local UAWC at 40X

3.1.4 Flower color

The investigated accessions show two flower colors, the violet and creamy (Fig. 11).

The violet flowers appeared in local Dura, wild Bani-Noaim and wild Dura accessions. On the other hand, the creamy flowers appeared in the rest of pea accessions.

Wild Dura flower Commercial Canada flower

Figure (11) Flower for wild Dura, and commercial Canada pea accessions

3.2 Physiological characteristics:

3.2.1 Chlorophyll content

Data showed that the highest chlorophyll content is for wild Bani-Noaim accessions, which is significantly higher than other accessions, except local Dahria (Fig. 12). All other accessions have no significant differences between them.

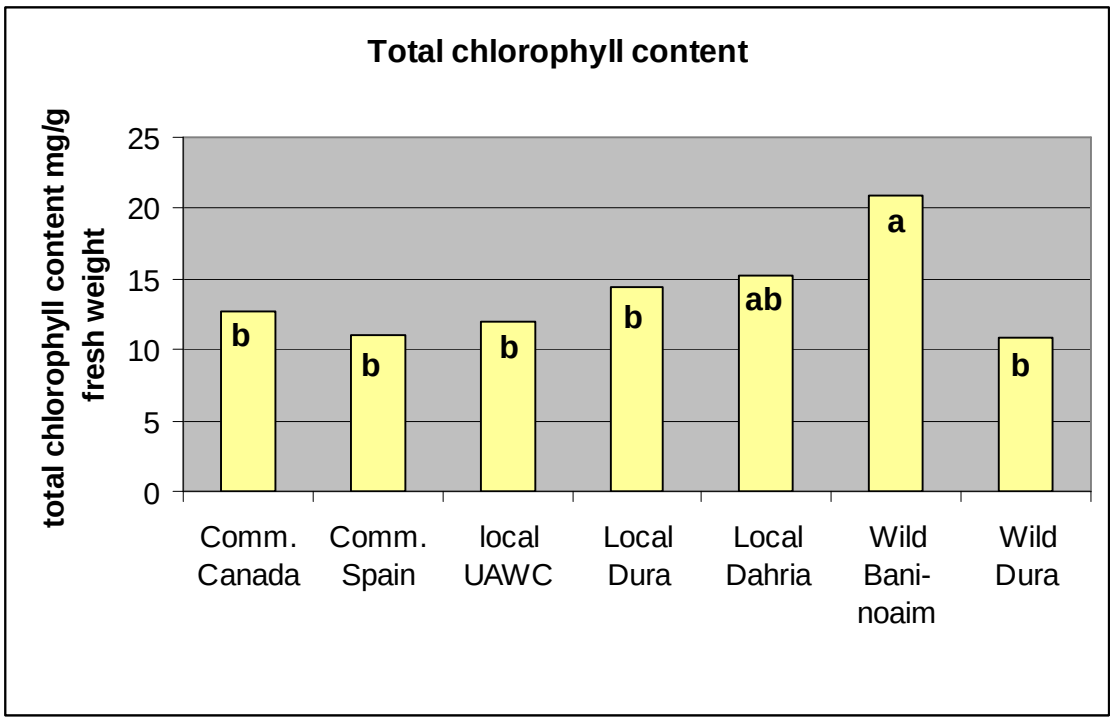


Figure (12) Total chlorophyll content (mg/g fresh weight) of the investigated accessions.

3.3 Relatedness of pea accessions according to morphological and physiological measurements:

At the 2-D graph score plot (Fig. 13) for relatedness, there is two clearly separated groups: the wild accessions in one group (cycles) and the local and commercial accessions in the other (squares and triangles), with an overlap between the local and commercial accessions (squares and triangles). These results indicate that wild accessions are morphologically and physiologically more related to each other than other accessions.

