

**ASSESSMENT OF GENETIC DIVERSITY AND
POPULATION GENETIC STRUCTURE AMONG THE
ACCESSIONS OF JEERA (*Nigella sativa* L.), FROM
AFGHANISTAN AND INDIA USING MORPHOLOGICAL,
MOLECULAR AND BIOCHEMICAL TRAITS**

SAYED ESMAIL

UHS12PGD56

**COLLEGE OF HORTICULTURE, BENGALURU
UNIVERSITY OF HORTICULTURAL SCIENCES
BAGALKOT**

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SAYED ESMAIL

UHS12PGD56

Thesis submitted to the

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In partial fulfillment of the requirements

for the award of the degree of

DOCTOR OF PHILOSOPHY

in

HORTICULTURE

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Dedicated

*To my beloved family and
Ministry of higher education
(Afghanistan)*

**UNIVERSITY OF HORTICULTURAL SCIENCES, BAGALKOT
COLLEGE OF HORTICULTURE, GKV POST, BENGALURU**

CERTIFICATE

This is to certify that the thesis entitled “**Assessment of genetic diversity and population genetic structure among the accessions of Jeera (*Nigella sativa* L.), from Afghanistan and India using morphological, molecular and biochemical traits**” submitted by **Mr. SAYED ESMAIL, ID No. UHS12 PGD5**, in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **HORTICULTURE** to the University of Horticultural Sciences, Bagalkot, is a bona-fide record of research work carried out by him during the period of his study in this University, under my guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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(Sayed Esmail)

ABSTRACT

ASSESSMENT OF GENETIC DIVERSITY AND POPULATION GENETIC STRUCTURE AMONG THE ACCESSIONS OF JEERA (*Nigella sativa* L.), FROM AFGHANISTAN AND INDIA USING MORPHOLOGICAL, MOLECULAR AND BIOCHEMICAL TRAITS

SAYED ESMAIL

Black cumin (*N. sativa* L.) is a crop of great medicinal value, but poorly studied and less tried for genetic improvement. Assessment of the genetic diversity is therefore of crucial importance for its genetic improvement. The present study was undertaken to assess the genetic diversity of the 37 accessions collected from Afghanistan and India. The accessions were sown during late *Rabi* 2014 and early *Rabi* 2015 and evaluated for 11 traits including yield and seed oil content. Black cumin accessions showed significant differences for all the characters including total yield and oil percentage during 2014. During 2015 the accessions did differ significantly for most characters except plant height, number of locules per capsule and seeds per capsule. PCA analysis based on the Morphological traits revealed that there is broad-scale geographic differentiation of the populations of *N sativa* and populations seem to be mixing well at a small geographic scale either because of human mediated commercial exploitation or because of natural out-crossing. RAPD primers were used for molecular diversity analysis of 12 accessions chosen to represent the diversity at phenotypic level. The number of bands produced by each primer ranged from 1 to 11. The highest number of loci were amplified in I.15 and BH.2 primers and the lowest in I.18; the gene diversity observed ranged from 0.00 in primer I.14 to 0.42 in primer I.1 with a mean gene diversity of 0.2. The Polymorphic information contents (PIC) of the 11 RAPD primers used varied between 0.00 and 0.35. Average PIC was 0.22. The genotypes from Afghanistan and India had similar similarity index values within each group (or country) than between them suggesting that short geographical isolation has low influence on the genetic diversity of the genotypes.

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K. N. GANESHAIAH
(Signature of Major Advisor)

ಸಾರಾಂಶ

ಆಫ್ಫಾನಿಸ್ತಾನ ಮತ್ತು ಭಾರತ ದೇಶಗಳಲ್ಲಿ ದೊರೆಯುವ ಜೀರಿಗೆ ಪ್ರಭೇದ (ನೈಜೆಲ್ಲ ಸಟ್ಟೆವ; ಎಲ್.; *Nigella sativa* L.) ದ ವಂಶತಳಿಗಳ ರೂಪ, ಅನುವಂಶಿಕ-ಧಾತು, ಮತ್ತು ಜೀವರಾಸಾಯನಿಕ ಗುಣಲಕ್ಷಣಗಳನ್ನಾಧರಿಸಿ ಅವುಗಳಲ್ಲಿನ ಅನುವಂಶಿಕ ವೈವಿಧ್ಯತೆ ಹಾಗೂ ಸಮೂಹ-ವಂಶ ರಚನೆಯ ಅಧ್ಯಯನ

ಸೈಯದ್ ಇಸ್ಮಾಯಿಲ್

ಕಪ್ಪು ಜೀರಿಗೆ (ನೈಜೆಲ್ಲ ಸಟ್ಟೆವ, ಎಲ್.; *Nigella sativa* L.)ಎಂದು ಕರೆಯಲ್ಪಡುವ ಪ್ರಭೇದವು ಒಂದು ಅಪೂರ್ವವಾದ ವೈದ್ಯಕೀಯ ಮೌಲ್ಯದ ಬೆಳೆಯಾದರೂ, ಕೃಷಿ ಅಧ್ಯಯನ ಮತ್ತು ತಳಿಗಳ ಅಭಿವೃದ್ಧಿ ಯೋಜನೆಗಳಿಂದ ನಿರ್ಲಕ್ಷ್ಯಕ್ಕೆ ಒಳಗಾಗಿರುವುದು ವಿಶಾದ. ಅಂತಹ ಪ್ರಭೇದಗಳಲ್ಲಿ ಅನುವಂಶಿಕ ವೈವಿಧ್ಯತೆಯ ಅಧ್ಯಯನವು ಅವುಗಳ ಅಭಿವೃದ್ಧಿಗೆ ಪೂರಕ ಹಾಗೂ ಅತ್ಯವಶ್ಯಕವೂ ಕೂಡ. ಹಾಗೆಂದೆ ಆಫ್ಫಾನಿಸ್ತಾನ ಮತ್ತು ಭಾರತದಲ್ಲಿ ದೊರೆಯುವ ಜೀರಿಗೆಯ 37 ತಳಿಗಳ ಅನುವಂಶಿಕ ವೈವಿಧ್ಯತೆಯನ್ನು ಅಳೆಯಲೆಂದು ಈ ಅಧ್ಯಯನವನ್ನು ಕೈಗೊಳ್ಳಲಾಯಿತು. ಈ 37 ತಳಿಗಳನ್ನು 2014ರಲ್ಲಿ ರಭಿಯ ಕೊನೆಯ ಕಾಲದಲ್ಲಿ ಮತ್ತು 2015ರಲ್ಲಿ ರಭಿಯ ಪ್ರಾರಂಭದಲ್ಲಿ ಬೆಳೆದು, ಅವುಗಳ ಇಳುವರಿಯ ಜೊತೆಗೆ ಇತರೆ ಹತ್ತು ರೂಪಲಕ್ಷಣಗಳ ಬಗ್ಗೆ ಮಾಹಿತಿಯನ್ನು ಪಡೆಯಲಾಯಿತು. 2014ನೇ ವರ್ಷದಲ್ಲಿ, ಜೀರಿಗೆಯ ತಳಿಗಳು ತಮ್ಮ ಇಳುವರಿ ಮಟ್ಟ, ಎಣ್ಣೆಯ ಪ್ರಮಾಣ ಸೇರಿ ಎಲ್ಲ ಗುಣಲಕ್ಷಣಗಳಲ್ಲಿಯೂ ಗಣನೀಯವಾದ ವ್ಯತ್ಯಾಸಗಳನ್ನು ತೋರಿದವು. 2015 ರಲ್ಲಿ ಸಸ್ಯಗಳ ಎತ್ತರ, ಕಾಯಿಗಳಲ್ಲಿನ ಕುಳಿಬಾಗಗಳು ಹಾಗೂ ಕಾಯಿಯಲ್ಲಿನ ಬೀಜಗಳ ಸಂಖ್ಯೆಯ ಹೊರತಾಗಿ, ಮಿಕ್ಕಲ್ಲ ಗುಣಲಕ್ಷಣಗಳಲ್ಲಿಯೂ ತಳಿಗಳು ಗಣನೀಯವಾದ ವ್ಯತ್ಯಾಸಗಳನ್ನು ತೋರಿದವು. ಈ ಎಲ್ಲ ರೂಪಲಕ್ಷಣಗಳನ್ನಾಧರಿಸಿ ಬಿಡಿಸಲಾದ ಪಿ ಸಿ ಎ (Principal Component Analysis) ವಿಶ್ಲೇಷಣೆಯಲ್ಲಿ, ಅಧ್ಯಯನಕ್ಕೊಳಪಡಿಸಿದ ತಳಿಗಳು ಅದರ ಚಿತ್ರದ ವಿಸ್ತಾರದಗಲಕ್ಕೂ ಹರಡಿಕೊಂಡಿದ್ದು, ದೂರ-ಪಾರದ ಜೀರಿಗೆ ತಳಿಗಳು ಅನುವಂಶಿಕ ವ್ಯತ್ಯಯಗಳನ್ನು ತೋರಿದವಾದರೂ, ಹತ್ತಿರದ ಬೂಪ್ರದೇಶದ ತಳಿಗಳಲ್ಲಿ ಧಾತುಗಳ ವಿನಿಮಯ ಹಾಸುಹೊಕ್ಕಾಗಿರುವ ಸಾಧ್ಯತೆಯನ್ನು ತೋರಿಸಿಕೊಟ್ಟವು. ಬಹುಷಃ ಮಾನವ ಪ್ರೇರಿತ ಬೀಜ ವಿನಿಮಯ ಮತ್ತು ಬೆಳೆಯ ವ್ಯಾಪಾರೀಕರಣದ ಫಲವಾಗಿ ಹತ್ತಿರದ ತಳಿಗಳು ಸಂಕರಣ ಕ್ರಿಯೆಯ ಮೂಲಕ ತಮ್ಮ ಧಾತುಗಳನ್ನು ವಿನಿಮಯ ಮಾಡಿಕೊಂಡಿರುವ ಸಾಧ್ಯತೆ ಇದೆ ಅನಿಸುತ್ತದೆ. ರೂಪಲಕ್ಷಣಗಳ ವೈವಿಧ್ಯತೆಯ ಆಧಾರದ ಮೇಲೆ 12 ವಿಭಿನ್ನವಾದ ತಳಿಗಳನ್ನು ಆರಿಸಿ, ವಂಶಧಾತು ವೈವಿಧ್ಯತೆಯ ಮತ್ತು ಧಾತು-ಸಂಯೋಜನೆಯ ಅಧ್ಯಯನಕ್ಕೆ ಒಳಪಡಿಸಲಾಯಿತು. Random Amplified Polymorphic DNA (RAPD ಆರ್ ಎ ಪಿ ಡಿ), ವಿಶ್ಲೇಷಣೆ, ಎಂಬ ತಂತ್ರ ವಿಧಾನದ ಮೂಲಕ ನಡೆಸಿದ ಈ ಅಧ್ಯಯನದಲ್ಲಿಯೂ ಇಂತದೇ ತೀರ್ಮಾನಗಳನ್ನು ಕಾಣಲು ಸಾಧ್ಯವಾಯಿತು: ಆಫ್ಫಾನಿಸ್ತಾನ ಮತ್ತು ಭಾರತದ ತಳಿಗಳು, ಆಯಾ ದೇಶದ ತಳಿಗಳ ನಡುವೆ, ಹೆಚ್ಚಿನ ಹೋಲಿಕೆಯನ್ನು ತೋರಿದವಾದರೂ, ಎರಡೂ ದೇಶದ ತಳಿಗಳ ನಡುವೆ, ದೂರವನ್ನಾಧರಿಸಿದ ವೈವಿಧ್ಯತೆಗಳು ಕಂಡು ಬಂದಿತು.

ಸೈಯದ್ ಇಸ್ಮಾಯಿಲ್
(ವಿದ್ಯಾರ್ಥಿಯ ಸಹಿ)

ಕೆ. ಎನ್. ಗಣೇಶಯ್ಯ
(ಪ್ರಮುಖ ಸಲಹೆಗಾರರ ಸಹಿ)

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I. INTRODUCTION

Medicinal plants have been used for curing diseases for many centuries in different indigenous systems of health care system. Moreover, medicinal plants are also used in the preparation of herbal medicines as they are considered to be safe compared to modern allopathic medicines (Debas *et al.*, 2006). Consequently, medicinal plants have come under focus of research for a thorough investigation of their medicinal value, chemical composition, mechanism of action, and toxicity. Despite this, hundreds of medicinal plants are yet to be investigated for their known potential as medicinal plants.

Black cumin *Nigella sativa* (*N. sativa* L. Family *Ranunculaceae*) is emerging as one such a miracle medicinal herb with rich historical and religious bases. The genus *Nigella* contains about 14 species of annual herbs of which *Nigella sativa*, is being used for healthcare from pre-historic times. It is commonly known as black cumin, fennel flower, Roman coriander and Kalonji (Weiss, 2002).

N. sativa is native to the geographic space associated with Southern Europe, North Africa and Southwest Asia and it is cultivated in many countries of Middle Eastern Mediterranean region, South Europe, and in India, Pakistan, Syria, Turkey, Saudi Arabia (Abdel- Fattah *et al.*, 2000). The seeds of *N. sativa* and their oil have been widely used for centuries in the treatment of various ailments throughout the world. It is an important drug in the Indian traditional system of medicine like Unani and Ayurveda (Abdel- Salam *et al.*, 1993 and Agarwal *et al.*, 1979). Avicenna (Abou Basha *et al.*, 1995) refers to black seeds in the "The Canon of Medicine", as seeds that stimulate the body's energy and helps recovery from fatigue and dispiritedness (Mukherjee and Wahile, 2006). Black seeds and their oil have a long history and folklore of usage as food and medicine in Indian and Arabian civilization (Al-Awadi *et al.*, 1991 and Badary *et al.*, 1997).

Owing to its claim in one of the Prophetic hadith (Juynboll, 2008) that black seed is the remedy for all diseases except death, the plant is considered as one of the greatest forms of healing among Muslims. It is also recommended for use on regular basis in Tibb-e-Nabwi Prophetic Medicine, (Abou Basha *et al.*, 1995). Black cumin provides a good source of some essential nutrients with diverse chemical composition comprising of essential fatty acids, amino acids, carbohydrates, fiber, and minerals such as calcium, iron, etc., (Gali- Muhtasib *et al.*, 2006 and Tauseef *et al.*, 2009). Traditionally the seeds and its oil are used for curing several diseases. The seeds are considered bitter, pungent, aromatic, appetizer, using stimulant, diuretic, emmenagogue, galactagogue, anthelmintic, acrid, thermogenic, carminative, anodyne, deodorant, digestive, constipating, sudorific, febrifuge, expectorant, purgative, abortifacient. They are used in ascites, cough, jaundice, hydrophobia, fever, paralysis, conjunctivitis, piles, skin diseases, anorexia, dyspepsia, flatulence, abdominal disorders, diarrhoea, dysentery, intrinsic hemorrhage and amenorrhea (Aboulela *et al.*, 1996 and Sharma *et al.*, 2005).

Black cumin oil is the spicy yellowish oil with an aromatic flavor; fresh flavored odor is extracted from black cumin seed (Hajhashemi *et al.*, 2004 and Ramadan, 2007). Most of the therapeutic properties of this plant are due to the presence of thymoquinone (TQ) which is a major active chemical component of the essential oil. Black seeds are also used as flavoring additive in the breads and pickles as it has very low of toxicity (Akhtar and Riffat, 1991).

Black cumin seeds contain some other compounds in trace amounts. Seeds contain two different types of alkaloids; i.e. isoquinoline alkaloids e.g. nigellicimine and nigellicimine-N-oxide, and pyrazol alkaloids or indazole ring bearing alkaloids which include nigellidine and nigellicine. Moreover, *N. sativa* seeds also contain alpha-hederin, a water soluble pentacyclic triterpene and saponin, a potential anticancer agent (Al-Ghamdi, 2001 and Al-Gharably *et al.*, 1997).

In spite of its great value in medicine, the crop is not well studied for the extent of variability in the available genetic resources and, for the influence of location and season on crop growth performance. In majority of the countries, the crop is grown in natural habitat under marginal soils without any agronomic and production practices. Knowledge about plant genetic resources enables plant breeders to create

novel plant gene combinations and select crop varieties suited to the needs of diverse agricultural systems (Glaszmann *et al.*, 2010).

Estimation of genetic diversity is essential for breeding programs and for the conservation of genetic resources (Karp, 1997). For any crop improvement program, a breeder depends on the variability present in the germplasm collections in order to advance productivity, bring about stability under different biotic and abiotic stresses (Ibrahim *et al.*, 2010). Genetic resource conservation activities require characterization of the diversity present both in the gene pools and the gene banks (Karp, 1997). Assessment of the genetic diversity in black cumin is therefore, of crucial importance for its genetic improvement.

Genetic diversity of a crop species has been investigated using either morphological or molecular markers. With the development of the polymerase chain reaction (PCR), several molecular techniques have been developed for estimating genetic diversity and for genomic analysis (Esayas and Bryngelsson, 2006). They are complementing the traditional morphological and agronomic characterization, since they virtually cover the whole genome, are not influenced by the environment, less time consuming and increased efficiencies in sampling variation (Esayas *et al.*, 2005).

Molecular techniques have also had critical roles in studies of phylogeny and species evolution, and have been applied to increase our understanding of the distribution and extent of genetic variation within and between species.

The choice of an appropriate molecular marker depends on many considerations, and there is no single approach that is optimal for studying variation in natural populations. Important classes of DNA-based molecular markers, which are used for such assessing as genetic-diversity, fingerprinting the cultivars, and phylogenetic analysis, include restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and Single Sequence Repeats (SSR); (Brown *et al.*, 1996; Heslop-Harrison and Schwarzhacher, 1996; Penner *et al.*, 2000; Reiter, 2001; De Vienne *et al.*, 2007 and Bhat *et al.*, 2004) Random Amplified Polymorphic DNA (RAPD) has served as a powerful tool for investigating the extent of genetic diversity of different crop plants. They offer numerous advantages over conventional, phenotype-based alternatives and are highly suitable in less understood and less studied crops such as black cumin where required molecular information and techniques are not readily available.