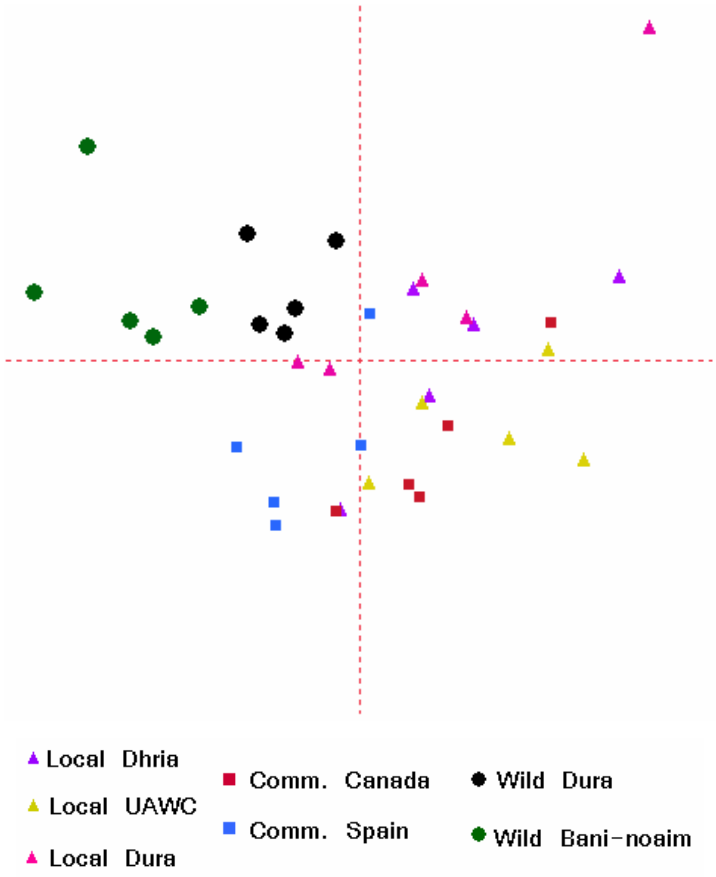


Figure (13) Score plot for commercial, local and wild accessions according to morphology and physiology results.

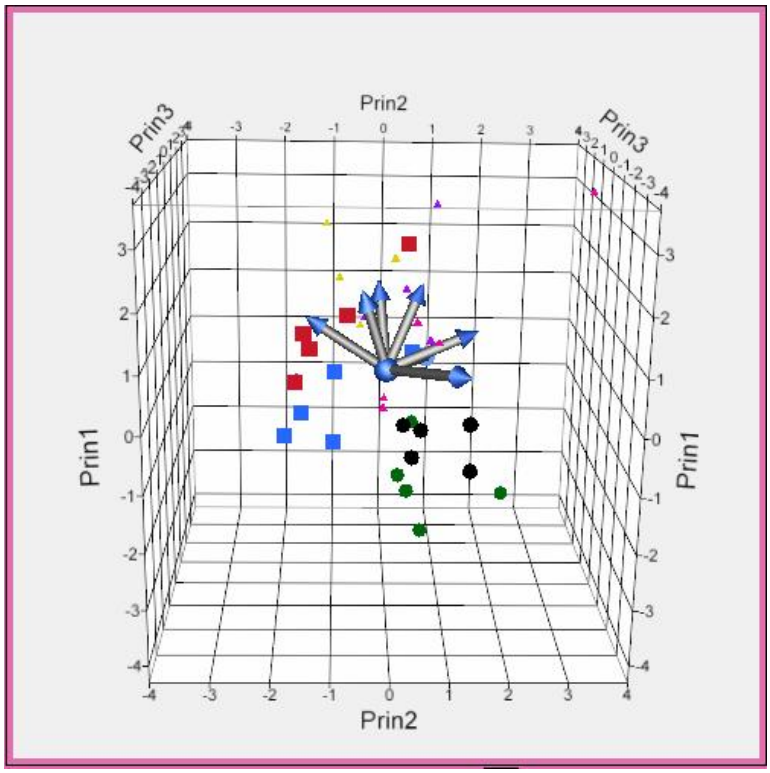


Figure (14) 3-D Scatter plot of investigated according to morphology and physiology results.

On the other hand, the 3-D graph (Fig. 14), shows that the relatedness within each group is high, but the three groups of accessions (wild, local and commercial) are less related to each other morphologically and physiologically.

3.4 Genetic variation and polymorphism within and among pea populations:

RAPD and SSR analyses were carried out to examine genetic diversity, population structure and polymorphism level among and within the seven populations of pea (Table 1).

Seventy plants were analyzed using 12 polymorphic primers (six RAPD and six SSR), and 90 polymorphic bands ranging in size from 140 to 1600 bp were revealed using the selected polymorphic primers (table 7, figure 15).

Table (7) Primer names, polymorphic band size range, total number of amplified bands, monomorphic and polymorphic bands and percentage of polymorphic bands for each primer

Primer code	Band size range (bp)	Total number of band	Monomorphic bands	Apomorphic bands	Polymorphic bands	Polymorphic (%)
RAPD						**55.9**
BC-305	550-1600	9	0	0	9	10.7
BC-329	300-1100	6	0	0	6	7.2
G10	400-1300	9	1	0	8	9.5
R11	380-1400	8	1	0	7	8.3
BC-318	350-1250	10	0	2	8	9.5
LEB-10	200-1500	11	1	1	9	10.7
SSR						**44.1**
PSMPSA148	180-900	10	0	1	9	10.7
PSMPD21	150-950	8	0	1	7	8.3
PSMPSAD141	140-1100	7	0	0	7	8.3
Y14273	200-800	5	1	0	4	4.8
PSMPSAD135	150-650	5	0	1	4	4.8
PSMPD23	180-600	6	0	0	6	7.2
Total		**94**	**4**	**6**	**84**	
Average		**7.8**	**0.33**	**0.5**	**7**	