In the context of the above the present study is aimed at understanding the genetic diversity existing among the collections of black cumin, from the Indian sub-continent, especially from Afghanistan and India. The study intends to address this by using both the traditional, morphology based tools and the recent DNA based techniques. In the process, the study also aims to assess the relationship among different plant features with the seed yield and oil content. The specific objectives of the study are as follows:

1. Assessment of genetic diversity among the accessions of *Nigella sativa* from Afghanistan and India using morphological traits and molecular markers.
2. Constructing phylogenetic relations among the Jeera accessions and identifying genetically diverse groups for breeding and hybridization in Jeera.
3. Assessing the role of spatial isolation among Jeera accessions on the genetic distance among them and associating them with the biogeography of their origin.
4. Resolving the taxonomic confusions among the Jeera species from Afghanistan.

II. REVIEW OF LITERATURE

2.1 Origin and distribution of black cumin

There are widely divergent opinions on the probable origin of *Nigella sativa*. Probably, it is indigenous to the Mediterranean region and the Middle East up to India (Naz, 2011). Black cumin has long been cultivated and, is mentioned in ancient Hebrew, Greek and Roman texts. Originally black cumin was the common name for *Bunium persicum* and later named as *Carum bulbocastanum*, which is now near extinction and slowly *Carum carvi* graduated to the name. Due to unavailability of the species later *N. sativa* L. was adopted from Portuguese or Turkish merchants (Zohary and Hopf, 2000).

In East Africa the former Soviet Union, Europe, North America and South-East Asia it is cultivated on a minor scale, mainly for medicinal use (Akhtar and Shah, 1993).

In India the species is cultivated and distributed especially in Punjab, Himachal Pradesh, Gangetic plains, Bihar, Bengal, Assam and Maharashtra (Paarakh, 2010).

2.2 Uses of Black Cumin

N. sativa contains more than 100 valuable bioactive compounds (Duke, 1992). It is used for food and medicinal purposes in India, Pakistan, Saudi Arabia, Syria, Iran, Egypt and many other countries (Fillippo *et al.*, 2002 and Nergiz and Ötles, 1993). *N. sativa* is used as a spice, whole or crushed seeds are mixed with dough or sprinkled on bread in Sri Lanka, India, the Middle East, Turkey, the former Soviet Union, Egypt and Ethiopia. Crushed seeds impart a black color to the bread. *N. sativa* is an essential ingredient of semi-sweet breakfast rolls and coffee cakes known as 'choereg' in the Middle East (Warrier *et al.*, 2004).

The seeds are used to flavor a variety of dishes ranging from sauces, curries, pickles and meat dishes to vegetables, fruit pies and other confectionaries and also in flavoring vinegar and alcoholic beverages. In Arab countries a few seeds mixed with honey is taken as a sweet (Thattet and Dahanukar 1989). In Islamic tradition *N. sativa* seeds are reputed to be a panacea. They also play an important role in Western herbal medicine, and consequently the seeds are traditionally used to treat numerous diseases, such as headache, rheumatic pains, asthma and coughs (Fillippo *et al.*, 2002 and Nergiz and Ötles, 1993).

Nowadays, the consumption of herbal based products are getting widely accepted among consumers because of the numerous beneficial therapeutic effects they could impart to our body and indirect help in sustaining a healthy condition. Recently, people have begun to realize the differences between traditional and modern medication technique.

N. sativa has been used traditionally for centuries in the Middle East, Northern Africa and India for the treatment of various diseases (Worthen, 1998; Burits and Bucar, 2000; Al-Ghamdi, 2001 and Gilani *et al.*, 2004). Extracts of the black cumin have many therapeutic effects such as anti-diabetes, anti-bacteria, and antitumor (Khan *et al.*, 2003 and Hussein *et al.*, 2006). Despite the voluminous research published about the great medicinal benefits of the black cumin, there is not much genetic analysis of the species, on the application of molecular markers to study the genetic variation of *N. sativa*. Further, whether the black seeds grown in different localities are different varieties of one species has also not been addressed.

Products from herbs, especially the Jintan Hitam (*Nigella Sativa*) are the right choice for treating certain kinds of ailments or diseases without side effects to our body if consumed based on scientific findings and research. In the religion of Islam, black cumin has been given a great importance because of

its wide uses. The Islamic prophet Muhammad stated that the black cumin can heal every disease except death (Al-Akili, 1993). In the Indian system of medicine the seeds are used as astringent, stimulant, emmenagogue, anthelmintic, for treating jaundice, intermittent fever, dyspepsia, paralysis, piles, skin diseases and many more (Warrier *et al.*, 2004).

Ancient traditions document the use of black cumin seed as an energy source, perhaps because of its rich nutritional value. The seeds are still believed to increase heat in the body, making metabolism more efficient. As a nutritional supplement in modern times, black cumin seed is used to treat respiratory conditions like bronchitis, asthma and emphysema. In addition, it is used to support stomach and intestinal health as well as kidney and liver function. And the seed is thought to have antihistamine-like properties that make it useful in treating congestion, and it is widely used as a general tonic to boost immune function and to help prevent cancer. Several skin conditions can be treated with black cumin seed, and it is also used to enhance blood circulation (Khan *et al.*, 2003, Kanter *et al.*, 2003, and Hussein *et al.*, 2006).

2.3 Plant description and floral biology

The species is an erect annual herb (Figure 1) attaining one to two feet at maturity; the plant bears about 4-10 primary branches and 6-20 secondary branches. Flowers are arranged alternately with phyllotaxy of 1-2. Pinnae of leaves are broad, with 5-6 pinnae per rachis; flower is hermaphrodite with determinate flowering patterns; main axis terminates with a solitary flower (Figure 2). Small flowers (2.74 cm × 2.78 cm) are of French blue (43/3 – Horticultural Color Chart); flowers are without any involucre of bracts, pedunculated and erect; whorl, 4-6 mostly 5 and characterized by the presence of nectaries; flower fertility 89.89%; stamens in 3 to 4 whorls (Figure 3), numerous (32 to 66; 49.6 ± 2.7) and shed their pollen as the filament bent outward during male phase; gynoecium 5, completely united follicles, each with a long indehiscent style and composed of variable number of multi ovule carpel, developing into a follicle after pollination; fruit single partially connected to form a capsule like structure (capsule 5 to 45; mean 20.0 ± 3.37 ; capsule fertility 94.5%) dehiscence through suture; fruits (length – 0.4 to 1.7 cm, mean $1.03 \text{ cm} \pm 0.13$; seta per capsule 4 to 8, mean 5.10 ± 0.10) with numerous seeds (59.29 ± 3.2 ; average seed production/plant - 935 ± 177.9 ; seed yield – 1.91 gm; seed viability 80% to 90%); seeds ovate, tetragonal, angles sharp, acute, more tapering at the end (Figure 4), colour black (000021 – British Atlas of Colour, 2007); seed size $2.33 \text{ mm} \pm 0.1 \times 1.14 \text{ mm} \pm 0.02$. The quantitative data of the species were provided from plants grown as *Rabi* crop (Datta and Saha, 2003).

2.4 Pollination biology

Onset of the male stage: stamens stand erect, curved outwards one by one, roughly in whorls and strictly reflecting the order of initiation; pollen grains released when anthers reach a horizontal position; male phase is initiated a few days before the stigmas become receptive and lasts for five days; anthesis occurs between 8.00 a.m. to 13.00 p.m. for one day only, male and female stages synchronized on the last day of the flowering. Anthers produce pollen grains of about 0.064 mg/flower. The volume of nectar 0.13 µl; Abu- Hammour, (2008) reported that one bee per flower visited in morning around 7.00 a.m.; high temperature enhances fertilization success by affecting stigma receptivity and accelerating ovule degeneration (Postweiler *et al.*, 1985).

2.5 Ecological and reproductive studies

Andersson (2005) suggested that increased allocation to perianths leads to reduced allocation to direct component of fitness. Plants both with and without perianths did not differ in fecundity of total flower number. Further, perianthless plants produced heavier seeds with earlier germination dates than the control plants. Removal of perianth didn't affect seed viability or the fecundity of plants. High seed mass and rate of germination had positive and independent effects on progeny fecundity. The author was of opinion that it is necessary to determine whether large conspicuous perianths enhance the amount of

cross pollination and in such case perianth is to be under stabilizing selection, the species is capable of setting seed without being cross pollinated, an advantageous feature in seed crop which should be under strong selection for increased seed production. Finally, the author concluded that resource trade-offs with seed mass and time to germination may facilitate evolutionary reduction in flower size.

2.6 Cultivation

In India *N. sativa* is mostly grown in plains once a year as *Rabi* crop during the months of October - November to March-April and rarely in hills during May-June (Chakraborty and Chakraborty, (1964).

1. Area of cultivation and production: In India it is cultivated in 6234600 ha with 254000 tonnes; in Turkey, it is cultivated in 8122010 ha and 689350 tonnes; in USA, 16420 ha, 11200 tonnes; in UK: 500 ha, 10 to 20 tonnes (Wiersema and Leon, 2016).
2. Climate: The crop grows well in cool-dry condition with light snowfall areas to warm-humid areas. Cool and humid weather favors flowering and seed setting.
3. Soil: Sandy, loam rich in microbial activity is the most suitable soil for cultivation. The sloppy lands of heavy rainfall areas and leveled and well drained soils of moderate rainfall areas are quite suitable for cultivation. Neutral pH 7.0 to 7.5 is favourable for cultivation.
4. Preparation of land: One ploughing followed by 2-3 harrowing and leveling will be suitable.
5. Method of sowing: Seed sowing or by replanting previous year root stocks is followed. Seed sowing is done during October-November by broadcasting (1.5 kg/hectare) or seed drill method or by line sowing keeping space between lines (30, 40 or 50 cm) and at the depth of 2 cm. After 20 days of sowing thinning of the plant to a distance of 20 cm is done. Sowing by bulbs (previous year root stock) is possible when soil moisture content of the field is favorable for deep ploughing i.e. neither too wet nor too dry.
6. Manure and fertilizer: NPK 5:3:2 kg/hectare is generally applied every year along the side of the planted bulbs.
7. Irrigation: One or two irrigations at flowering and seed formation stage are helpful to increase grain size and oil content.
8. Harvesting: Black cumin is grown as *Rabi* crop in West Bengal plains are generally harvested late March to first week of April. The crop harvested before shedding at little green stage gives high aromatic oil contents providing good market. Black cumin retains its seed viability for few years when it is full ripe. It is rather essential that harvesting is done before shedding as shattering of fruits is a major problem; therefore 2 to 3 or more pickings can be done to avoid loss of seeds due to shattering of the capsules. The harvested crop is dried under Sun and threshed by beating with the stick.
10. Postharvest management: *N. sativa* requires extensive labor in collection and harvest as the capsules (fruit) tend to shatter at maturity. Post-harvest management of the fruits usually involves their harvesting one by one, by hand and dry storage till natural dehiscence. The mature fruits do not require much attention as they are self-preserving and their essential oil is a great deterrent to fungal attack, insect attack as well as rodent infestation (Larrousse , 2007).

2.6.1 Shelf life

The seeds of *N. sativa* can be stored well for one year as planting material; as a spice, they can be stored in airtight conditions to prevent the loss of aroma. As a spice, it is recommended to be stored away from other species as the species has an overbearing flavor and aroma and disturb the flavor of other species.

2.6.2 Diseases

Sinha and Singh (1994), reported *Macrophomina phaseolina* infection in roots causing its deformation. It grows along the seedling and the leaves and branches look light green in colour, leaves shed and plant dries up. It can be control by spraying Dithane M-45 0.2% or Dithane Z-78 or Blitox 5 w.p. at 15 days interval. Rotting of bulbs emit a special odour that be controlled by dipping bulbs in 0.3% bavistin for 30 mins. Before planting, field shall be kept free from stagnant water. Early report by Mc-Rae and Shaw (1979), also suggested Fusarium wilt in the species. Prolonged survival of *F. udum* for up to 8 years was reported in roots.

2.6.3 Pest

1. Caterpillar: Makes holes in the bulbs and cuts down seedlings. Control: Dust the soil at the sowing or hoeing with 5% Aldrine, 10% BHC at the rate of 25 kg per hectare; application of well-decomposed farmyard manure.
2. Armyworm and semi-looper: Feed on the flowers, seeds, and damage the crop. Control: Spray with 0.05% methyl parathion – 1 ml/l water or Thiodian or Endosal 35EC, 1 ml/l of water at 15 days interval.

2.7 Biochemical constituents

Constituents of *N. sativa* seeds are fixed oil – 32 to 40% (saturated fatty acids- about 30%; palmitic acid, stearic and myristic acid; unsaturated fatty acids: arachidonic, eicosadienoic – 3%, linoleic – 50 to 60%; oleic acid – 20%; dihomolinoleic fatty acids – 10%), volatile oil- 0.4 to 0.45% (nigellone, thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, and -pinene, dlimonene, d-citrlnellol, p-cymene), proteins 16-19.9% (arginine, glutamic acid, leucine, lysine, methionine, tyrosine, proline, threonine), minerals 1.79-3.74% (calcium, phosphorus, potassium, sodium, iron), carbohydrate 33.9%, fiber 5.50% and water 6.0% 25 (Aggarwal and Kunnumakkara 2009). Ramadan and Morsel (2002) reported that apart from physical constants: 2% w/w, foreign matter; 6% w/w, total ash; 0.2% w/w, acid insoluble ash; 20% w/w, alcohol soluble extractive; 15% w/w, water soluble extractive; 3.91% w/w organic matter; 4% w/w, loss on drying (Sharma *et al.*, 2005). The seeds contain carotene, which is converted to vitamin A in liver (Al-Jassir 1992). Acetylated triterpene saponin (penta hydroxyl pentocyclic triterpene) has been isolated from the species (Mehta *et al.*, 2009).

2.7.1 Seed oil

The seed oil contains cholesterol, campesterol, stigmasterol, -sitosterol, -spinasterol, (+)-citronellol, (+)-limonene, p-cymene, citronellyl acetate, carvone (Rastogi and Mehrotra 1993), nigellone, arachidic, linolenic, linoleic, myristic, oleic, palmitic, palmitoleic and stearic acids. Fixed oil: linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid (12.5%). Volatile oil: trans-anethole (38.3%), p-cymene (14.8%), limonene (4.3%), and carvone (4.0%) Nickavar *et al.*, 2003), 2-(2-methoxypropyl)5-methyl-1, 4-benzenediol, thymol and carvacrol (Enomoto *et al.*, 2001). Root and shoot are reported to contain vanillic acid (Bourgou *et al.*, 2008).

2.7.2 Extraction methods of essential oil

The oil content in seeds was determined using Soxtherm apparatus on the principle of solvent extraction (Spiro and Chen, 1994).

2.8 Genetic Diversity

2.8.1 Genetic variability

A critical estimate of genetic variability is pre-requisite for initiating appropriate improvement programs in a population. Greater the variability in a population, greater is the chance for effective selection of desirable types (Vavilov, 1951).

The variability observed in many populations could be due to two factors, viz., the genetic and environmental. The genetic and environmental components of variation were discussed in the early part of last century by Johannsen (1909), who attributed the variation within pure line to environmental factors. Nelson-Ehle (1909) and East (1916) later confirmed Johannsen's work and showed that continuous variation also confirms to Mendelian genetics.

The phenotypic variability is a measure of variability due to genotype, environment and their interaction. The genetic variability is the real measure of variability concealed in a population. It indicates the relative magnitude of genetic diversity existing in the population and helps to capture the genetic variability present for different characters.

Selection for quantitative characters is less efficient, if it is based on poor phenotypic expression with less genetic control. Hence it is necessary to assess the relative extent of genetic and non-genetic variability exhibited by individual character. So, partitioning of overall variability into heritable and non-heritable components by calculating genetic parameters such as Genotypic Coefficient of Variation (GCV) and Phenotypic Coefficient of Variation (PCV) is necessary (Chadha and Sindhu, 1983 and Sinha and Singh, 1994).

Kaicker *et al.*, (1982) reported stability of 10 cultivars of opium poppy for morphine content and found existence of genetically conditioned differences in the stability of cultivars. Techniques employed to determine the adaptive potential relative stability and environmental interaction showed significant varietal differences when the cultivars' mean square was tested over pooled deviations (Finlay and Wilkinson, 1963 and Eberhart and Russel (1966). Significant $V \times E$ (linear) component suggests the existence of genetically conditional differences in the stability of cultivars. Highest morphine content was observed in cultivar H and least in R (Finlay and Wilkinson, 1963 and Eberhart and Russel (1966), four cultivars exceeded the grand mean.

2.8.2 Heritability

Heritability is the portion of phenotypic variation which is transmitted from parent to progeny. Higher the heritable variation greater will be the possibility of fixing the characters by selection methods. Therefore, heritability studies are of foremost important to factorize the observed variation for a particular character into genotype and to environment influence. The ratio of genotypic variance to total variance in non-segregating population is known as heritability in broad sense, whereas the ratio of additive to the total or phenotypic variance is referred to as narrow sense heritability (Hanson *et al.*, 1956). Thus, heritability denotes the proportion of phenotypic variance that is due to genotypic which is heritable.

2.8.3 Character association and path analysis

The correlation coefficient measures the strength of mutual relationship between characters and also indicates the component traits on which selection can be applied on, to improve the yield. There are three types of correlations viz., phenotypic, genotypic and environmental correlations. Phenotypic correlation is the observable correlation between two variables and includes both genotypic and environmental effects. Genotypic correlation on other hand is the inherent association between two variables which may be either due to pleiotropic action of genes or linkage or more likely both are developmentally induced relationships (Harland 1972). Genotypic correlation measures the magnitude of selection that can be applied for improvement in yield (Johannson 1955).

The path analysis is a standardized partial regression coefficient and measures the direct influence of one variable upon other and permits the separation of correlation coefficient into components of direct and indirect effects of a set of independent variables on a dependent variable.