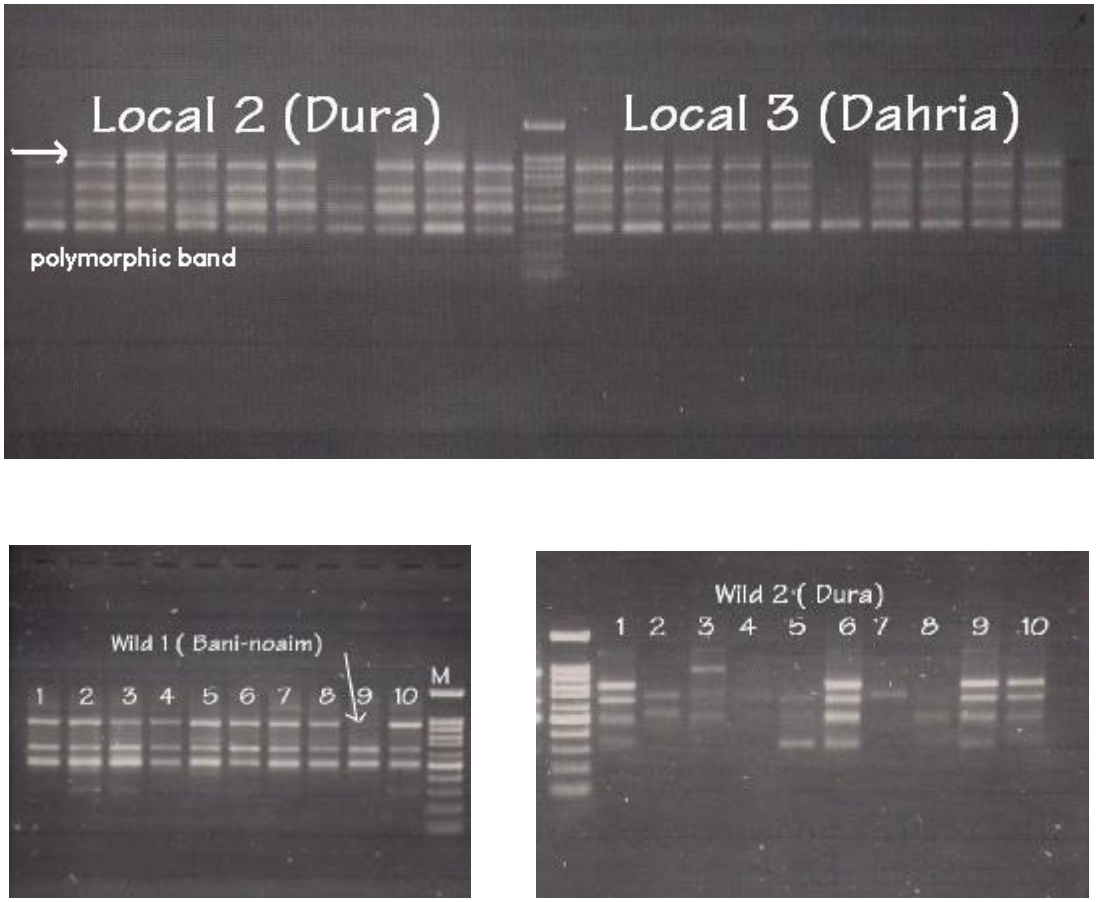


Figure (15) Results of BC-318 primer, show polymorphic bands in wild and local accessions

3.4.1 Principal coordinates analysis (PCO)

The 3-D plot of the principal coordinate analysis (PCO) of the squared Euclidean distances between all 70 pea accessions shows clearly four major groups. The first three coordinates accounted for 44.72%, 14.20% and 9.87% of the total variance (Fig. 16).