The concept of path analysis was developed by Wright (1921), but the technique was first used for plant selection by Dewey and Lu (1959) as a means of separating direct and indirect contribution of various characters. Basically the technique aims to understand the behavior of dependent character like yield when the independent characters have a significant relation in desirable direction and positive direct effect or indirect effect through other traits on the dependent characters. The use of this technique requires identifying cause and effect situation among the variables (Singh and Chaudhary, 1985). If the correlation coefficient between a causal factor and the effect is almost equal to its direct effects, then correlation explains the true relationship. If the correlation coefficient is positive, but the direct effect is negative or negligible, the direct effects seem to be cause of positive correlation. In such situations, the indirect causal factors are to be considered simultaneously for selection. If the correlation coefficient may be negative but the direct effects is positive and high. Under these circumstances, a restricted simultaneous selection model is to be followed i.e restrictions to be imposed to mollify the undesirable indirect effects in order to make use of the direct effect. If the correlation coefficient is negative and direct effect is also negative, then we have to drop the selection based on that character. The residual effect determines how best the casual factors account for the variability of the dependent factor. If residual effect is high, some other factors, which have not been considered here, need to be included in this analysis to account fully for variation in yield.

Biswas (1970) observed positive correlation between number of branches and root yield per plant in clonal population of *Rauwolfia serpentina*.

Shivanna *et al.* (2007) studied genotypic and phenotypic correlations and path coefficients for 12 economic traits in 17 genotypes of makoi (*Solanum nigrum* L.). The genotypic correlation coefficients were higher than phenotypic correlation coefficients for most of the traits. The total alkaloid yield had highly significant positive genotypic and phenotypic association with dry herbage yield per plant. Days to flower bud initiation and days to 50 per cent flowering had significant positive association at the genotypic level. Path coefficient analysis revealed maximum direct effect of herbage yield on total alkaloid yield.

Kumar *et al.*, (2008) studied genetic variability among 31 genotypes using nine characters of *Chlorophytum borivillianum*. Correlation analysis for root yield (dependent variable) and the remaining seven plant traits (independent variables) revealed that leaf number, leaf length and finger number had significant associations with root yield.

Singh *et al.*, (2008) studied variability and association of yield and its contributing morphometrical traits over two micro-environmental conditions in Safed musli (*Chlorophytum borivillianum*). They revealed the scope for genetic improvement in major yield attributes with some limitations for root length and diameter. An interrelationship among the yield and yield attributes indicated that improvement in yield can be attained through improvement in root numbers, root length, and diameter; however, more emphasis must be paid on root diameter for organic conditions. The cause and effect study on the interrelationship exhibited the importance of productive root yield as causation to improvement in yield.

Chitra and Rajamani (2010) studied character association and path analysis for 13 morpho-economic traits using 18 genotypes of *Gloriosa superba* L. Plant height, number of leaves per plant, number of branches per plant, days to 50% flowering, number of flowers per plant, number of pods per plant, number of seeds per plant, fresh seed weight per plant, fresh seed yield per plant and fresh seed recovery were found to have positive association with dry seed yield per plant. Fresh seed yield per plant had highest positive effect on seed yield followed by number of pods per plant and fresh seed weight per pod. These associated yield components suggested that they may be good selection criteria to improve seed yield of glory lily (*Gloriosa superba*) crop.

Karuppaiah and Kumar (2010) investigated 34 genotypes of African marigold to assess association of yield components and their direct and indirect effects on flower yield. Results of correlation analysis indicated flower yield per plant to be significantly and positively correlated with number of branches per plant, flower size, flower weight, number of flowers per plant and xanthophyll content. Days to first flowering showed negative association with flower yield per plant. Path analysis revealed high positive direct effects of number of flowers per plant and xanthophyll content. Medium level of direct effect was recorded by flower diameter. Remaining characters recorded low to very low direct effects.

Das *et al.*, (2011) studied genetic variability, character association and path coefficient analysis for yield (seed / leaf / root) and 11 yield related (including chemical parameters) traits in 6 genotypes (parental cultivar and 5 macromutant lines) each of high yielding recommended varieties Poshita and Jawahar 22 of *Withania somnifera* (L.) Dun. (Ashwagandha; Family: *Solanaceae*; potential plant species of commerce for therapeutic uses) for undermining attributes maximizing yield for efficient breeding and further improvement of the elite genotypes for commerce. They found that root length and total number of berries / plant are important selection criteria for economic yield irrespective of the parental cultivars of *W. somnifera*.

2.8.4 Genetic divergences

The magnitude of divergence between two groups provided by several parameters, such as Pearson difference measure and D^2 statistic developed by Mahalanobis (1936). Mahalanobis used the estimate to study the morphometric divergence among Asian human races. For the first time, D^2 statistic was applied for biological population by Nair and Mukherji (1960) to classify the natural and plantation teak tree types. Its application was extended later to taxonomic studies also. Murthy *et al.*, (1962) observed that D^2 analysis can be extended to the situations where overlapping species need to be discriminated and also when the discrimination at subspecies level is needed. This technique was subsequently used in different crops. through these techniques were initially used for morphological features, later they were also applied to molecular and environmental differences.

Typically D^2 estimate measures the geometric differences between any two populations based on their mean differences in diverse traits. Once D^2 estimates are obtained the populations can be clustered in to group with similar types. PCA (Principal Component Analysis) Analysis is another graphical method of plotting populations in a space defines by several traits. The technique involves extracting several variables in to a minimum set of axes that represent most variability. Plotting populations with in the space of those axes helps grouping them.

Kumar *et al.*, (2007) studied six phenotypic characters and three *withanolide* markers in 25 accessions of *Withania somnifera* collected from different states of India for studying genetic variability. The variability ranges observed at phenotypic and chemotypic levels were polymorphic. Based on D^2 values and PCA (Principal Component Analysis) of phenotypic traits like plant height, number of branches per plant, number of seeds per berry, root length, root diameter and root yield, these 25 accessions were grouped in to five clusters. Five accessions AGB002, AGB003, AGB004, AGB006 and AGB009 representing clusters 2 and 4 exhibited maximum intra and inter-cluster divergence. Cluster 5 representing accession AGB053 was having mixed traits. Chemically most of the accessions in cluster 3 showed uniformity in presence of three marker withanolides Withaferin A, Withanone and Withanolide A in the leaves.

Kumar *et al.*, (2008) studied genetic divergence among 31 genotypes of *Chlorophytum borivilianum* using nine characters. The genotypes were grouped into eight clusters. Intra-cluster distance was largest for cluster 8 (nine genotypes), followed by cluster one (six genotypes). Inter-cluster D^2 values recorded where high between cluster two and three and cluster three and six, indicating the possibility of raising transgressed hybrids from cross hybridization programs using parents of these four clusters. The clustering pattern indicated that geographical diversity was not necessarily related with genetic diversity. Leaf number contributed most toward divergence (15.8%), followed by finger number, length and width

(each with at least 13% contribution) and leaf length (12.1%). Thul *et al.*, (2009) evaluated 24 lines of *Capsicum* species for 12 quantitative and qualitative traits and grouped all 24 accessions into six clusters. Cluster one consisted of 33% of accessions (8) and clusters two of 29.17% of accessions (7), while clusters five and six consisted of single genotype. The three characteristics that played the greatest role in differentiation were fruit diameter, number of fruits per plant and leaf diameter.

2.8.5 Genetic Markers

A genetic marker is a measurable character that can detect variation in either protein or DNA sequence. A difference, whether phenotypic or genotypic, may act as a genetic marker if it identifies characteristics of an individual's genotype or phenotype, and if its inheritance can be followed through different generations (De Vicente and Fulton, 2003 a). A number of markers exist that can be used as tool to discern genetic variability among populations. These markers fall into one of the three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers). These markers are widely used by breeders and conservationists to study genetic diversity and association mapping. Each marker system has their own advantages and disadvantages in terms of ease of use and/or the degree of information provided (De Vicente and Fulton, 2003 a).

2.9 Morphological Markers

The earliest studies of genetic characterization and divergence are based on morphological markers such as qualitative and quantitative traits (Arriel *et al.*, 2007). These markers are inexpensive and simple to score as they are based on distinct phenotypes such as plant colour, plant height, seed characteristics, etc.. The apparent disadvantage of such markers is that in studies of genetic diversity the expression of the phenotypes is highly influenced by environmental conditions. These traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation.

Dispensable traits in studies of genetic diversity are relatively invariant ones, highly influenced by the environment or redundant for being correlated to other traits. In other words, those that contribute most to the divergence must be weakly correlated (Arriel *et al.*, 2007). Lack of adequate genome coverage because of limitation of the number of markers and problems of dominance can also be mentioned as a weakness of morphological markers (Brown, 1978). Furthermore, the expression of these characters which are influenced by the environment may require that plants be grown to a suitable stage before certain characters can be scored.

2.10 Biochemical Markers

These are also termed as isozyme/allozyme markers or simply protein markers. Isozymes represent different molecular forms of an enzyme system with the same catalytic functions. They are variants of an enzyme that can be differentiated by electrophoretic mobility. They originate through amino acid alterations which cause changes in net charge or conformation of an enzyme and hence in electrophoretic mobility. The electrophoretic verification of such amino acid alterations provides a means of monitoring changes in the nucleotide sequence of the respective coding gene. They are distinguished mostly by horizontal gel electrophoresis that separates proteins primarily on the basis of charge and size (Weeden, 1989). The limitation of the isozyme marker is that it is based on the phenotype and has inadequate genotype coverage, so this type of marker is highly influenced by the environment (De Vicente and Fulton, 2003 b).

Biochemical analysis is based on the separation of proteins into specific banding patterns. It is a fast method which requires only small amounts of biological material. However, only a limited number of enzymes are available and thus, the resolution of diversity is limited. The use of biochemical markers

involves the analysis of seed storage proteins and isozymes. This technique uses enzymatic functions and is a comparatively inexpensive yet powerful method of measuring allele frequencies for specific genes (Winter and Kahl, 1995).

2.10.1 Molecular Markers

Molecular markers are identifiable DNA sequences, found at specific locations of the genome, and transmitted from one generation to the next. Molecular or DNA markers are relatively recent but most robust tools for genetic diversity analysis. It overcomes most of the shortcomings of morphological and biochemical markers. When compared with other markers systems, DNA based diversity estimates were shown to reflect the actual differences between genotypes, relatively simple to detect, abundant throughout the genome, completely independent of environmental conditions and can be detected at any stage of plant development (Barrett and Kidwell, 1998).

Based on the method of analysis two basic types of DNA marker systems are available: those that rely on hybridization between a probe and homologous DNA segment such as restriction fragment length polymorphism (RFLPs) and those that use the polymerase chain reaction (PCR) to exponentially amplify genome segments between arbitrary or specific oligonucleotide priming sites such as inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR; Semagn *et al.*, 2006). The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. No molecular markers are said to be ideal and fulfill all requirements needed by researchers. Thus one can choose among the variety of molecular techniques, according to the kind of study to be undertaken. Each of these markers systems have their own strengths and weaknesses. Thus one has to choose the kind of markers that best suits the crop and population. To be called a suitable molecular marker it should be highly polymorphic, have co-dominant inheritance, frequent occurrence and show even distribution throughout the genome, selectively neutral behavior, easy access, easy and fast assay, low cost and high throughput, high reproducibility, and transferability between laboratories, populations and/or species (Behara *et al.*, 2008; Semagn *et al.*, 2006 and Levi *et al.*, 2004).

RFLP is the most widely used hybridization-based molecular marker. RFLP analysis has been used in the detection of genetic diversity, selection for traits of agronomic importance, and segregation analyses of progenies (Brettschneider 1998). The analysis involves digestion of complex genomic DNA into small DNA fragments using restriction enzymes such as restriction endonucleases that recognize and cut specific short sequences of DNA followed by separation of these fragments by gel electrophoresis. They differentiate genotypes based on mutations in endonuclease restriction sites across the genome in addition to genome rearrangements.

However, the low level of DNA polymorphism exhibited by RFLP markers, time consumption, laborious, expensive and its requirement of radioactively labeled probes limited its application (Levi *et al.*, 2004; Semagn *et al.*, 2006 and Behara *et al.*, 2008).

PCR based markers such as RAPD, ISSR, AFLP and SSR are superior to RFLP in that they show high diversity even in autogamous crops with a narrow genetic base eg; soybean and wheat (Russel *et al.*, 1997). These markers are currently widely employed in diversity and mapping studies (Milbourne *et al.*, 1998) particularly AFLP and RAPD are more preferred markers for diversity analysis.

2.10.2 Applications of molecular markers in herbal drug technology

DNA-based molecular markers have proved their utility in fields like taxonomy, physiology, embryology, genetics, etc. As the science of plant genetics progressed, researchers have tried to explore these molecular marker techniques for their applications in commercially important plants such as food

crops, horticultural plants (Zhang *et al.*, 2001 and Chen *et al.*, 2001) and recently in pharmacognostic characterization of herbal medicine.

2.10.2.1 Genetic variation using molecular markers

It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles (Oleszek *et al.*, 2002). Many researchers have studied geographical variation at the genetic level. Estimates of genetic diversity are also important in designing crop improvement programs for management of germplasm and evolving conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana* (Shasany *et al.*, 1999), neem (Farooqui, *et al.*, 1998), *Juniperus communis* L. (Adams *et al.*, 2002), *Codonopsis pilosula* (Fu *et al.*, 1999), *Allium schoenoprasum* L. (Friesen and Blattner, 1999), *Andrographis paniculata* (Padmesh *et al.*, 1999) collected from different geographical regions. Similarly, different accessions of *Cannabis sativa* (Kojoma *et al.*, 2002) have been discriminated using ISSR markers and those of *Arabidopsis thaliana* L. Heynh (Barth *et al.*, 2002) have been differentiated using cleaved amplified polymorphic sequence and ISSR markers. Inter- and intra-species variation has also been studied using DNA-based molecular markers. Interspecies variation has been studied using RFLP and RAPD in different genera such as *Glycyrrhiza* (Yamazaki *et al.*, 1994), *Echinacea* (Kapteyn *et al.*, 2002), *Curcuma* (Chen *et al.*, 1999) and *Arabidopsis* (Lind *et al.*, 2002). RAPD and RFLP have also been applied for characterization of *Epimedium* (Nakai *et al.*, 1996) species at the genetic level. Members of three different species of *Scutellaria* (Hosokawa *et al.*, 2000), Chinese medicinal plants and three subspecies of *Melissa officinalis* (Wolf *et al.*, 1999) have been discriminated using RAPD. Varietal characterization of Kenaf (*Hibiscus cannabinus* L. Cheng *et al.*, 2002) has been done with the help of agronomical and RAPD data. Varietal identification and genetic purity test in pepper and *Capsicum annuum* were carried out using RAPD markers (Hulya, 2003). RFLP technique was used for interspecific genetic variation within the genus *Capsicum* and also for DNA fingerprinting of pepper cultivars (Prince *et al.*, 1995). RAPD has served as a tool for the detection of variability in Jojoba (*Simmondsia chinensis* L. Schneider; Amarger and Mercier 1995), *Vitis vinifera* L. (Tessier *et al.*, 1999) and tea (*Camellia sinensis*; Wachira *et al.*, 1995). An attempt has been made to understand population structure of *Podophyllum peltatum* to establish commercial level propagation of useful secondary metabolites using molecular markers (Lata *et al.*, 2002). Also, high genetic diversity has been shown in *Podophyllum hexandrum* species from Himachal Pradesh, India (Singh *et al.*, 1999). Genetic variation and relationships among and within *Withania* species, Negi *et al.* 2000 and genetic relationships among papaya and its wild relatives (*Caricaceae*; Van *et al.*, 2002) have been revealed using AFLP markers. Genetic variation within *Brassica campestris* cultivars has been studied using AFLP and RAPD markers (Das *et al.*, 2010).

Phylogenetic relationship has been studied among citrus and its relatives using SSR markers (Pang *et al.*, 2003). RAPD has been used to construct genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* (Grattapaglia and Sederoff, 1994). RAPD markers have been developed for genetic mapping of Pacific yew (*Taxus brevifolia* Nutt; Gocmen *et al.*, 1996). An attempt has been made to develop a physical AFLP map of the complex *Arabidopsis* genome by combining gel-based AFLP analysis with in silico restriction fragment analysis using the published genome sequence (Peters *et al.*, 2001).

2.11 Authentication of medicinal plants

DNA-based techniques have been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable. Dried fruit samples of *Lycium barbarum* were differentiated from its related species using RAPD markers (Zhang *et al.*, 2001). The RAPD technique has also been used for determining the components of a Chinese herbal prescription, yu-pingfeng san. In this study the presence of three herbs (*Astragalus membranaceus* (Fisch.) Bge., *Ledebouriella seseloides* Wolff and *Atractylodes macrocephala* Koidz) in the formulation have been detected using a single RAPD primer (Cheng *et al.*, 1998).

Three rapid primers have been identified that could successfully discriminate between three species of *Atractylodes*, from Chinese formulation purchased from local markets (Chen *et al.*, 2001). In another study, three random primers were used to reveal the genetic variability of *Astragalus* medicine materials sold in Taiwan market. SSCP analysis was also conducted on PCR products from the ITS-1 region of rDNA in order to differentiate the two *Astragalus* species (Cheng *et al.*, 2002). Primers have been designed for hybridization with the hypervariable ends of microsatellite loci that could reveal DNA-polymorphism among five Eucalyptus species (Matsuda *et al.*, 1997). DAF has been used to identify the Chinese traditional medicine, *Magnoliae officinalis*, its counterfeits and substitutes (Wang *et al.*, 2001). An RAPD primer that is selective for an elite strain Aizu K-111 of *Panax ginseng*, including its cultured tissues has been identified (Yukiko *et al.*, 2001). RAPD and PCR–RFLP analysis have been used for authentication of *P. ginseng* among ginseng populations (Um *et al.*, 2001). Some researchers have used a new approach called Direct Amplification of Length Polymorphism (DALP) for authentication of *Panax ginseng* and *Panax quinquefolius* (Ha *et al.*, 2001). Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of rDNA has been done successfully (Lau *et al.*, 2001). A DNA microarray for detecting processed medicinal *Dendrobium* species (*Herba dendrobii*) was constructed by incorporating the ITS1-5.8s-ITS2 sequences of *Dendrobium* species on a glass slide. The established microarray could detect the presence of *D. nobile* in a Chinese medicinal formulation containing nine herbal components (Zhang *et al.*, 2003). Molecular authentication of *Atractylodes*-derived crude drugs (Jutsu) was done with the help of PCR–RFLP and direct sequencing of chloroplast trnK. Two inside the chloroplast trnK were selected as molecular markers for identification and discrimination of *Atractylodes rhizome* (Byaku-jutsu) and *Atractylodes Lancea* rhizome (So-jutsu). Based on polymorphism in the restriction site for Hinf1 in Region1 fragment (260 bp), it was possible to discriminate between the two species. By direct sequencing of Region 2 (436 bp) and comparison of the nucleotide sequence datasets, we could not only discriminate Byaku-jutsu and Sojutsu, but also identify the original plant species of each crude drug specimen (Mizukami *et al.*, 2003).