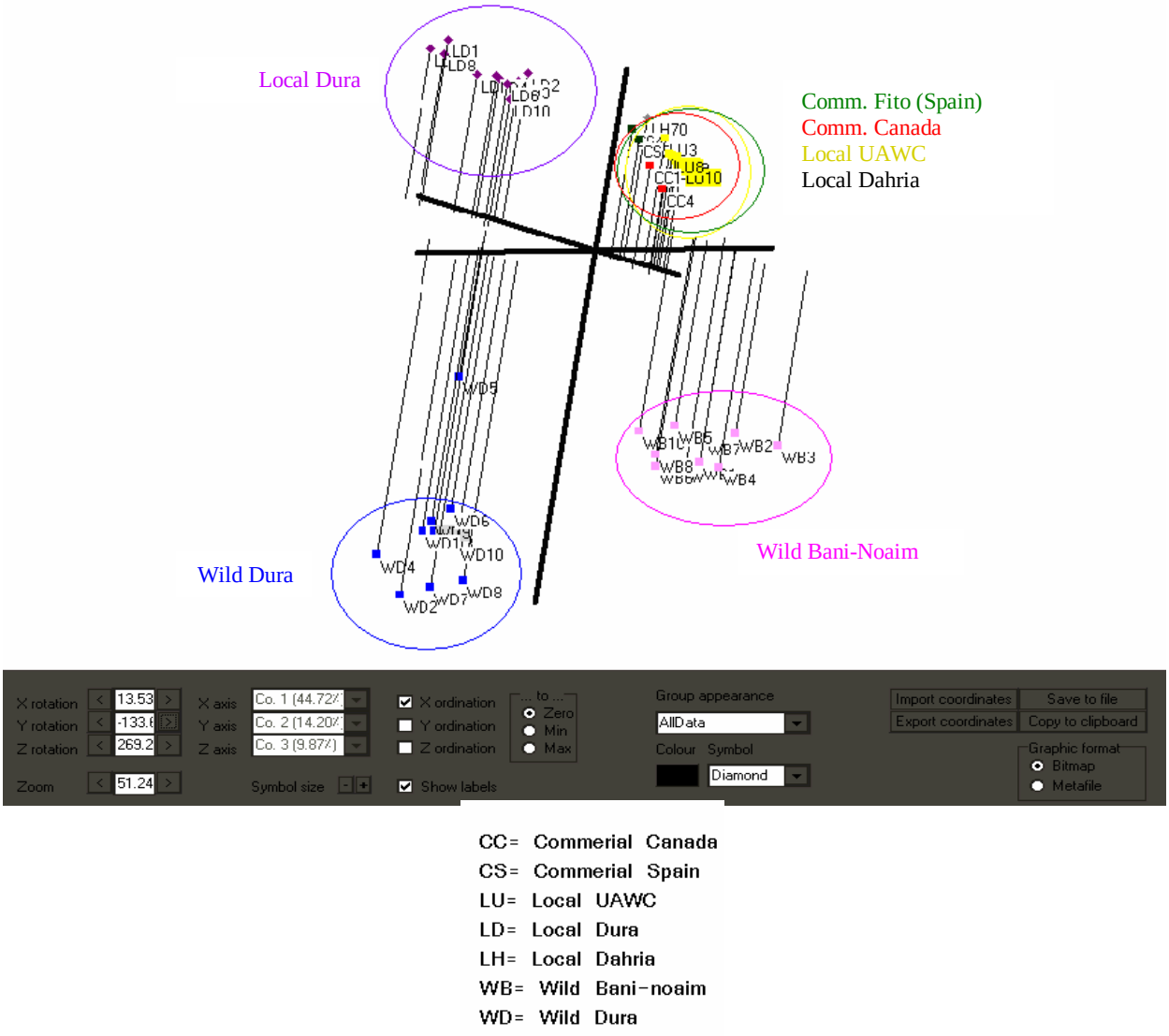


Figure (16) 3-D plot of the principal coordinate analysis for 70 accessions according to RAPD and SSR results analysis

In this 3-D plot four groups appeared clearly, namely the wild Bani-Noaim accessions, the wild Dura accessions, local Dura accessions, and the rest of accessions.