2.12 Detection of adulteration/substitution

RAPD technique was adopted to identify eight types of dried *Coptis* rhizomes and one type of *Picrorrhiza rhizome*, a substitute for the former in the Chinese herbal market. *P. ginseng* is often substituted by (Cheng *et al.*, 1997) *P. quinquefolius* (American ginseng). Sequence characterized amplified region (SCAR), AP–PCR, RAPD and RFLP have been successfully applied for differentiation of these plants and to detect substitution by other closely related species (Shaw and 1995; Wang *et al.*, 2001 and Mihalov *et al.*, 2000). Characterization of *Echinacea* species and detection of possible adulterations have been done using RAPD technique (Wolf *et al.*, 1999). DNA fingerprinting and polymorphism in the Chinese drug ‘Ku-Di-Dan’ (*herba elephantopi*) and its substitutes were studied using AP–PCR and RAPD. The results were used for authentication of ‘KuDi-Dan’ and its substitutes (Cao *et al.*, 1996). DNA fingerprinting of *Taraxacum mongolicum* (*herba taraxaci*) and its adulterants of six species of Compositae was demonstrated using AP–PCR and RAPD (Cao *et al.*, 1997). Bulb of *Fritillaria cirrhosa*, an official drug of Chinese Pharmacopoeia (Zhu and Woerdenbag 1995), is commonly used as an antitussive and expectorant. It has often been adulterated with similar bulbs of other related species. Specific DNA-based primers have been designed for authentication of *F. cirrhosa* at the genomic level (Li *et al.*, 2003). A molecular marker that is specific to medicinal rhubarb-based on chloroplast trnL/trnF sequence which is absent in its adulterants has been identified (Yang *et al.*, 2001). DNA sequence analysis of rDNA ITS and PCR–RFLP were explored for their application in differentiating four medicinal *Codonopsis* species from their related adulterants, *Campanumoea javania* and *Platycodon grandiflorus*. The technique allowed effective and reliable differentiation of *Codonopsis* from the adulterants (Farooqui *et al.*, 1998).



Figure 1: Normal *N. sativa* plant



Figure 2: Flower before pollination



Figure 3: Flower after pollination



Figure 4: Seeds of black cumin



Figure 4a: General view of evaluation plot during late *Rabi* 2014 at initial growing stage in 37 accessions of *Nigella sativa* L.



Figure 4b: General view of evaluation plot during late *Rabi* 2014 at maturity stage in 37 accessions of *Nigella sativa* L.



Figure 4c: Seedling of 37 accessions of *Nigella sativa* L. under protected condition early *Rabi* 2015.



Figure 4d: General view of evaluation plot during early *Rabi* 2015 at initial growing stage in 37 accessions of *Nigella sativa* L.

III. MATERIAL AND METHODS

This study was carried out in the Department of Plantation, Spices, Medicinal, and Aromatic Crops, College of Horticulture, (University of Horticultural Sciences), GKVK, Bengaluru- 65, India. Two experiments were conducted for the present study.

1. Genetic variability studies in the accessions of black Jeera (*Nigella sativa*) collected from different provinces of Afghanistan and India.
2. Molecular diversity analysis among selected divers accessions

3.1 Expt. I. Genetic variability studies in the accessions of Jeera (*Nigella sativa*) collected from different provinces of Afghanistan and India

The experiment was taken up twice with first crop sown on November, 28, 2014 (Late *Rabi*) and the second crop sown on 5th October of 2015 (Early *Rabi* season). The material and methods used are described below.

3.1.1 Geographical location of experimental plot

The experimental plot for the year 2014 was located at field of the Department of Genetics and Plant Breeding (13°05'06.6"N 77°34'30.7"E), University of Agricultural Sciences, GKVK, Bengaluru - 65.

The experimental plot for the year 2015 was located at of the Department of Plantation, Spices, Medicinal, and Aromatic Crops (13°05'45.5"N 77°33'54.0"E), College of Horticulture, GKVK, Bengaluru - 65.

3.1.2 Genetic variability analysis

This experiment was conducted to study the variability existing among the genotypes and to assess the contribution of several quantitative characters to yield and oil content. Thirty seven genotypes collected from different places from Afghanistan (28 accessions) and India (Nine accessions) were included in this study. The accessions from Afghanistan were collected from the field visits to different places of cultivation (Figure 5), while the accessions from India were procured from the markets at Bengaluru. Therefore the exact location of origin of the later cannot be known. The geographical locations of all the accessions are listed in the Appendix-I.

3.1.3 Experiment details

Year I (late <i>Rabi</i> 2014)		Year II (early <i>Rabi</i> 2015)	
Replications	3	Replications	2
Number of accessions	37	Number of accessions	37

For both the years Randomised Complete Block design was followed

3.1.4 Cultural practices

The space between row to row and between plants within the row was 40 cm respectively for both the years. Thus there were 12 plants in each row in the year 2014; for recording field observations 6 plants were chosen randomly and for the year 2015; each row accommodated 6 plants of which 3 plants were chosen randomly for recording field observations. The experimental site was ploughed three times and brought to a fine tilth and the land was divided into plots of required size and number; provision was made for bunds and irrigation channels. The fertilizer (at 5:3:2 kg/ha of N: P: K) was provided while sowing. The seeds were sown on 28th of November in 40 cm wide rows at 1 cm depth; furrows were properly covered after 25 days of sowing; excess seedlings were manually thinned to maintain only one seedling per hill at 40cm X 40 cm spacing. The plot was kept free from weeds by hand weeding at 40 and 70 days after sowing. Protective irrigation was given during the whole cropping period depending on the soil moisture condition. To protect the crop from root rot caused by *Sclerotium rolfsii*, the crop was given 1 per cent Bavistin drench twice at 40 and 60 days after sowing. The crop took about 110 to 120 days to reach maturity. The crop was harvested when the plants as well as the capsules (pods) turned yellow in colour and when split capsules discharged deep black coloured seeds with pleasant characteristic aroma. The whole plant was cut and dried under shade. The weight of ten capsules per each plant was recorded; seeds

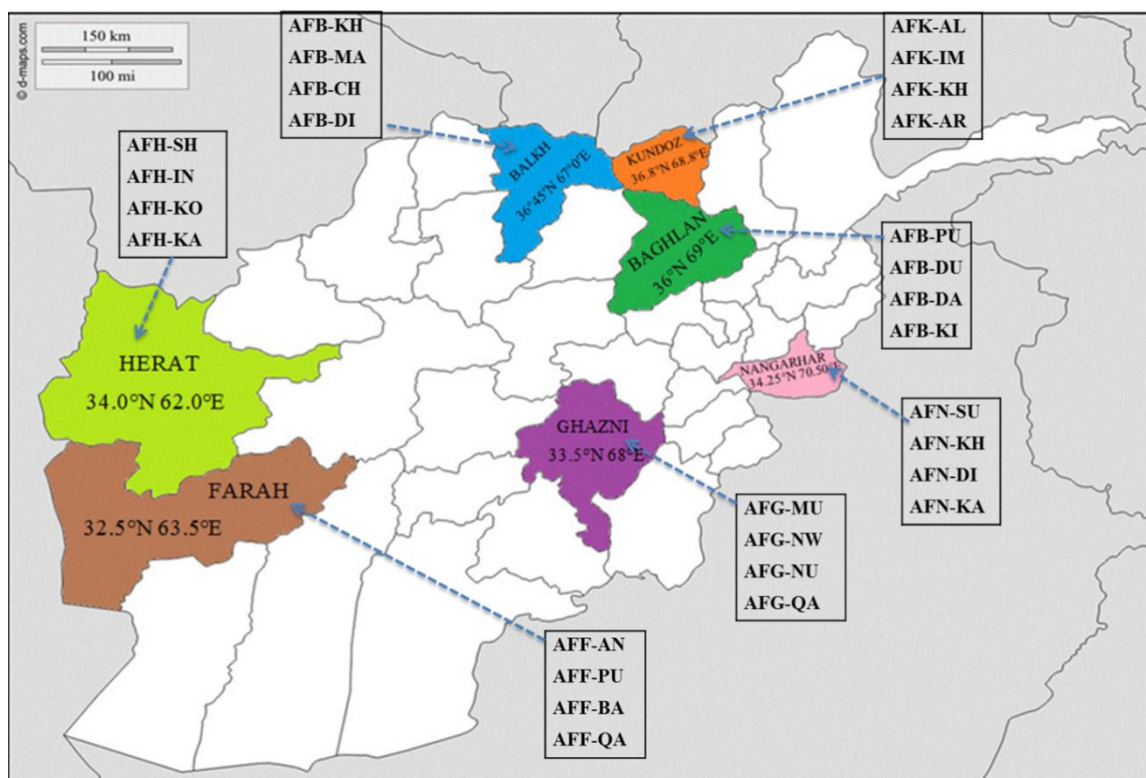


Figure 5: Collection areas of *Nigella sativa* L. accessions in Afghanistan. For the accessions collected from each district see Appendix I.

were then separated by threshing and winnowing of the dried herb. The produce was cleaned and the weight of the seeds per plant was recorded.

3.1.5 Recording the observations

The following observations were recorded on the selected plants in each replication and average was calculated.

1. **Days to first flowering:** First day of flowering of any plant in the accession after the sowing was recorded
2. **Days to 50 % flowering:** Days taken for 50 per cent of plants to show flowering within the accession from the date of sowing were recorded.
3. **Plant height:** The height of plant from collar level to the tip was recorded in centimetres, when 50 per cent of the plants in a plot showed flowering.
4. **Number of branches per plant:** All the primary and secondary branches were counted for each plant and averaged.
5. **Number of capsules per plant:** Total number of capsules at harvest was recorded for each plant.
6. **Number of locules per capsule:** Ten capsules were chosen at random from each plant and total number of locules was recorded and the average was calculated.
7. **Number of seeds per capsule:** Ten capsules were chosen at random from each plant and total number of seeds per capsule was counted and the average calculated.
8. **Ten capsules weight:** Ten harvested capsules were chosen at random and weight was recorded.
9. **Seed yield per plant:** The capsules were threshed and seeds weighed for all the plant and averaged.
10. **1000 seeds weight:** Weight of 1000 randomly selected seeds was recorded for each accession.
11. **Oil content (%):** The oil content in seeds was determined using Soxtherm apparatus on the principle of solvent extraction.

Apparatus required: Mixer grinder, Soxtherm apparatus (Gerhardt, Germany) (Figure 6).

Chemicals and reagents required: Petroleum ether (40-60⁰ C) and Calcium carbonate (Ca CO₃).

Procedure:

1. Sun dried seeds were powdered in mixer grinder.
2. Three grams of powder was weighed with precision to third decimal places.
3. Powdered seeds samples were taken in a cotton thimble and plugged with white cotton and placed in pre-weighed Soxtherm jar containing boiling stones.
4. About 100 mL petroleum ether was added to these jars and placed in Soxtherm apparatus.
5. Oil was extracted by running the pre-programed Soxtherm apparatus for four hours and 35 minutes.
6. After completion of extraction, the remaining petroleum ether and moisture was removed by keeping it in hot air oven at 110⁰ C for one hour.

7. The jars were removed from the oven and placed in desiccators containing and calcium carbonate (Ca CO_3).
8. The oil content in the seeds was determined using following formula.

$$\text{Oil content percentage} = \frac{W_2 - W_1}{W} \times 100$$

where, W, weight of powdered seeds sample: W_1 , weight of Soxtherm jar along with boiling stones, W_2 , final weight of Soxtherm jar along boiling stones and extracted oil.



Figure 6: Soxtherm apparatus

3.1.6 Statistical analysis

3.1.6.1 Analyses of variance

The data was analysed as Randomized Complete Block Design, RCBD (Cochran and cox 1957). The analysis of variance for different characters was carried out using the mean data in order to partition variability due to different sources by following Panse and Sukhatme (1961).

Sources of variation	d.f.	MSS	Expected value of MSS	F value
Replication	(r-1)	MSS r=M ₁	-	
Genotypes	(g-1)	MSS g=M ₂	$\sigma^2e + r\sigma^2g$	M ₂ /M ₃
Error	(r-1) (g-1)	EMSS=M ₃	σ^2e	
Total	(rg-1)	M ₁ +M ₂ +M ₃		

Where,

g = number of genotypes

r = number of replications

3.1.6.2 Estimation of genetic parameters

In order to assess and quantify the genetic variability among the accessions for the characters under study, the following parameters were estimated.

a. Estimation of variance components

Phenotypic and genotypic variances were estimated using the following formula (Singh and Chaudhary, 1985).

$$\text{Genotypic variance } (\sigma^2g) = \frac{\text{MSS (genotype)} - \text{MSS (error)}}{\text{Number of replications}} = \frac{M_2 - M_3}{R}$$

$$\text{Phenotypic variance } (\sigma^2p) = \sigma^2g + \text{MSS (error)} = \frac{M_2 - M_3}{R} + M_3$$

b. Coefficient of variability

Both genotypic and phenotypic coefficients of variability were computed as per the method suggested by Burton and Devane (1953).

(i) Genotypic coefficient of variability (GCV)

$$\text{GCV} = \frac{\sqrt{\sigma^2g}}{\bar{x}} \times 100$$

(ii) Phenotypic coefficient of variability (PCV)

$$PCV = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100$$

Where,

σ^2_g = genotypic variance

σ^2_p = phenotypic variance

$\bar{x}$ = General mean of the characters

The GCV and PCV values were classified as described by Sivasubramanian and Menon (1973).

GCV and PCV values	Classification
0 to 10%	Low
10 to 20%	Medium
20% and above	High

3.1.6.3 Estimation of heritability and genetic advance:

Heritability in broad sense was computed as the ratio of genetic variance to the total phenotypic variance as suggested by Hanson *et al.* (1956) and expressed as percentage.

$$h^2 = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

Where,

σ^2_g = genotypic variance

σ^2_p = phenotypic variance

Heritability estimates were classified into low, moderate and high by following Hanson *et al.* (1956).

Heritability	Classification
0 to 30%	Low
30 to 60%	Medium
60% and above	High

Genetic advance (GA)

Genetic advance was estimated by using the formula given by Johnson *et al.* (1955).

$$GA = h^2 K \sigma_p$$

Where,

h^2 = Heritability in broad sense

K = Selection differential which is equal to 2.06 at 5 per cent intensity of selection (Lush, 1949).
 σ_p = Phenotypic standard deviation

Genetic advance as per cent of mean (GAM)

$$GAM = \frac{GA}{\bar{x}} \times 100$$

Where,

GA = Genetic advance

$\bar{x}$ = General mean of the character

GAM was categorized as follows by Johnson *et al.* (1955).

GAM	Classification
0 to 10%	Low
10 to 20%	Medium
20% and above	High

3.1.6.4 Estimation of correlations

The correlation coefficient was worked out to determine the degree of association of a quantitative character with yield and also among the yield components for each season separately. Phenotypic correlation was computed by using the formula given by Weber and Moorthy (1952).

$$\text{Where } r_p = \frac{\text{Cov } (x_p y_p)}{\sqrt{\text{Var } (x_p) \text{ Var } (y_p)}}$$

Where, r_p = Phenotypic correlation

Cov $x_p y_p$ = Phenotypic covariance between the characters 'x' and 'y'

Var(x_p) and Var(y_p) = Phenotypic variance of the character 'x' and 'y' respectively

The genotypic correlation coefficients were also calculated as phenotypic correlation coefficients using the formula given below.

$$r_g = \frac{\text{Cov } (x_g y_g)}{\sqrt{\text{Var } (x)_g \text{ Var } (y)_g}}$$

Where, r_g = Genotypic correlation

Cov $x_g y_g$ = Genotypic covariance between the characters 'x' and 'y'

Var(x_g) and Var(y_g) = Genotypic variance of the character 'x' and 'y' respectively

Both genotypic and phenotypic correlation coefficients were compared against table 'r' values given in Fisher and Yates (1963) table at (n-2) degrees of freedom at the probability levels of 0.05 and 0.01 to test their significance.

3.1.6.5 Path coefficient analysis

The path coefficient analysis was carried out by using the correlation coefficient to know the direct and indirect effects of the component characters on seed yield for each season separately as suggested by Wright (1921) and illustrated by Dewey and Lu (1959).

Path coefficients were obtained by solving the simultaneous equations which express the basic relationship between correlations and path coefficients. The equations were as follows.

$$\begin{aligned} r_{1,y} &= P_{1,y} + r_{1,2}P_{2,y} + r_{1,3}P_{3,y} + \dots + r_{1,7}P_{7,y} \\ r_{2,y} &= P_{2,y} + r_{2,1}P_{1,y} + r_{2,3}P_{3,y} + \dots + r_{2,7}P_{7,y} \\ r_{3,y} &= P_{3,y} + r_{3,2}P_{2,y} + r_{3,4}P_{4,y} + \dots + r_{3,7}P_{7,y} \\ &\dots \\ &\dots \\ r_{6,y} &= P_{6,y} + r_{6,2}P_{2,y} + r_{6,3}P_{3,y} + \dots + r_{6,6}P_{6,y} \end{aligned}$$

Where,

$r_{1,y}$ to $r_{6,y}$ denote the correlation coefficients between independent characters 1 to 6 and dependent characters 'y', $r_{1,2}$ to $r_{5,6}$ denote the correlation coefficients between all possible combination of independent characters $P_{1,y}$ to $P_{6,y}$ denote the effects of characters 1 to 6 on character y.

3.1.6.6 Multivariate analysis of variance

A simultaneous test of significance was conducted considering all the characters together. The data on individual plant character was subjected for the computation of variance and co-variance by following the details set up in the table given below.

Source of variation	Mean sum of squares (X)	Mean sum of squares (Y)	Mean sum of squares (XY)
Treatment	M. S. S. (X)	M. S. S. (Y)	M. S. P. (XY)
Error	E. S. S. (X)	M. S. S. (Y)	E. S. P. (XY)

For these variances and co-variances among all the characters, the dispersion matrices were constructed for both treatments and error components. The order of each matrix was 11X11 since all the 11 characters were considered.