3.4.2 UPGMA analysis

3.4.2.1 RAPD primer result unrooted tree

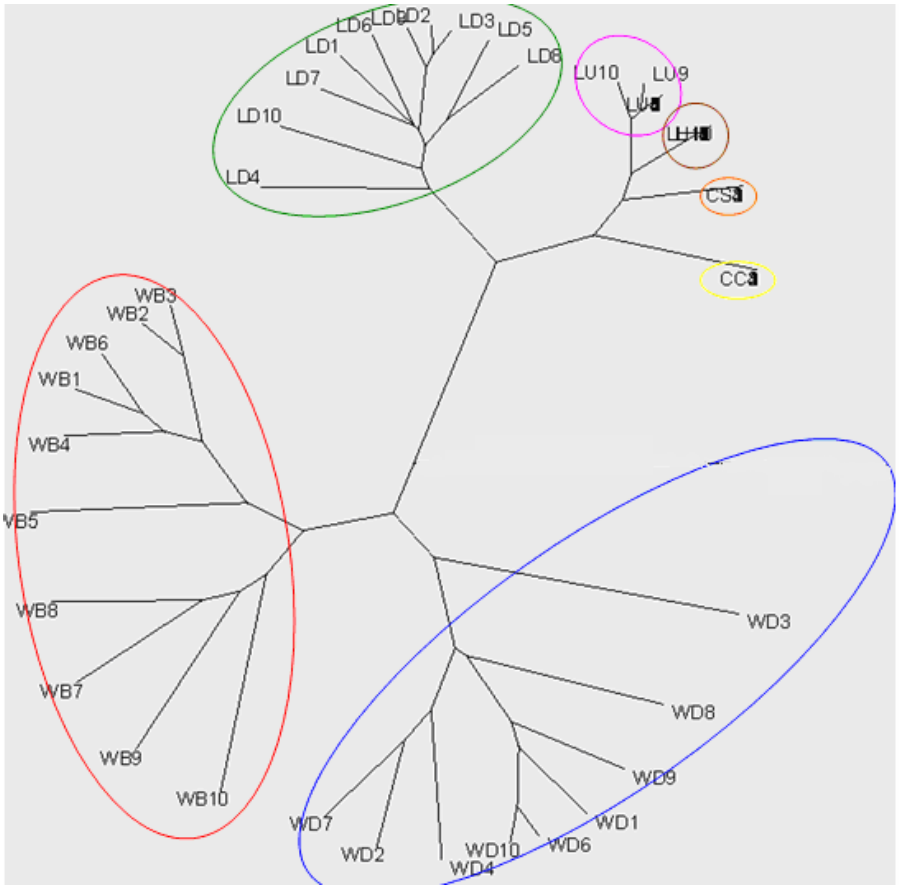
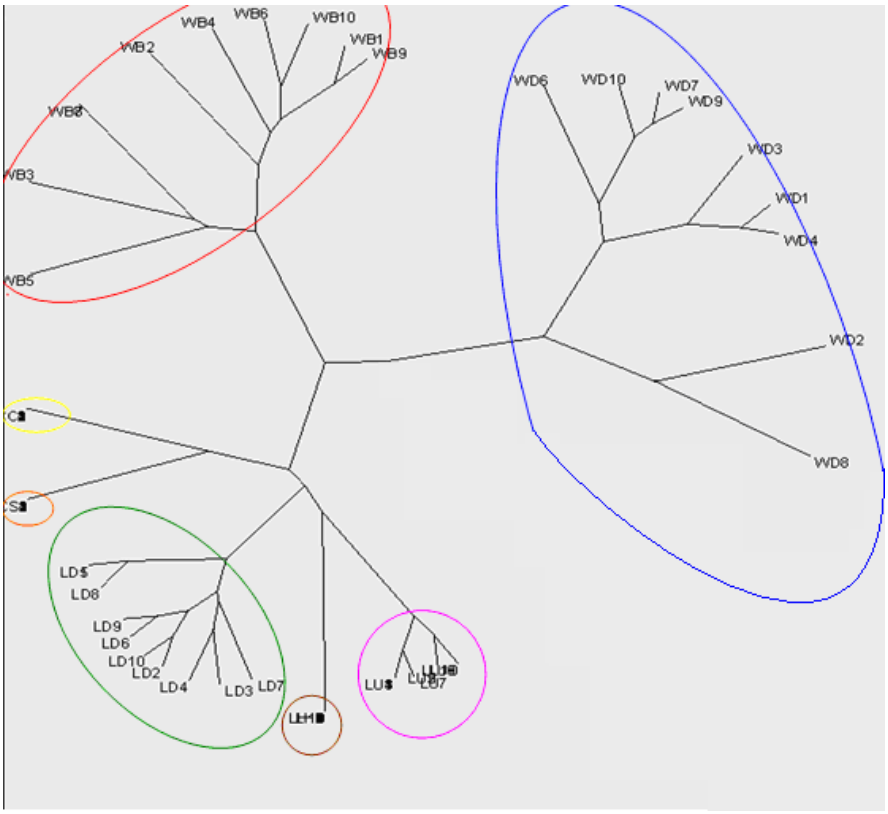


Figure (17) Unrooted UPGMA tree based on 53 polymorphic bands obtained from six RAPD primers.

Accessions were divided into four major clusters, where each wild group has its own cluster, as well as local Dura accessions. The rest of the groups were grouped in one cluster (Fig. 17). The polymorphic percent of this primer was 55.9%.