3.1.6.7 Mahalanobis's D^2 analysis:

The square of the Mahalanobis's generalized distance between any two populations is given by the formula.

$$D^2_p = d^1 S^{-1} d \quad \text{where}$$

D^2_p = square of the distance considering p variables.

d = vector of differences of the mean values among all the characters of two accessions.

S^{-1} = inverse of variance –covariance Matrix

Since inverting the matrix is complicated especially when the numbers of variables under study are large, the original correlated variables (X_1) were transformed to non-correlated variables (Y_1) so that

the computation of D^2 values reduce to simple summation of the squares of the differences between values of transformed variables of the two accessions.

This transformation can be done by pivotal condensation method. From these newly transformed un-correlated variables, the square of distance was computed by using the formula.

$$D^2 = \sum (X_{i1}^- - X_{i2}^-)^2 \text{ for accession 1 and 2}$$

X_i^- = vector of transformed mean values.

The square root of these D^2 values gives the generalized distance between the two accessions; the D^2 values were arranged in a matrix form.

3.1.6.8 Clustering accessions using D^2 values:

All the $\frac{n(n-1)}{2}$ D^2 values were calculated and used for clustering by Tocher's method (Rao, 1952).

The two genotypes having lowest D^2 value between them were selected as the members of a cluster and third genotype which on an average added the smallest D^2 value for the first two was included in to cluster. Similarly others were added as long as their added D^2 value is small to within cluster distance among its members. However, if at any stage increase in average D^2 value because of the addition of a new genotype exceeded the average of those already included, then that genotype was taken out and the cluster was closed. The procedure was repeated for other genotypes omitting those that are already included in the former clusters.

The average distance of all the genotypes with in the cluster (intra-cluster distance) and average distance between and two clusters (inter-cluster distance) were calculated.

The D^2 values were used to construct the dendrogram.

3.1.6.9 Canonical variate analysis:

This analysis was followed to represent the number of uncorrelated variables by lesser number of canonical variates. These are obtained as a linear combinations of a number of uncorrelated variables. The co-efficient ($1_1, 1_2, 1_3, \dots, 1_p$) are obtained by solving the equation

$$I(A - \lambda I) = 0$$

where I = the coefficient vector,

λ = root of matrix 'A'

The method follows the construction of the matrix of sum of squares and sum of products for the transformed mean values. The suitable power of the original matrix was obtained, and canonical vectors were obtained by iteration starting from a trial vector (1, 1, 1 ...1).

The canonical roots were also obtained by subtracting the product of (1) (i^{th} element $\times j^{\text{th}}$ element) of the first vector, from the power matrix. Then the process of iteration was repeated to get subsequent roots (2) and vectors (1_2). The roots are obtained in the decreasing order of magnitude. The Z alveus were calculated for each genotype from the mean values of (Y_1) transformed variables through the formula:

$$Z = (X) (Y)$$

where I = canonical vector

Y = transformed variables in a vector.

Two (Z) values were computed for each genotype corresponding to (Y) respectively using their corresponding vectors. These 'Z' values were plotted on a two dimensional graph taking end. The group constellations were marked arbitrarily. The percentage contribution for each the canonical root was calculated by the formula:

$$\text{The percentage contribution from each of the canonical root} = \frac{\lambda}{\text{Total of trace in the dispersion matrix}} \times 100$$

3.2 Expt 2: Molecular diversity analysis

After germinating seeds and growing them for four weeks, young fresh leaves were collected separately from randomly selected individual plants from each accession and washed three times in sterile distilled water. Further, the leaf samples were frozen in liquid nitrogen and kept at -80 °C until further use.

The genomic DNA was extracted from leaf samples collected from 12 accessions of *Nigella sativa*. The genomic DNA was prepared by following method given by Krishna and Jawali, (1997) with minor modifications as follows;

3.2.1 Selection of accessions for DNA Study

Based on data collected in two years clustering was done for all the 37 accessions. The 12 accessions were selected from those using the following objective criteria;

- a. All clusters were represented by at least one genotype (as far as possible).
- b. Number of genotypes from each cluster was also a function of the size of the cluster such that diversity is well represented.
- c. There is consistence of genotypes in clustering with other genotype in both seasons. Accordingly the 12 accessions studied are listed (Table 6) and used for DNA analysis.

3.2.2 Genomic DNA isolation

The seedling of 12 accessions was grown in small caps, the 3.5 g of leaf samples were collected from 30 days old seedlings for DNA isolation the leaf samples were immediately frozen in liquid nitrogen. The DNA was isolated using CTAB method and the protocol followed is given below.

1. Frozen tissue sample (3 g) was grind into fine powder in liquid nitrogen, using autoclaved mortar and pestle.
2. The fine powder was transferred to 2.0 ml Eppendorf tube containing 1.0 ml of extraction buffer (SDS).
3. The solution mixed well and incubated at 65 °C for 1 hour with intermittent shaking.
4. The contents were cooled on ice.
5. Potassium acetate (300 µl) was added and mixed thoroughly.
6. Then centrifuged for 20 min at 13000 rpm at 4 °C.
7. Supernatant was collected to fresh.
8. Isopropanol – ammonium acetate mixture (600 µl) was added for precipitate of DNA.
9. The mixture was centrifuged at 13000 rpm for 20 min at 4 °C.

10. Supernatant was discarded and pellet obtained was washed with 70 per cent alcohol.
11. Pellet was air dried.
12. The air dried pellet was dissolved in 200 µl of TE buffer.
13. RNAase (10 mg/ml) was added, mixed well and incubated 37 °C for 30 min.
14. The DNA was precipitated by adding 1/10th volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol.
15. The contents were gently mixed and incubated at 4 °C for 30 min.
16. Centrifuged for 1 min at 3000 rpm at 4⁰ C.
17. Supernatant was discarded and the pellet was washed with 70% ethanol.
18. Pellet was air dried and dissolved in 100 µl TE buffer.

3.2.3 Purification and quantification of extracted genomic DNA

1. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added to each tube and the contents were mixed by inverting.
2. The contents were spun at 2500 rpm for 10 min and supernatant was transferred to fresh tubes. Supernatant was removed with wide-bore tip (cut off blue tip) to clean tube, care was taken to get clear supernatant, though may be coloured.
3. Step 1 and 2 were repeated.
4. To the above supernatant, 10 µl of 3 M sodium acetate (1/10 volume of aqueous layer) and 2.5 volumes of chilled absolute ethanol was added, mixed gently and incubated at -20 °C for 15-20 min.
5. The DNA was spooled in 1.5 ml eppendorf tubes using a glass hook, washed with 70 per cent alcohol and dried.
6. DNA was dissolved in 100 µl TE buffer and kept at -20 °C till its further use.

The amount of DNA in each sample was quantified by taking the readings at 260 / 280nm in the Nano Drop (UV Technologies, USA).

1. The instrument was switched on and the blank was set with the TE buffer (3 µl).
2. The quantity of DNA was measured at 260 / 280nm with 3 µl on Nano Drop spectrophotometer pedestal.
3. The readings obtained were quantified in ng/µl and OD value for each sample was recorded.

The ratio between the readings at 260 and 280 nm (OD 260/OD 280) was used as an estimate of the purity of the DNA samples. Pure preparations of DNA have 260/ 280 nm OD ratio between 1.7 and 1.8 (Sambrook and Russel, 2001). Computed OD values were used to dilute the DNA samples to the working concentrations of 5ng/µl. The stock DNA solutions were diluted to 5 ng/µl of 100 ng/µl working solution for PCR. Then the amount of stock DNA solution to be taken for dilution was calculated using the following formula, where M1 is the stock DNA concentration (for example, 100 ng/µl), V1 is the volume of stock to be diluted, M2 is the concentration of working solution (5 ng/µl) and V2 is the volume of working solution to be prepared (for example, 100 µl).

$$M1V1 = M2V2$$

$$(100 \text{ ng/}\mu\text{l}) V1 = (5 \text{ ng/}\mu\text{l}) (100 \mu\text{l})$$

$$V1 = (5 \text{ ng/}\mu\text{l}) (100 \mu\text{l}) / (100 \text{ ng/}\mu\text{l})$$

V1 = 5 µl

The appropriate volume from the stock was transferred to 0.5 ml micro-centrifuge tube and the volume was made to 100 µl using distilled water. The DNA working solutions were stored at -20 °C till further use.

3.2.4 PCR optimization for RAPD

Three concentrations each of primer (2.5, 3.5, 5.0 pmol/µl), Mg²⁺ (1.0, 2.0, 5.0 mM), and (1U/µl) *Taq* polymerase enzyme (GeNei, India), and two concentrations of DNA (50, 100 ng/µl) and dNTP (5.0, 10 mM, Eppendorf, USA) were varied in different combinations and the combination that gave good amplification was selected to carry out further experiments. Amplifications were performed in a final volume of 20 µl with 1X assay buffer. Three different annealing temperatures of 30°C, 33°C and 35°C were used for standardization of annealing temperatures. Suitable annealing temperature was standardized which produced sharp amplicons without much spurious products.

Amplification reaction mixture for RAPD markers was prepared following components in a total volume of 20 µl.

Components	Quantity (µl/reaction)
Genomic DNA (50 ng/µl)	1.0
dNTPs mix (10 mM each)	1.0
(RAPD) Random primer (5 pmol/µl)	1.0
MgCl ₂ (25 mM)	1.0
Assay buffer (10x)	2.0
<i>Taq</i> DNA polymerase (3U/µl)	0.4
Sterile water	13.6

PCR reaction was carried out using Master Cycler gradient 5331-Eppendorf version 2.30.31-09, Germany. The cycler was programmed as follows;

Step	Temperature (°C)	Duration	No. of cycles
Initial denaturation temperature	: 95	: 4 min.	1
Denaturation	: 94	: 1 min.	} 35
Primer annealing	: 33	: 2 min	
Primer extension	: 72	: 2 min	
Complete primer extension	: 72	: 20 min	1
Dump	: 4	: up to removed	1

Separation and visualization of RAPD products was done on 1.5 per cent agarose. The markers, which exhibited polymorphism in agarose, were genotyped on 1.5 per cent agarose gel.

3.2.5 Gel Electrophoresis

RAPD fragments were separated electrophoretically on 1.5 % agarose gels in 1×TBE buffer, stained with ethidium bromide, and photographed on a UV trans illuminator using a digital camera. DNA from each plant was amplified and the banding patterns were compared. All visible and unambiguously scorable fragments were recorded and polymorphic bands were scored as '1' present and '0' absent.

3.2.6 Genetic diversity analysis

The allelic data obtained from the RAPD markers were used to visualize the genetic relationships among twelve accessions of Jeera. The similarity was calculated using the simple matching (SM) coefficient (Sneath and Sokal, 1973). Similarity matrices were obtained using the subprogram SIMQUAL to generate pair-wise Jaccard's similarity coefficient (NTSYS-pc, version 2.0), (Rohlf, 2000).

$$\text{Jaccard's coefficient} = N_{AB} / (N_{AB} + N_A + N_B),$$

Where, N_{AB} is the number of fragments shared by samples, N_A represents fragments amplified in sample A and N_B is fragments amplified in sample B. Finally using similarity indices, the clusters were built by Unweighted Pair Group Method Using Arithmetic Average (UPGMA) procedure. After scoring and computing the allelic data, a dendrogram was constructed by using NTSYSpc, version 2.0. The informativeness of given primer combinations i.e. polymorphism information content (PIC) was calculated. PIC was calculated for all the primer combinations (Botstein *et al.*, 1980) using POWERMARKER program version, 3.0 (Liu and Muse, 2005).

$$\text{PIC} = 1 - \left[\sum_{i=1}^n p_i^2 \right] - \left[\sum_{i=1}^{n-1} \sum_{j=i+1}^n 2 p_i^2 p_j^2 \right]$$

Where, p_i is the frequency of i^{th} allele and p_j is the frequency of $(i+1)^{\text{th}}$ allele.

IV. EXPERIMENTAL RESULTS

The results of the experiment conducted using 37 accessions collected from Afghanistan and India are presented below.

4.1 Genetic variability studies in the accession of black Jeera

4.1.1 Analysis of variance (ANOVA)

The mean sum of squares due to various sources of variation for 11 characters of 37 genotypes is presented in Table 1-a, for late *Rabi* 2014 and in Table 1-b for early *Rabi* 2015. Highly significant difference was observed among accessions for all the characters during late *Rabi* 2014.

The mean performance of all accessions was though similar during the both seasons for many traits, the plants grew taller (62.83 cm) during early 2015 compared to late *Rabi* 2014 (38.57 cm). Average yield and oil percentage were very different between two years; the average yield was more (2.31 g) during late *Rabi* 2014 compared to 2015 (1.93 g) and the average oil percentage was 23.25% during late *Rabi* 2014 and it was 21.33% during *Rabi* 2015.

Day to first flowering ranged from 53.20 to 56.06 days, days to 50% flowering ranged from 66.66 to 78.33 days, plant height ranged from 28.81 to 46.4 cm, number of branches per plant ranged from 16.85 to 28.26, number of capsules per plant ranged from 18.76 to 31.93, ten capsules weight ranged from 1.31 to 2.19 g, number of locules per capsule ranged from 5.43 to 6.38, number of seeds per capsule ranged from 71.27 to 90.30, 1000 seeds weight ranged from 1534.58 mg to 1680.41mg, seed yield per Plant ranged from 1.44g to 3.35g, oil percentage ranged from 13.76% to 30.12% during late *Rabi* 2014.

During early *Rabi* 2015, except four traits viz; days to first flowering, plant height, number of locules per capsule and seeds per capsule all other traits showed significant differences. The seed yield per yield manifested highest (F) ratio (21.05), followed by 1000 seeds weight (16.12) and 10 capsules weight (10.16). The days to first

Table 1a: Analysis of variance for different traits among the selected genotypes in late *Rabi* 2014

	Days to first flowering	Days to 50% flowering	Plant height (cm)	Number of branches per plant	Number of capsules per plant	10 capsules weight	Number of locules per capsule	Number of Seeds per Capsule	1000 seeds weight (mg)	Seed yield Per plant (g)	Oil content %
Mean	54.58	73.12	38.57	22.83	25.54	1.87	5.88	80.72	1587.13	2.31	23.25
C.V.	1.04	3.19	4.33	7.51	5.85	8.72	5.55	5.01	1.26	10.42	3.95
F ratio	6.00 ^{**}	3.82 ^{**}	15.36 ^{**}	7.23 ^{**}	9.77 ^{**}	3.81 ^{**}	1.64 [*]	5.26 ^{**}	10.00 ^{**}	12.16 ^{**}	68.53 ^{**}
S.E.	0.33	1.35	0.96	0.99	0.86	0.09	0.19	2.34	11.51	0.14	0.53
C.D. 5%	0.92	3.80	2.72	2.79	2.43	0.26	0.53	6.58	32.44	0.39	1.49
C.D. 1%	1.23	5.04	3.61	3.70	3.23	0.35	0.71	8.74	43.06	0.52	1.98
Range	53.20-56.06	66.66-78.33	28.81-46.44	16.85-28.26	18.76-31.93	1.31-2.19	5.43-6.38	71.27-90.30	1534.58-1680.41	1.44-3.35	13.76-30.12

Table 1b: Analysis of variance for different traits among the selected genotypes in early *Rabi* 2015

	Days to first flowering	Days to 50% flowering	Plant height (cm)	Number of branches per plant	Number of capsules per plant	10 capsules weight	Number of locules per capsule	Number of Seeds per Capsule	1000 seeds weight (mg)	Seed yield Per plant (g)	Oil content %
Mean	61.30	72.89	62.83	24.37	21.84	1.85	5.38	78.64	1583.88	1.93	21.33
C.V.	1.79	1.85	462.22	4.53	5.02	4.66	4.71	860.23	0.64	3.75	3.28
F ratio	1.49 ^{NS}	3.86 ^{**}	1.00 ^{NS}	7.72 ^{**}	8.64 ^{**}	10.16 ^{**}	1.33 ^{NS}	1.00 ^{NS}	16.12 ^{**}	21.05 ^{**}	44.74 ^{**}
S.E.	0.78	0.95	205.35	0.78	0.78	0.06	0.7 206	2.07	7.12	0.05	0.49
C.D. 5%	-	2.73	-	2.24	2.23	0.17	-	-	20.42	0.15	1.42
C.D. 1%	-	3.66	-	3.00	2.98	0.23	-	-	27.39	0.20	1.90
Range	59.30-63.20	69.50-77.00	21.08-27.81	19.85-28.55	17.37-26.55	1.38-2.24	4.38-5.28	69.91-83.33	1517.62-1651.00	1.28-2.23	13.63-26.62

flowering ranged from 59.30 to 63.20 days, days to 50% flowering ranged from 69.50 to 77.00 days, plants height ranged from 21.08 to 27.81 cm, number of branches per plant ranged from 19.85 to 28.55, number of capsules per plant ranged from 17.37 to 26.55, ten capsules weight ranged from 1.38 to 2.24 g, number of locules per capsule ranged from 4.38 to 5.28, number of seeds per capsule ranged from 69.91 to 83.33, 1000 seeds weight ranged from 1517.62 to 1651.00 mg, seed yield per plant ranged from 1.28 to 2.23g, oil percentage ranged from 13.63 to 26.62. The results of the two years of study on 37 accessions are presented below and the performance of individual accessions is presented in Appendix 2.

4.1.2 Genetic variability parametric

The genetic variability parameters viz., Genotypic Coefficient Variability, Phenotypic Coefficient Variability, H^2 (Broad Sense of heritable), Genetic Advance, Genetic Advancement as % of Mean are presented in Table 2.

Days to first flowering: The number of days to first flowering ranged from 53.20 days to 56.06 days with an overall mean of 54.58 days during late *Rabi* 2014. The range was 59.30 days to 63.20 days during early *Rabi* 2015 with a mean of 61.30 days. The trait showed low GCV (1.34 and 0.88) and PCV (1.69 and 2.00) values in both the years. The heritability estimate was high (62.00%) during late *Rabi* 2014 while it was low during 2015 season. The genetic advance over mean values is also low in both the seasons.

Days to 50% flowering: The mean and range observed for this trait were more or less same in both the years. It ranged from 66.66 days to 78.33 days with a mean of 73.12 days in the year 2014 while it ranges from 69.50 days to 77.00 days with a mean of 72.89 days in *Rabi* 2015. The GCV (3.09 and 2.20) and PCV (4.44 and 2.88) values were also low in both the years. Low heritability of 48.00% and 59.00% was observed in both seasons. Low percentage of genetic advance over mean was seen in both seasons (4.44 and 3.49). The heritability values were moderate in both the seasons

Plant height: The plant height showed a wider range during 2015 (21.08 cm to 178.12 cm) and a higher mean of 62.83 cm compared to late *Rabi* 2014 season which had a mean

Table 2: Genetic parameters for different traits across seasons (late *Rabi* 2014: early *Rabi* 2015)

		Genotypic Coefficient Variability	Phenotypic Coefficient Variability	H ² (Broad Sense)	Genetic Advance	Genetic Advancement as % of Mean
Days to first flowering	2014	1.34	1.69	62%	1.19	2.19
	2015	0.88	2.00	20%	0.50	0.81
Days to 50% flowering	2014	3.09	4.44	48%	3.24	4.44
	2015	2.20	2.88	59%	2.54	3.49
Plants height (Cm)	2014	9.47	10.41	83%	6.85	17.75
	2015	5.50	4.62	78%	0.08	0.14
Number of branches per plant	2014	10.82	13.17	75%	4.55	17.80
	2015	8.31	9.46	77%	3.66	15.03
Number of capsules per plant	2014	10.01	11.59	68%	4.18	18.32
	2015	9.82	11.03	79%	3.93	18.01
Ten capsules weight	2014	8.43	12.13	48%	0.23	12.09
	2015	9.97	11.00	82%	0.34	18.61
Number of locules per capsule	2014	2.57	6.12	18%	0.13	2.23
	2015	1.90	5.08	14%	0.09	1.47
Number of seeds per capsule	2014	5.97	7.79	59%	7.61	9.42
	2015	0.01	8.60	60%	4.06	2.90
1000 seeds weight (mg)	2014	2.17	2.51	75%	61.60	3.88
	2015	1.74	1.86	88%	53.61	3.39
Seed yield per plant (g)	2014	20.08	22.62	79%	0.85	36.74
	2015	11.85	12.43	91%	0.45	23.29
Oil content %	2014	18.73	19.41	96%	8.78	37.75
	2015	15.32	15.67	96%	6.59	30.88

of 22.83 cm and a range of 28.81 cm to 46.44 cm. The GCV (9.47 and 5.50) values were low in both the years. However the PCV and GAM values were moderate during late *Rabi* 2014 season while it was low in early *Rabi* 2015. The heritability values were high in both the seasons.

Number of branches per plant: The range observed for this trait during late *Rabi* 2014 was 16.85 to 28.26 with a mean of 22.83. The range was 19.85 to 28.55 during early *Rabi* 2015 with a mean of 24.37. The GCV and PCV values observed during 2014 were moderate (10.82 and 13.17) while it was low during 2015 (8.31 and 9.46). Moderate GAM (17.80 and 15.03) values were observed in both seasons. High heritability was observed in both the seasons (75.00% and 77.00%).

Number of capsules per plant: Moderate and low GCV (10.01 and 9.82) values were observed in late *Rabi* 2014 and early *Rabi* 2015 respectively. The PCV (11.59 and 11.03) values were moderate in both the seasons. High heritability of 68.00% and 79.00% were observed in both seasons for this trait. Moderate GAM (18.32 and 18.01) values were observed in both seasons. The number of capsules showed a range of 18.76 to 31.93 and 17.37 to 26.55 during 2014 and 2015 seasons respectively

Ten capsules weight: The GCV (8.43 and 9.97) were low and the PCV (12.13 and 11.00) were moderate for this trait during late *Rabi* 2014 and early *Rabi* 2015 respectively. Moderate heritability of 48.00% was observed in late *Rabi* 2014 whereas high heritability 82.00% was reported in early *Rabi* 2015. The GAM (12.09 and 18.61) values were moderate in both the seasons. The mean (1.87 and 1.85) and range (1.31 to 2.19 in 2014 and 1.38 to 2.24 in 2015) were more or less same in both the seasons.

Number of locules per capsule: The mean number of locules was 5.88 in late *Rabi* 2014 and 5.38 in early *Rabi* 2015. The GCV (2.57 and 1.90) and PCV (6.12 and 5.08) were low in both the seasons. Low heritability of (18.00% and 14.00%) and low GAM (2.23 and 1.47) were observed in both the seasons. The range observed for this trait was also low in both the seasons.

Number of seeds per capsule: The mean number of seeds per capsule was 80.72 in late *Rabi* 2014 and 78.64 in early *Rabi* 2015 with a range of 71.27 to 90.30 in 2014 and 69.91 to 83.33 in 2015 seasons. Low GCV (5.97 and 0.01) and PCV (7.79 and 8.60) were observed in both the seasons. The heritability estimate (59.00%) was moderate in late *Rabi* 2014 whereas it was high (60.00%) in early *Rabi* 2015. The GAM (9.42 and 0.00) values were low in both the seasons.

1000 seeds weight: Low GCV (2.17 and 1.74) and PCV (2.51 and 1.86) in both the seasons combined with high heritability values (75.00% and 88.00%) were observed in both seasons. The GAM (3.88 and 3.39) values observed were also low in both the seasons. The mean (1587.13mg and 1583.88mg) and range (1534.58 to 1680.41 and 1517.62 and 1651.00) were more or less same for both the seasons.

Seed yield per plant: High GCV (20.08 and 11.85) and PCV (22.62 and 12.43) values were observed in both the seasons. The heritability (79.00% and 91.00%) and GAM (36.74 and 23.29) values were also high in both the seasons for this trait. Higher mean seed yield (2.13g / plant) was observed during late *Rabi* 2014 compared to early *Rabi* 2015 (1.93g / plant). The range (1.44g to 3.35g) was also wide during late *Rabi* 2014 compared to early *Rabi* 2015 (1.28g to 2.23g).

Oil content (%): Higher mean oil content (23.25%) and range (13.76 to 31.12) was observed during late *Rabi* 2014 compared to early *Rabi* 2015 which had a mean of 21.33% and a range of 13.63 to 26.62. Moderate GCV (18.73 and 15.32) and PCV (19.41 and 15.67) values were consistently observed in both the seasons. High heritability (96.00% and 96.00%) combined with high GAM (37.75 and 30.88) values was observed in both the seasons.

4.1.3 Differential performance of accessions during the two seasons

Accessions were found to exhibit differences in their performance for different traits during the two seasons. This could be captured effectively by plotting the performance of the accessions during one season (2015) against that during another season (2014) and comparing the pattern of distribution with the line that depicts a linear relation of slope = 1.00. If the altered performance is purely due to the seasonal effect, with no genotype X environment interaction, then all the accessions would fall around the line (of slope = 1.00) or parallel to it depending upon the effect of season. Any deviation from such linearity would suggest differential performance. Accordingly it was found that for yield two types of accessions could be identified: Those that performed linearly, with no G X E effect (enclosed in black shape) and those that were highly affected by G x E effect (enclosed in a dashed shape).

However the accessions behaved differently for different traits. A linear relationship was observed for the oil per centage (Figure 7). 1000 seed weight (Figure 8); ten capsule weight (Figure 9), number of branches per plant (Figure 10), plant height (Figure 11) and days to first flowering (Figure 12). Number of seeds per capsule also showed a linear relationship except for three accessions AFK-IM, AFB-DA and AFH-IN (Figure 13)., Total yield per plant also showed a linear relation except for the accessions INB-M1, INB-M2, INB-M3, INB-M5, INB-M7, AFG-NW, AFH-IN, AFF-AN, AFK-IM and AFK-A (Figure 15), two traits viz., number of locules per capsule (Figure 14) and days to first flower did not show any variation during the (2015) while the accessions showed a wide variation during 2014; consequently their relation was horizontal with a slope of -0.26, which is not significantly deviant from slope 0.0 (horizontal) as indicated by R^2 value of 0.04.

4.1.4 Correlation studies

In late *Rabi* 2014 days to first flowering was correlated strongly and positively with days to 50% flowering (0.39), moderately with plant height (0.2651), number of branches per plant (0.25), number of capsules per plant (0.24) and oil content % (0.24) where as it showed negative correlation with ten capsules weight (-0.1921). The plant height was positively correlated with number of capsules per plant (0.64), number of branches per plant (0.57) and number of locules per capsule (0.40) where as it showed negative correlation with oil content %. The number of capsules per plant showed positive correlation with total number of branches per plant (0.94) and moderately with number of locules per capsule (0.27). The number of branches per plant was positively correlated with number of locules per capsule (0.22). Ten capsules weight manifested significantly positive effect on number of seeds per capsule (0.21). The number of seeds per capsule showed significantly moderate positive correlation with oil content % (0.27), (Table 3).

In early *Rabi* 2015, the number of branches per plant correlated strongly and positively with the number of capsules per plant (0.96). Ten capsules weight was positively correlated with days to first flowering (0.29). Number of locules per capsule showed positive correlation with number of capsules per plant (0.96) and number of branches per plant, (Table 3).

The diagrams depict the strength of correlation in the colors hues shows that there are two sets of traits that show strong association among themselves in 2014 (Figure 16) and 2015 (Figure 17). The plant height, number of branches per plant, number of capsules per plant and seed yield exhibit very strong positive association among themselves during both the years. Where another set of traits namely 1000 seed weight, days to first flowering, days to 5% flowering and oil content % exhibit a strong negative association among themselves during 2015. This negative association was not evident clearly during 2014.

4.1.5 Path-coefficient analyses

The path-coefficient analysis for late *Rabi* 2014 (Table 4-a) and early *Rabi* 2015 (Table 4-b) was carried out to discern direct and indirect effects of yield attributing traits on total yield.

Number of capsules per plant had the highest positive direct effect on (0.31) on seed yield per plant followed by days to first flower, plant height and total branches (all had 0.21) during 2014 season. The number of branches and plant height contributed substantially but indirectly through number of capsules per plant (0.20 and 0.21)

There appeared to be seasonal effect on the way the yield is determined by the characters. The data from 2015 showed that number of capsules per plant and 100 seeds

Table 3: Phenotypic correlation values among different variables studied during late *Rabi* 2014 (below the diagonal) and early *Rabi* 2015 (above the diagonal)

	Days to first flowering	Days to 50% flowering	Plant height (cm)	Number of branches per plant	Number of capsules per plant	Ten capsules weight	Number of locules per capsule	Number of Seeds per capsule	1000 seeds weight (mg)	Seed yield per plant (g)	Oil content %
Days to first flowering	1.00	-0.16	0.10	0.11	0.10	0.29 *	0.10	-0.04	-0.01	-0.13	-0.07
Days to 50% flowering	0.39 ***	1.00	0.03	-0.04	-0.11	-0.08	-0.11	0.03	-0.01	-0.11	0.03
Plant height (cm)	0.26 **	0.07	1.00	-0.06	-0.04	0.15	-0.04	-0.00	0.06	0.12	-0.20
Number of capsules per plant	0.24 *	0.08	0.64 ***	1.00	0.96 ***	0.19	0.96 ***	0.03	0.11	-0.18	0.58***
Number of branches per plant	0.25 **	0.13	0.57 ***	0.94 ***	1.00	0.19	1.00 ***	0.04	0.09	-0.15	0.55***
Ten capsules weight	-0.19 *	-0.06	-0.13	-0.03	-0.00	1.00	0.19	0.12	-0.01	-0.15	0.16*
Number of locules per capsule	0.11	0.02	0.40 ***	0.27**	0.22 *	0.03	1.00	0.04	0.09	-0.15	0.55***
Number of seeds per capsule	0.16	0.04	0.03	0.06	0.08	0.21 *	0.09	1.00	0.10	0.08	0.05
1000 seeds weight (mg)	-0.14	0.03	-0.17	-0.02	0.04	0.10	-0.09	-0.10	1.00	-0.16	0.28**
Oil content %	0.24 *	0.03	-0.23 *	-0.09	-0.18	-0.05	0.00	0.27 **	-0.16	1.00	-0.10
Seed yield per plant (g)	0.31	0.09	0.57	0.69	0.69	0.08	0.26	0.11	0.09	-0.18	1.00

Table 4a: Path matrix of total yield per plant during late *Rabi* 2014

[illegible]

Table 4b: Path matrix of Total yield per plant during early *Rabi* 2015

[illegible]

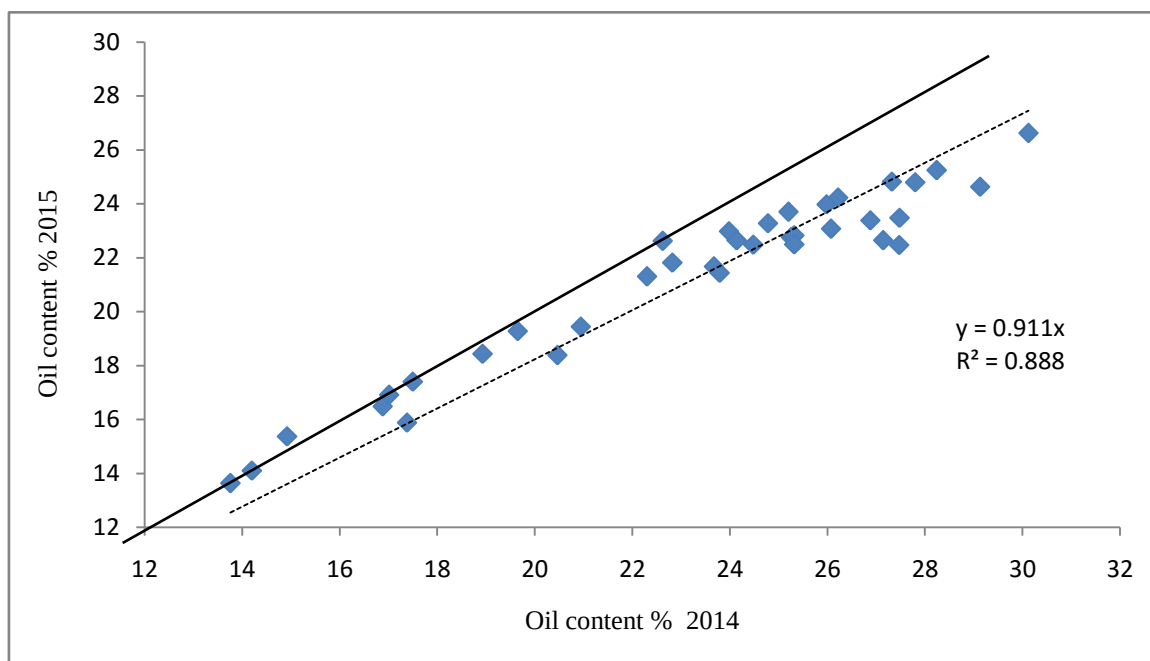


Figure 7: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the oil content %. The line shows a linear relation with slope = 1.00. The dotted line shows the best fit line

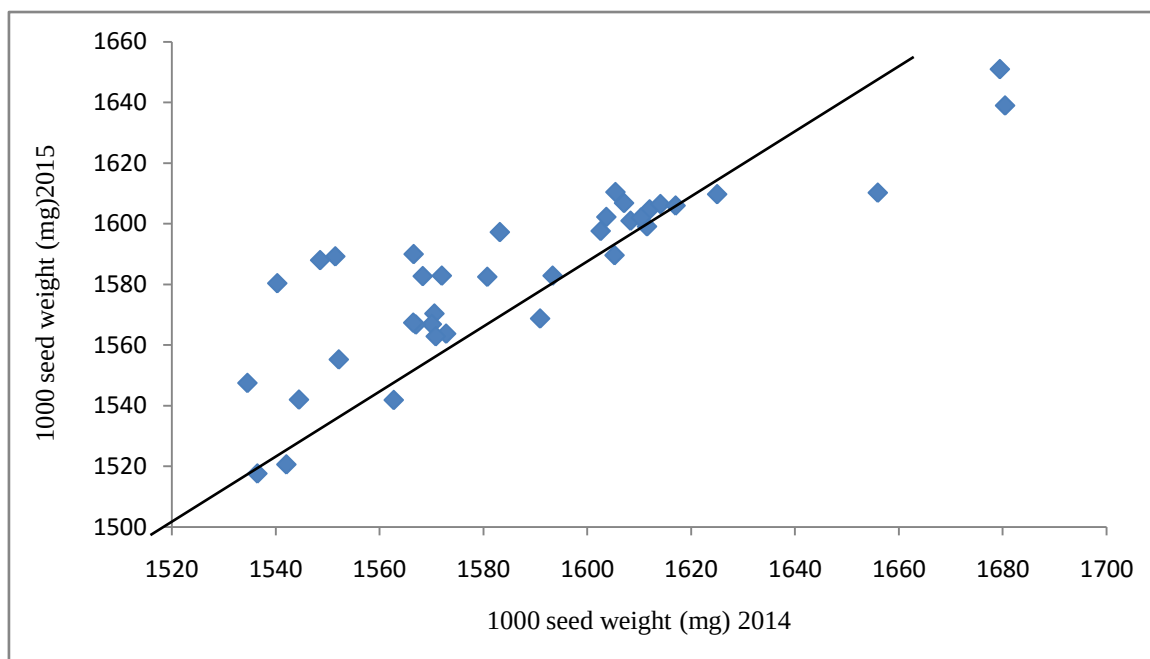


Figure 8: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the 1000 seeds weight. The line shows a linear relation with slope = 1.00

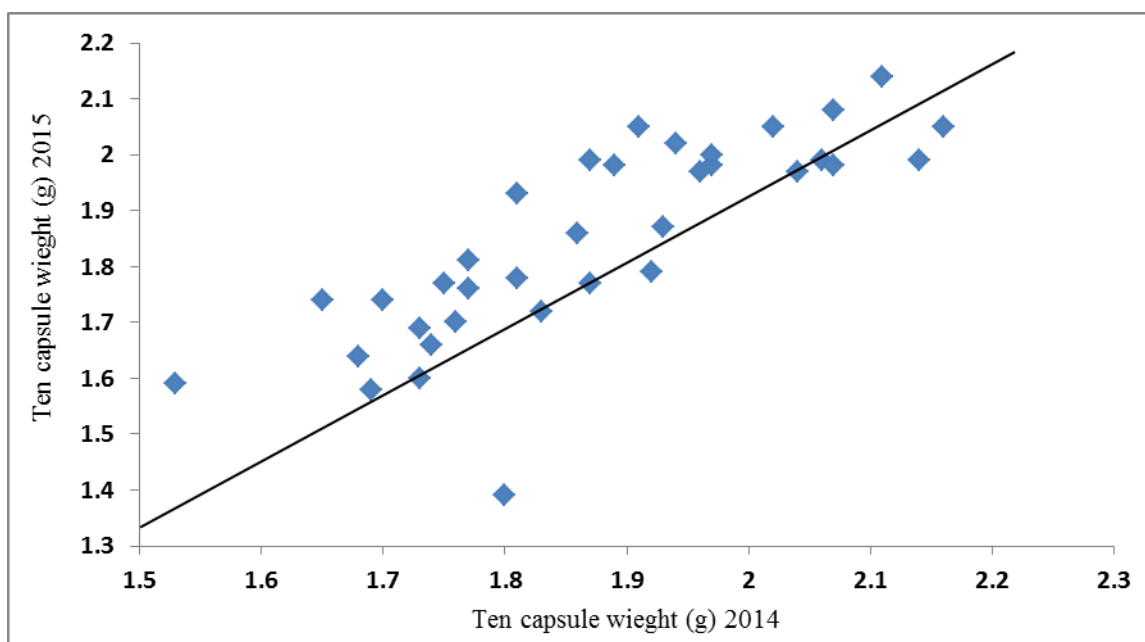


Figure 9: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the ten capsule weight. The line shows a linear relation with slope = 1.00.