3.4.2.2 SSR polymorphism and UPGMA analysis

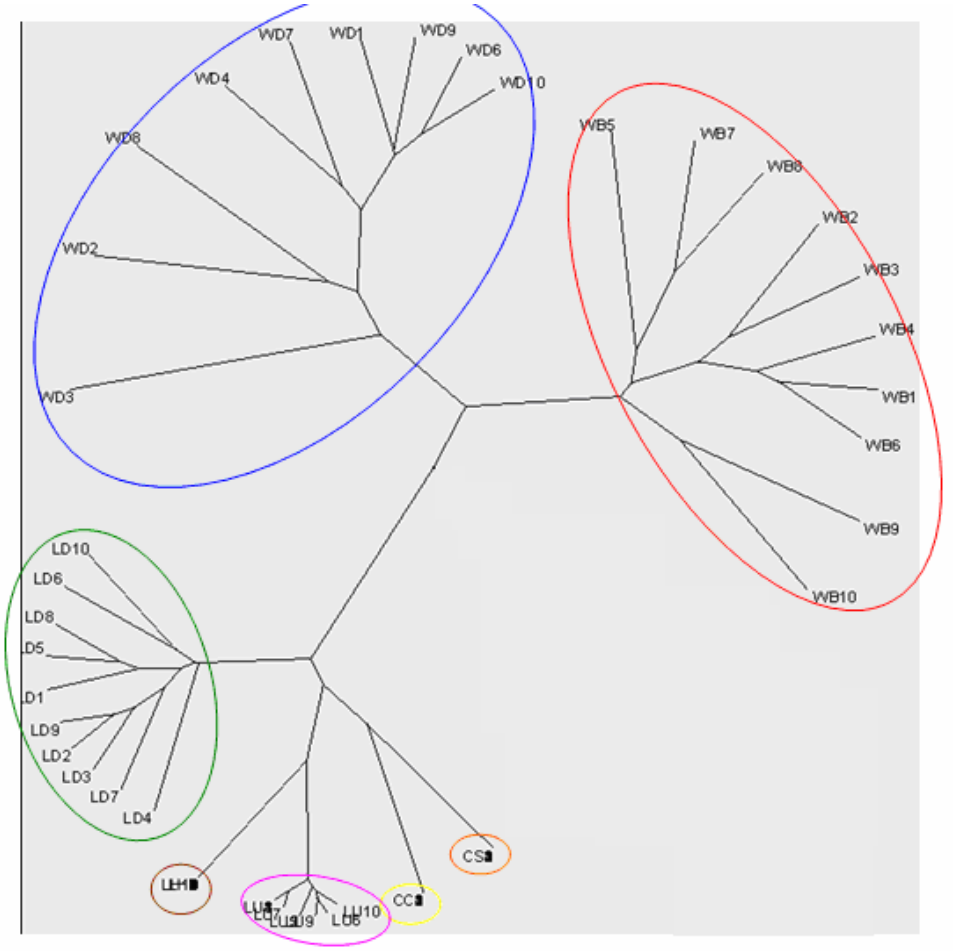


CC= Commerial Canada
CS= Commerial Spain
LU= Local UAWC
LD= Local Dura
LH= Local Dahria
WB= Wild Bani-noaim
WD= Wild Dura

Figure (18) Unrooted UPGMA tree based on 41 bands obtain from six SSR primers.

Results with SSR primers were similar to RAPD. Three major clusters were found, wild Bani-Noaim accessions cluster, wild Dura accessions cluster, and the local- commercial accessions cluster (Fig. 18). Their was no overlap also in this tree, and the polymorphic percent of this primer was 44.1%.

3.4.2.3 Combined data of RAPD and SSR



CC= Commerial Canada
CS= Commerial Spain
LU= Local UAWC
LD= Local Dura
LH= Local Dahria
WB= Wild Bani-noaim
WD= Wild Dura

Figure (19) Unrooted UPGMA tree based on 94 bands obtain from the combination of RAPD and SSR primers.

The combination obtained from RAPD and SSR primers results showed four major clusters wild Bani-Noaim accessions cluster, wild Dura accessions

cluster, local Dura accessions cluster, and the commercial and the rest of the local accessions cluster (Fig. 19), with no overlap between these clusters.

3.4.2 Internal Transcribed Spacer (ITS)

Seven accessions of *Pisum sativum*, in addition to the AY83940.1 from gene bank were included in the analysis. After alignment, the total length of the ITS region (18S+5.8S+26S) was 596 bp (ITS1 = 290 bp, 5.8S = 141 bp, and ITS2 = 165 bp).

Table (8) Number of mutations in each segment and their percentage

No.	Segment	Number of mutations	Percent
1	ITS1	22	81.5%
2	5.8S	0	0%
3	ITS2	5	18.5%
Total		25	100%

Chapter Four

Discussion

4.1 Morphological variation between accessions of *Pisum sativum*

Their were a diversity in morphological characteristics between accession groups and even within the same group, in particular within wild groups.
It should be mentioned that the commercial and local accessions were grown under irrigation, while the wild not.

4.1.1 Growth characteristics

Results showed that wild and local Dura populations have significantly the highest stem length compared with other accessions (Fig. 6). This is may be due to a wave of hot weather that occurred during the growing season affect more the rest of accessions, which may indicate that wild and local Dura are more adapted to heat stress.