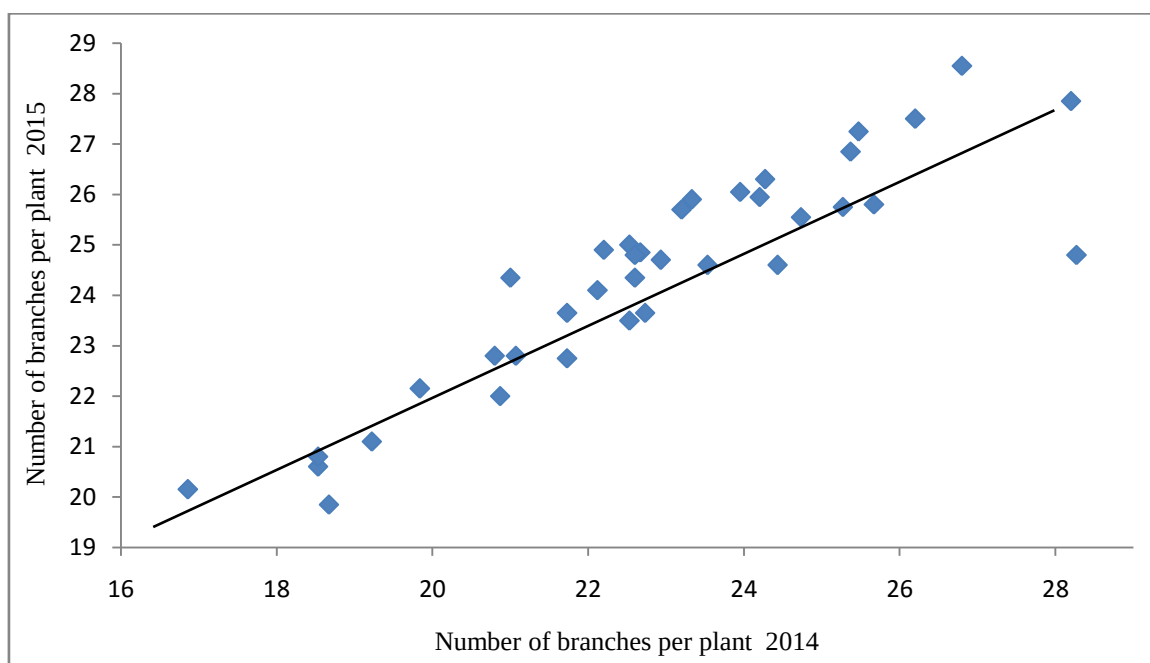


Figure 10: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the number of branches per plant. The line shows a linear relation with slope = 1.00.

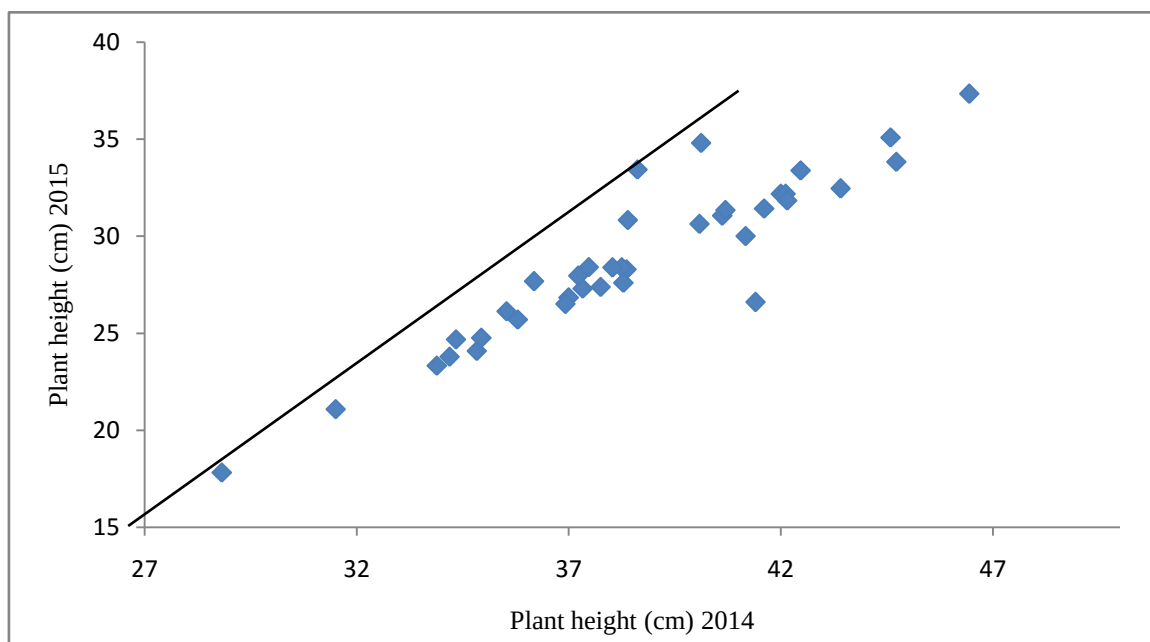


Figure 11: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the plant height (cm). The line shows a linear relation with slope = 1.00.

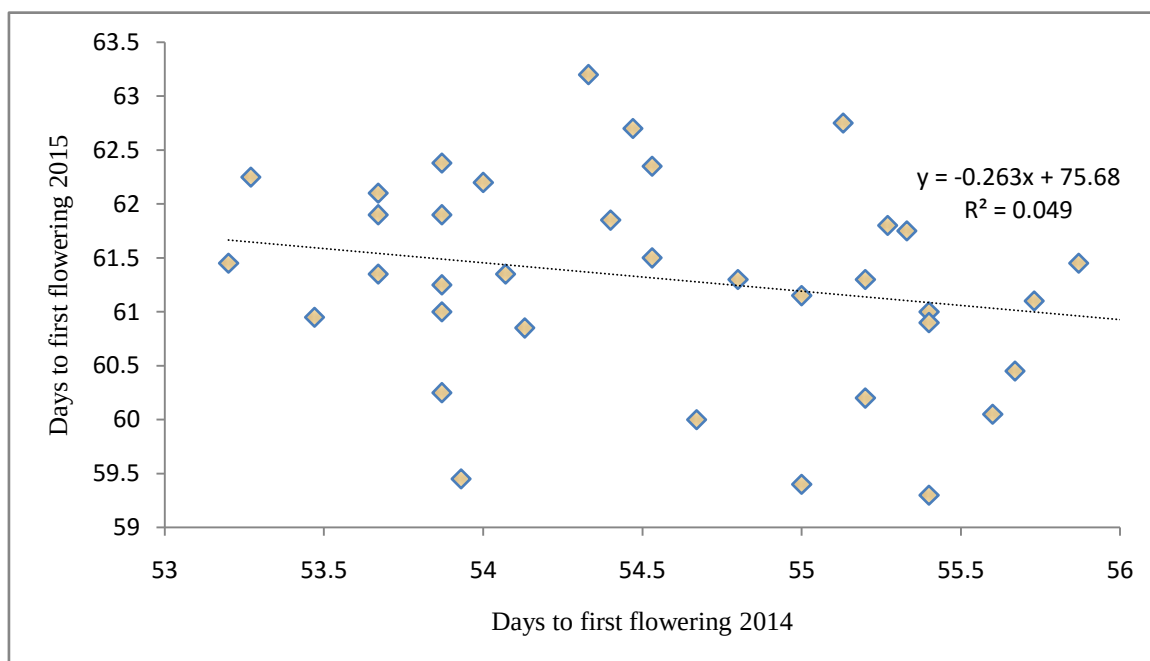


Figure 12: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the days to first flowering. The line shows a linear relation with slope = 1.00. The dotted line shows the best fit line.

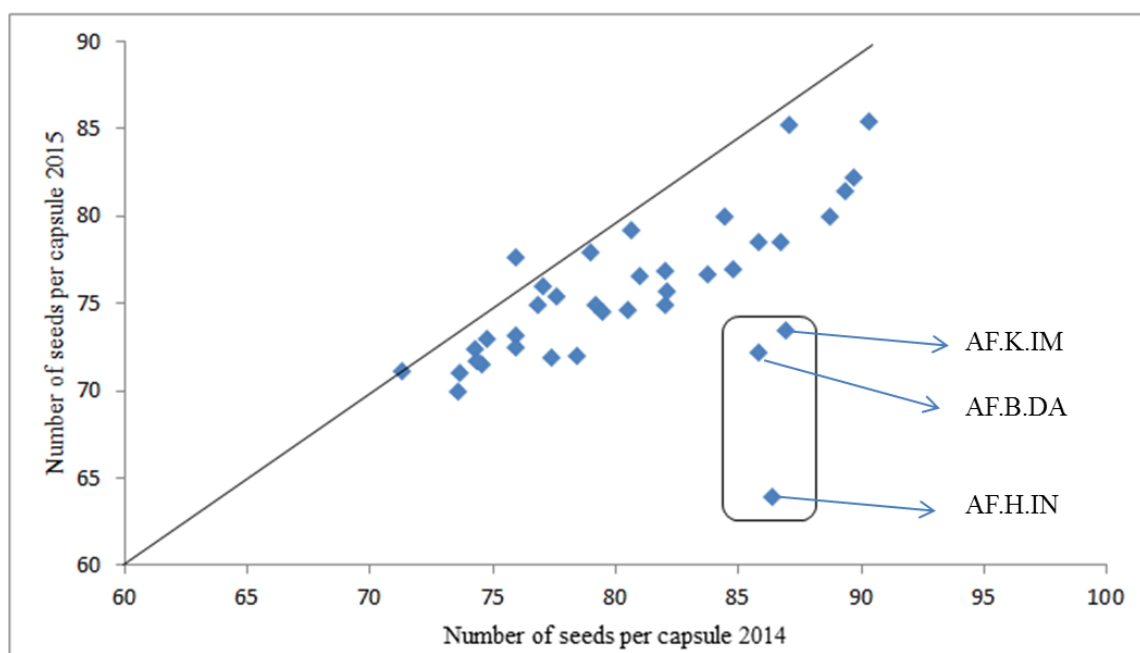


Figure 13: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the number of seeds per capsule. The line shows a linear relation with slope = 1.00.

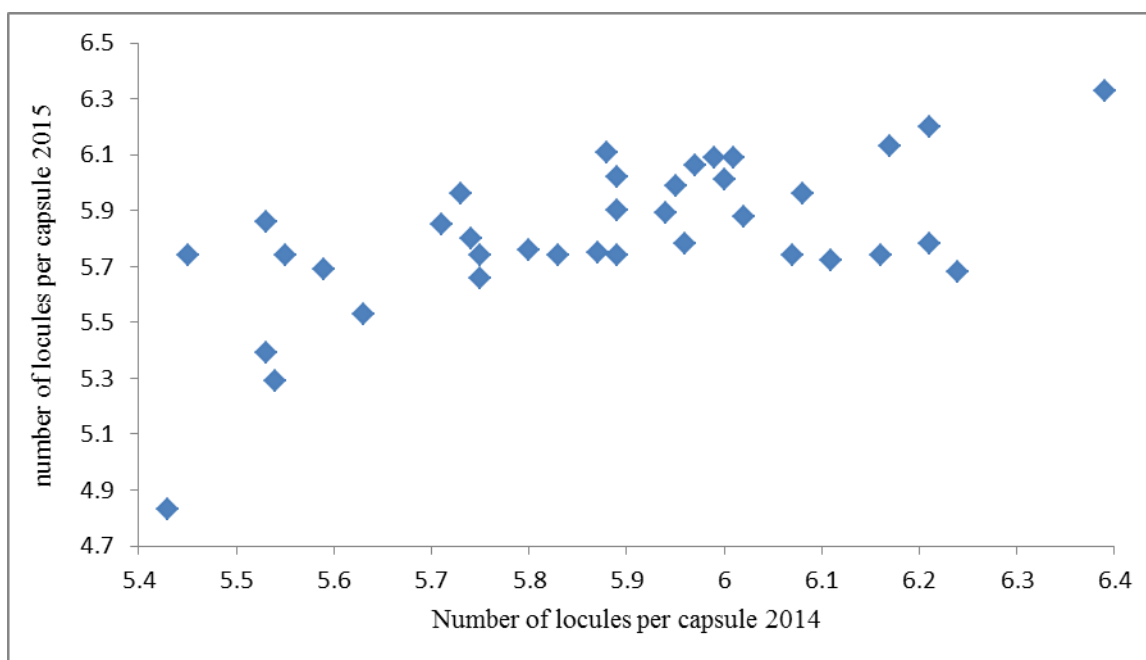


Figure 14: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the number of locules per capsule. The line shows a linear relation with slope = 1.00.

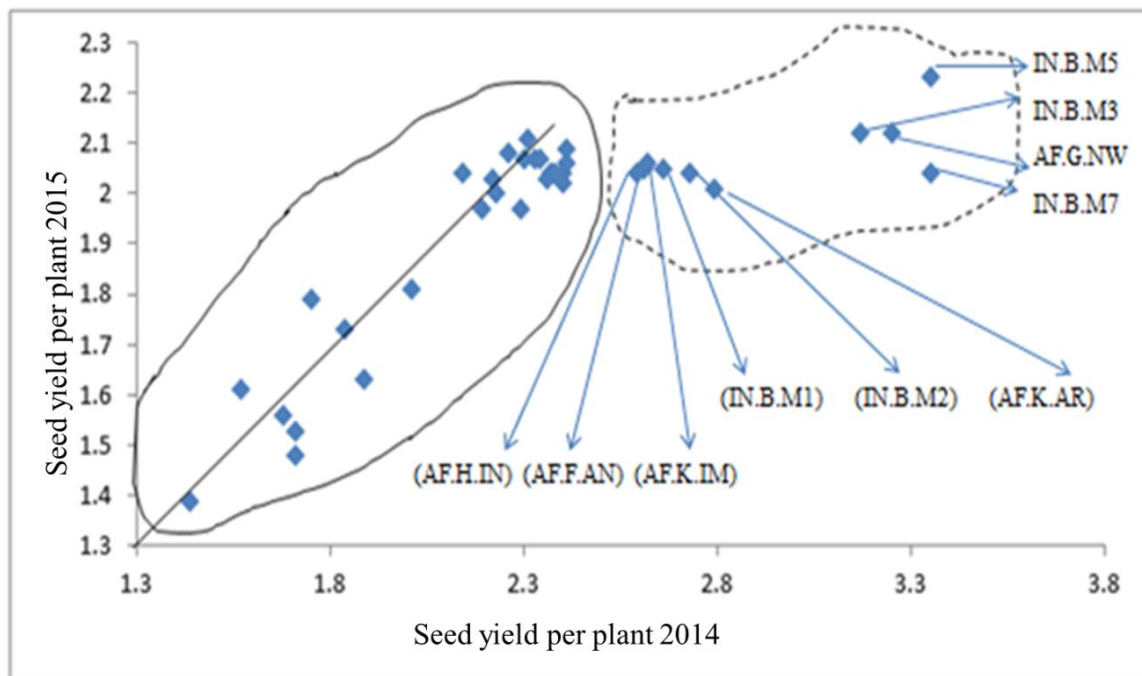


Figure 15: Relation between the performance of accessions during late *Rabi* 2014 (X axis) and early *Rabi* 2015 (Y axis) for the seed yield per plant. The line shows a linear relation with slope = 1.00.