For fresh weight the results show that the local accessions have the highest fresh weight (Fig. 7), whereas wild accessions were the lowest, which can be explained as the influence of differences in environmental condition between the locations.

These results agree with Okcu et al. (2005), they found that shoot length, and fresh weight of *Pisum sativum* decline with salt and drought stress. Another study done by Ayodele et al. (1990) found that whole plant fresh and dry weights and seed dry weight were significantly reduced by drought stress.

4.1.2 Fruit and seed production

Plants have evolved a fascinating array of reproductive strategies in order to survive and increase, it may produce and disperse hundreds or even millions of seeds in order that a few seedlings might survive to maturity, or it may produce few seeds (Gurevitch, 2002).

Commercial and local accessions had significantly higher number of pods than wild accessions (table 6). It is known that commercial and local accessions go through breeding improvement to increase quantity and quality of yield. Clua et al. (2006) results in *Lotus glaber* showed also a significant reduction in number of total pods under high temperature, compared with control treatment.

Results regarding number of seeds per pod (table 6) show that wild accessions had significantly the lowest number of seeds per pod.

Soil water deficit that occur during the reproductive growth is considered to have the most adverse effect on crop yield (Costa-Franca et al., 2000; Baigorri et al., 1999). Several studies also showed that water deficits during the reproductive development of dry beans decreased the number of pods and number of seeds per pod (Xia, 1997; Loss & Siddique, 1997).

Moreover Yadav et al. (1999) indicated that drought after flowering of pear millet decreased seed yield through reduction of number of ear per m^2, seed per ear and seed weight.

A significant difference regarding the weight of seeds (table 6) was recorded between and within commercial, local, and wild accessions.

These results are due to the differences in the environmental condition and to the genotype differences between these accessions as shown in molecular analysis.

4.1.3 Stomatal density

The amount of water in soil structure causes change in stomatal density (Brownlee, 2001). Bozoglu, (2006) highlighted that light level had also an effect on stomatal density. In our results, the stomatal densities with wild accessions were the lowest (Fig. 9). That may be attributed to the fact that many plants native to arid habitats evolved several mechanisms to withstand water stress condition, which include modify their stomata number, arrangement, and size (Bozoglu, 2006). Another mechanism is to have sunken stomata to increase the leaf's resistance to water loss (Gurevitch, 2002).

The reliability of these parameters (stomata density and chlorophyll content) to distinguish between stress tolerant and susceptible genotype seems to depend on the species under consideration (Colom and Vazzana, 2003; O'Neill et al., 2006; Rong-hua et al., 2006) since different species may possess different mechanisms of stress resistance and/or tolerance (Silva, 2007).

4.1.4 Flower color

Results show two flower color violet and creamy (Fig. 11), in accordance with available literature (Sardana, 2007; Ghafoor, 2005). The violet flowers appeared in local Dura accessions, wild Bani-noaim and Dura accessions. Genetic results showed that local Dura are the closest accession to wild accessions.

4.2 Physiological parameters

4.2.1 Chlorophyll content

Numerous authors demonstrate rapid change in chlorophyll content in response to internal as well as external factors (Ferus, 2001). Data showed that the highest chlorophyll content was found in wild Bani-Noaim accessions (the driest location), (Fig. 12). Paknejad et al. (2007) found with wheat plants, significant differences in chlorophyll content between plants that received different irrigation levels. The Mediterranean-type climate offers contrasting challenges for plant growth that oscillate between hot, arid summers with high irradiance and cool winters, irregular rainfall and episodic frosts. Plants growing in this environment should, in principle, represent a good test of the ecological utility of field measurements of chlorophyll fluorescence. Year-to- year variations in the abundance of many Mediterranean- grassland species can be correlated with similar variations in the accumulated rainfall over the growing season (Figueroa & Davy 1991). Increased light induced the formation of leaves with a higher density of stomata, veins, and chlorophyll (Goryshina, 1980, 1989). For the photosynthetic process to be accomplished affectively within short period during dry condition.

The ability to maintain Fv/Fm high (variable-to-maximum chlorophyll fluorescence ratio) Fv/Fm under drought stress indicates a high efficiency of radiation use, possibly for photochemistry and carbon assimilation (Silva, 2007), which may open the door for more studies using the wild accessions to assess their radiation use efficiency.

4.3 Genetic polymorphism

4.3.1 RAPD analysis

The polymorphic percent of RAPD primer was 55.9% (table 7). Compared with other studies (Choudhury et al. (2007)) this percentage located in the normal range; they worked with *Pisum sativum* using 60 RAPD primers.

Samec et al. (1998) also conducted the cultivars identification and relationships in *Pisum sativum* L. based on RAPD primers and iso-enzymes, with a polymorphic percentage of 69.9%.

4.3.2 SSR

The great advantages of SSR analysis are: accuracy, high polymorphism owing to multiallelism, possibility of genetic mapping, and co-dominance (Nybom 2004). Because SSR markers are highly variable they can provide some interesting information on the genetic diversity among *P. sativum* cultivated types and they may be useful tools for marker-assisted selection (Burstin, 2001). Results using SSR primer show no overlap, the polymorphic percent was 44.1% (Table 7), and three major branches appeared (wild Dura, wild Bani-Noaim, and the two commercials with the three local in separated group). On the other hand high uniformity was found, as expected, between commercial accessions. Three hundreds and nine SSR markers were tested for level of polymorphism, and the results shown 73% of the markers were polymorphic (Loridon et al. 2005). Similar results were found also by Tar'an et al. (2005), 25 SSRs were used to analyze 65 varieties and 21 wild accessions, in which 54 bands (51%) were polymorphic.

Another study done by Choudhury (2006) using 24 of the most popular and widely adapted varieties of pea (*Pisum sativum*), which were subjected to SSR analysis to find out the genetic relatedness among the under investigated accessions. All primers used were polymorphic and seven of them showed 100% polymorphism. Out of 579 amplified products, 433 were polymorphism (74.8%).

4.3.3 ITS

The ITS primers were used to make sure the samples that used in the study were *Pisum sativum*.

4.4 Relatedness of pea accessions according to morphological, physiological measurements and genetic variation

In general, results based on morphological, physiological characteristics, as well as molecular markers are complementary; each wild accessions stay in separated cluster from other clusters.

In the graph which combined both morphological and physiological parameters, three groups (the wild, local and the commercial) clearly appeared; the same result can be obtained from unrooted trees. In PCO 3-D plot a new group was appeared (the local Dura accessions). This group is more related to wild accessions in morphological and molecular analysis. In unrooted trees there was diversity between the clusters and within, except two commercial clusters and local Dahria, which have high similarity within, and they were more related in morphology also.

Wild accession groups have low similarity with other groups and between them, which mean that gene flow between them is limited, as they represent two different ecotypes. It seems that each one is adapted to its habitat.
Concerning the commercial accessions, which were affected badly by hot wave, they encountered during the growing season, results revealed their sensitivity to unfavorable growth conditions, where the local accessions appeared to be more adapted to stress conditions.

In conclusion, results clearly suggest the existence of pure wild pea plants that are endangered, as just few of these accessions could be found.
The Palestinian institutions, either governmental or non-governmental, are asked to develop and initiate programs to conserve and propagate these wild accessions; their high adaptability to drought stress is highly appreciated and will benefit Palestinian farmers in the long run.

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الملخص باللغة العربية

الصفات الشكلية و الفيسيولوجية و التنوع الوراثي بين نباتات البازيلاء (*Pisum sativum*) البرية و المزروعة

تمت دراسة عدة اصناف من نبات البازيلاء صنفين بريين جمعا من بني نعيم و دورا، و ثلاث اصناف محلية من الظاهرية و دورا و بذور لبازيلاء برية من بنك البذور التابع لاتحاد لجان العمل الزراعي و صنفين تجاريين مستوردة من اسبانيا و كندا.

الصفات الشكلية التي تم اخذ قراءاتها طول و وزن النبات و لون الزهرة و عدد التفرعات و عدد القرون و عدد البذور في القرون و وزن كل مئة بذرة، بالاضافة لعدد الثغور في سطح الورقة السفلي.

محتوى الورقة من الكلوروفيل الكلي تم اعتمادة كصفة فيسيولوجية تم قياسها.

التنوع الوراثي تم قياسه باستخدام عدة برايمرات و هي RAPD, SSR, ITS و كانت نتائج هذه الاختبارات على شكل شجرة قرابة للاصناف.

اظهرت النتائج ثلاث مجموعات منفصلة المجموعة الاولى الاصناف البرية و المجموعة الثانية الاصناف المحلية و الثالثة الاصناف التجارية.

ظهرت المجموعتان بشكل منفصل دون اي تداخل بينهما و هذا دليل على عدم حدوث اختلاط وراثي بينهما مما يجعل كلاهما مصدراً نادراً لكثير من الصفات التي لم تظهر بباقي الاصناف. الاصناف المحلية اظهرت تنوع بالنتائج مما يدل على تلوث بعضها بالاصناف التجارية.

استخدام الصفات الشكلية و الفيسيولوجية بالاضافة للقرابة الوراثية اعطت نتائج واضحة و متكامل اكثر من استخدام كل طريقة على حده.