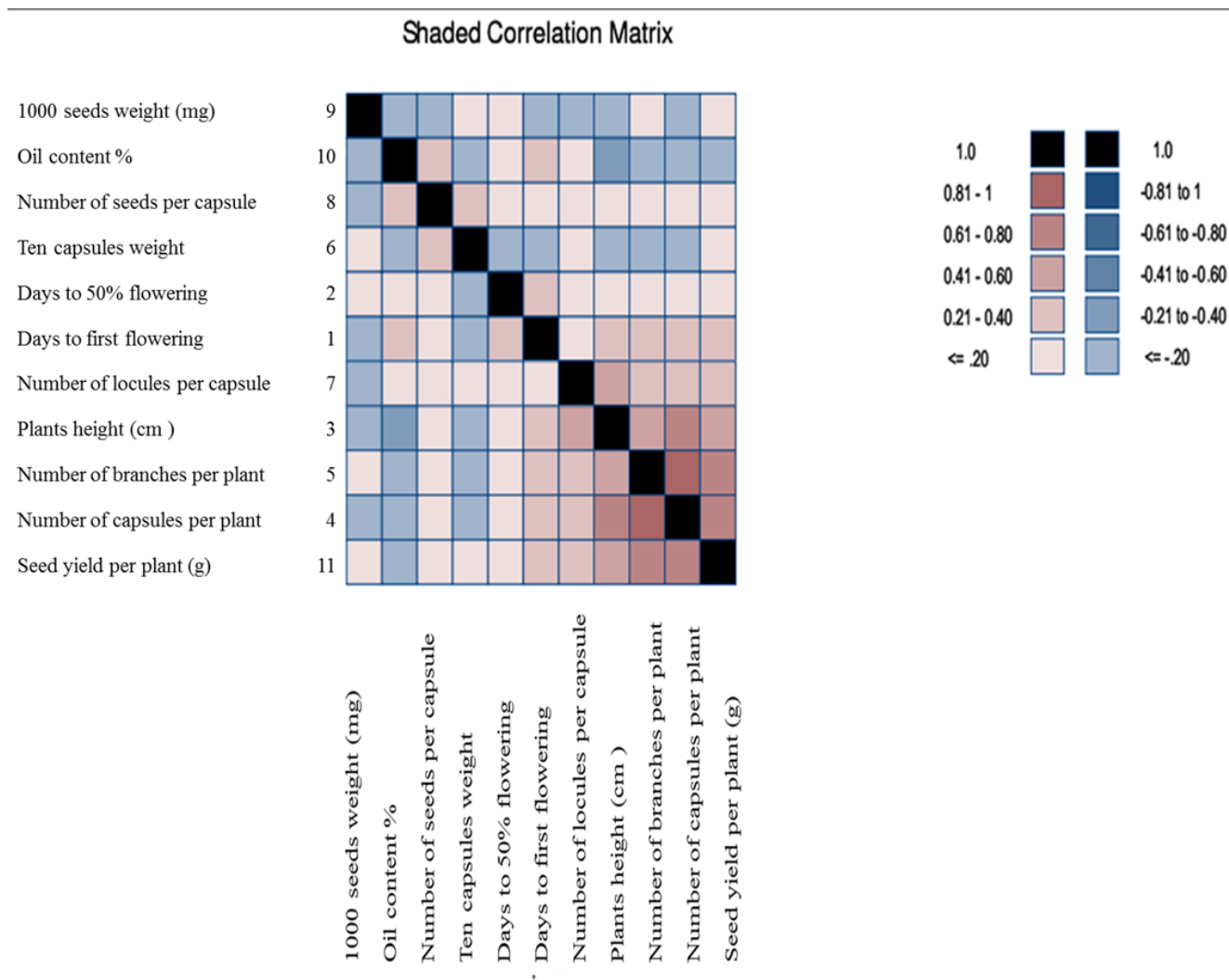


Figure 16: Correlation among different traits studied during late *Rabi* 2014.

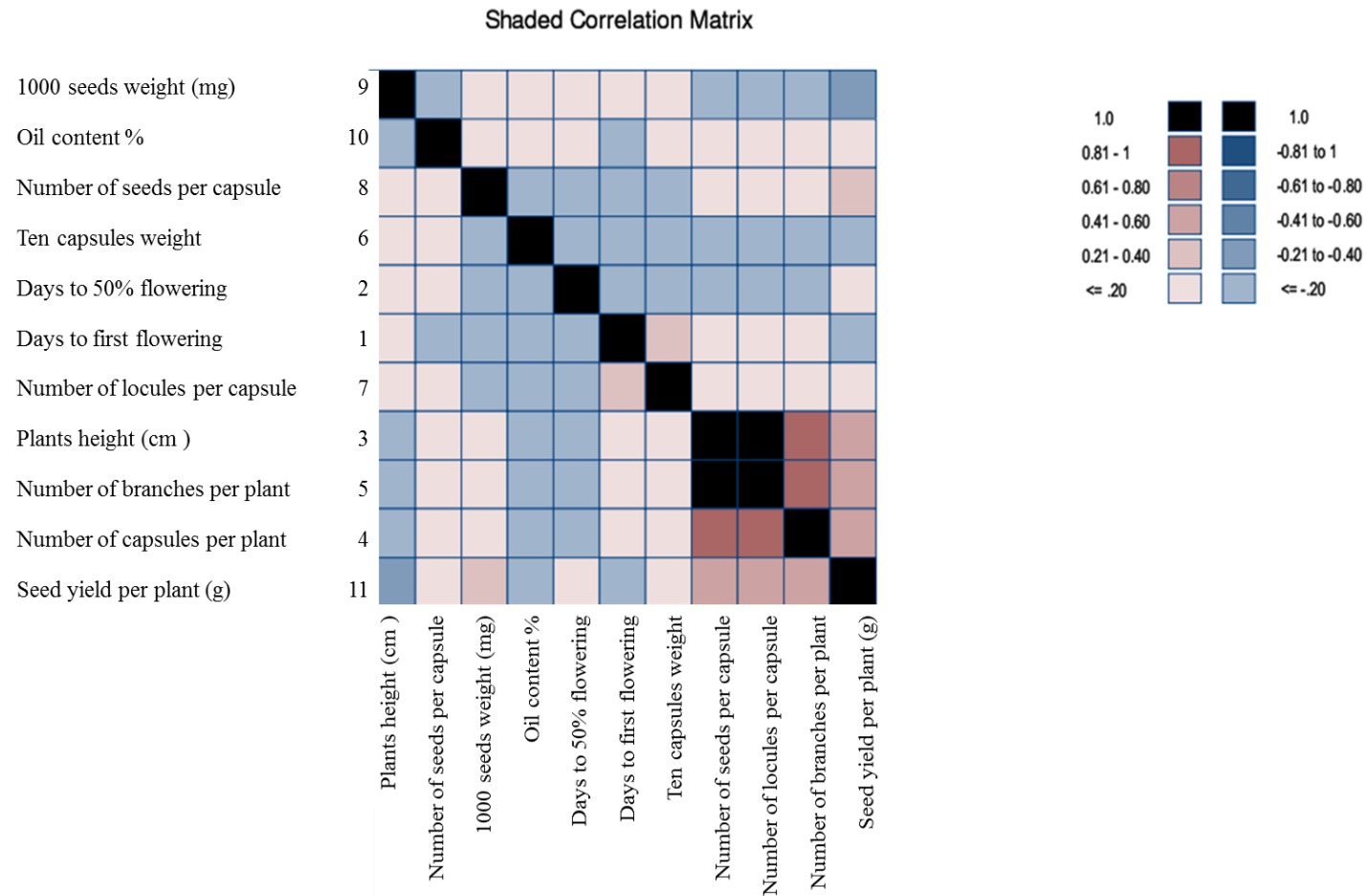


Figure 17: Correlation among different traits studied during early *Rabi* 2015.

weight has high direct effect (0.56 and 0.25) respectively on seed yield per plant. Therefore unlike in 2014, where in plant height had negative direct effect (-0.27)

Number of branches per plant had higher indirect effect through number of capsules per plant (0.54). Total number of locules per capsule had the highest positive indirect effect through number of capsules (0.54) on seed yield per plant during *Rabi* 2015. In both the years the residual effect was more.

4.1.6 Principal Component Analyses

The Principal Component Analysis could extract only 84.18 and 84.12 % of the variability among the accessions for all the traits. We used only the first three iterations as they together extracted more than 50 % variability. These first three Principal Component Axes (PCA) could extract 55.79 % during 2014 and 53.78 during 2015. Again the extent of each of the Axes extracted was also similar during both the years. During the years 2014 and 2015, the first PCA extracted 21.41 and 21.77 %, the second PCA extracted 18.66 and 17.22% and the third PCA 15.72 and 14.79 % respectively.

Even in the weights assigned to different traits also there was a similarity between the two years, especially when the first two PC Axes are considered. For example the most important traits that contributed through the first Axis during 2014 were 1000 seed weight (-0.52), plant height (0.50), and number of locules per capsule (-0.48) while through the second axis, number of branches per plant (0.48) and seed yield per plant (0.48) contributed the most. During the 2015 season, the traits that contributed the most were number of capsules per plant (0.51), number of branches per plant (0.42) and 10 capsule weight (0.37) through the first PC Axis while seed yield per plant (-0.58), oil per cent (0.44) and days to 50 % flowering (-0.43) contributed through the second PC Axis (Figure 18 and Figure 19).

The Separation of the accessions in the space between the first two axes was also very similar in both the years. There are three distinct patterns that can be discerned from the spatial separation of the accessions between the first two PC Axes:

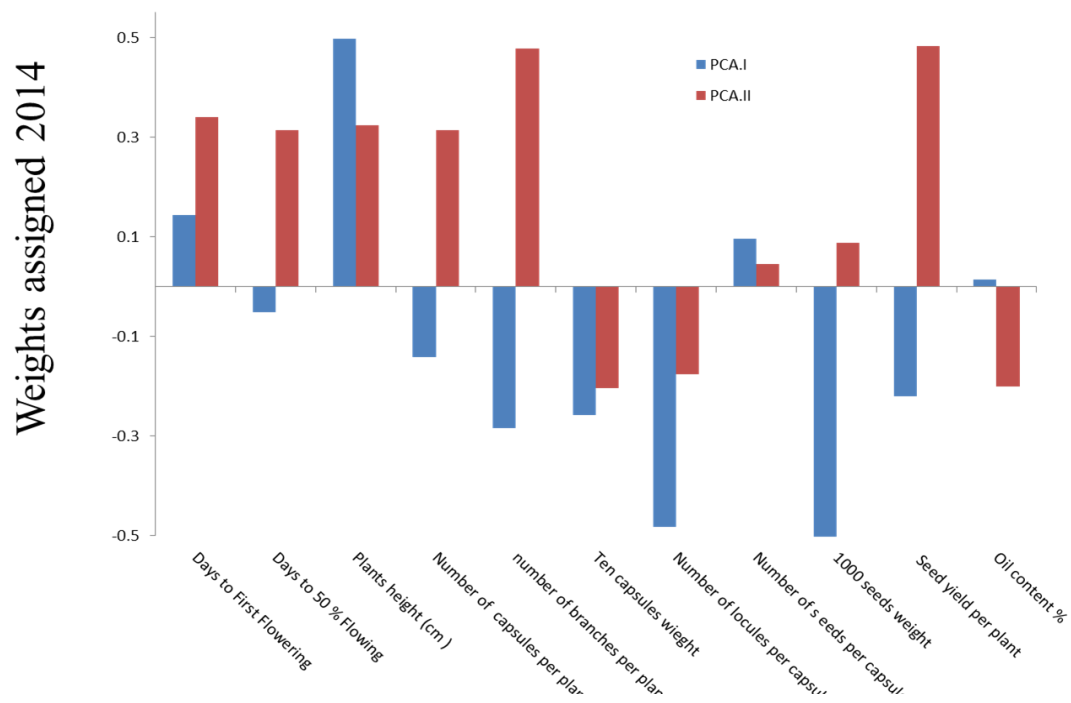


Figure 18: Graphical representation of PCA weightages assigned to variables late *Rabi* 2014.

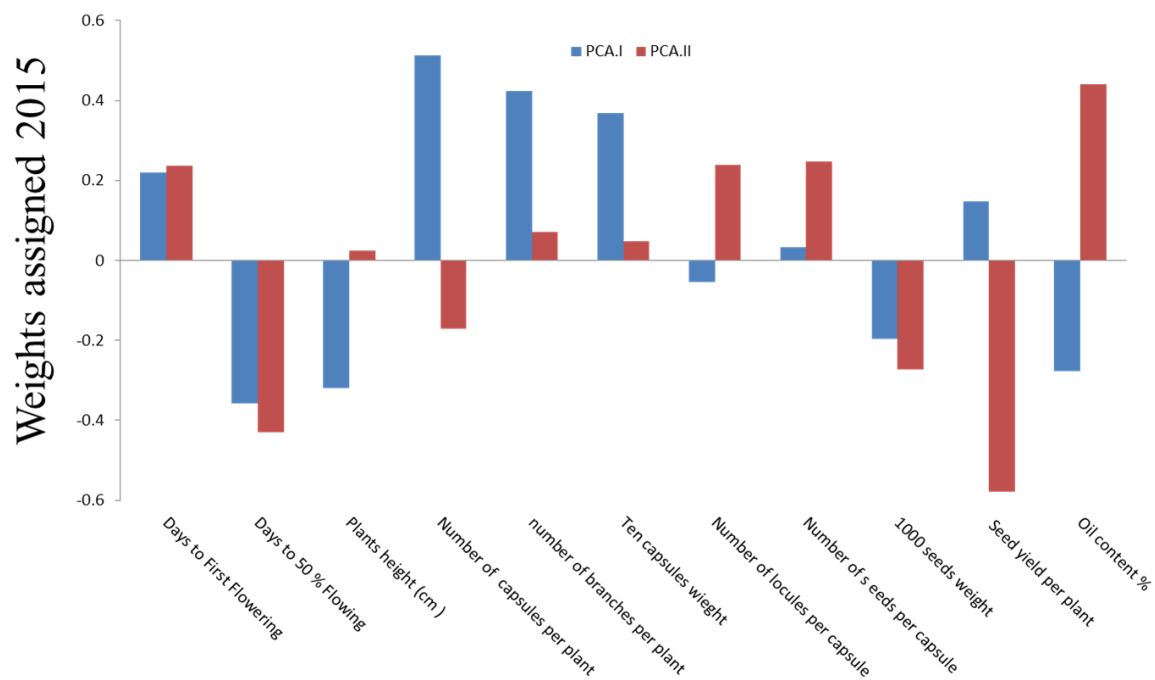


Figure 19: Graphical representation of PCA weightages assigned to variables early *Rabi* 2015.

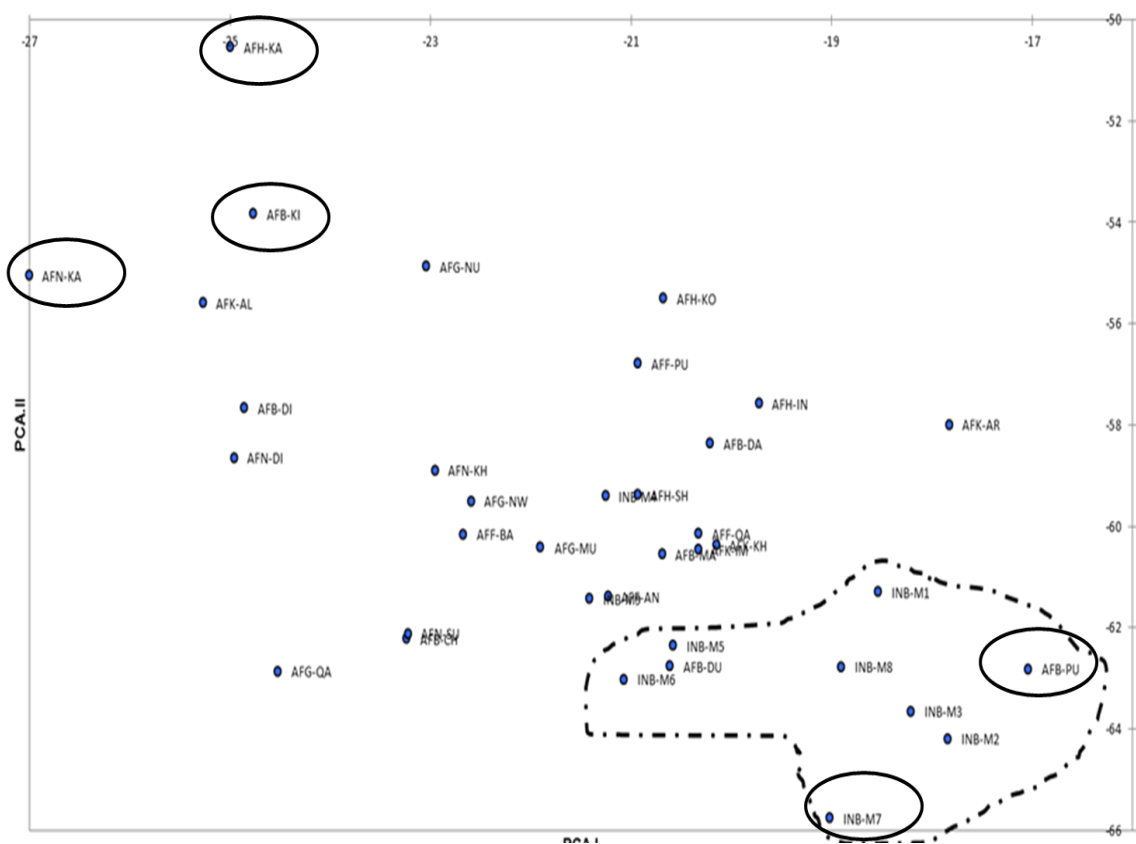


Figure 21: Distribution of accessions between the first two PC Axes (early *Rabi* 2015). The circled accessions are distinct types. The dotted shape encloses accessions mostly from India.

1. The 37 accessions separated widely during both the years suggesting that the collections from India and Afghanistan are clearly distinct accessions with less genetic overlap among them.
2. The Indian and Afghanistan accessions showed a clear separation on the PCA space. All the Indian accession can be seen to be congregated in the space defined by the mid values of PCA- I and high values of PCA II, as shown enclosed in the marked shape in Figure 20. Similarly, the 2015 analysis also showed a clear separation in to the space defined by the higher values of PCA- I and low values of PCA-II. Rarely did the Afghan collections were mixed with the Indian accessions except for INB-M4. In fact INB-M4 that grouped with Afghan accessions did so during both the years (Figure 21).
3. The pattern of separation of accessions was consistent in both the years: For example Accessions AFN-KA and AFB-PU were the most divergent accessions in the PCA analysis of both the seasons. The two occupied distinctly opposite spectra of the space. Further some of the accessions that grouped together during the 2014 such as AFK-IM and AFF-QA occupied the neighborhood during both the seasons.

4.1.7 Mahalanobis's D^2 Analysis

Dendrograms constructed using Mahalanobis's D^2 values resulted in the 37 accessions grouped in to four major clusters during 2014 and five major clusters during 2015 (Figure 22 and figure 23).

The major cluster formed during 2014 had 19 accessions in cluster-1, with 12 and 7 distributed in to its sub-clusters (1a and 1b). Cluster -2 had 10 accession with sub-cluster 2a having 6 accessions all from India, and 2b having four with one from India. Clusters- 3 and 4 had four accessions each.

The major cluster formed during 2015 had 23 accessions with 14 in one sub-cluster (1a) and 9 accessions all from India in another sub-cluster (1b). Cluster-3 had seven accessions, four in cluster 2 and, two and one in clusters 4 and 5 respectively.

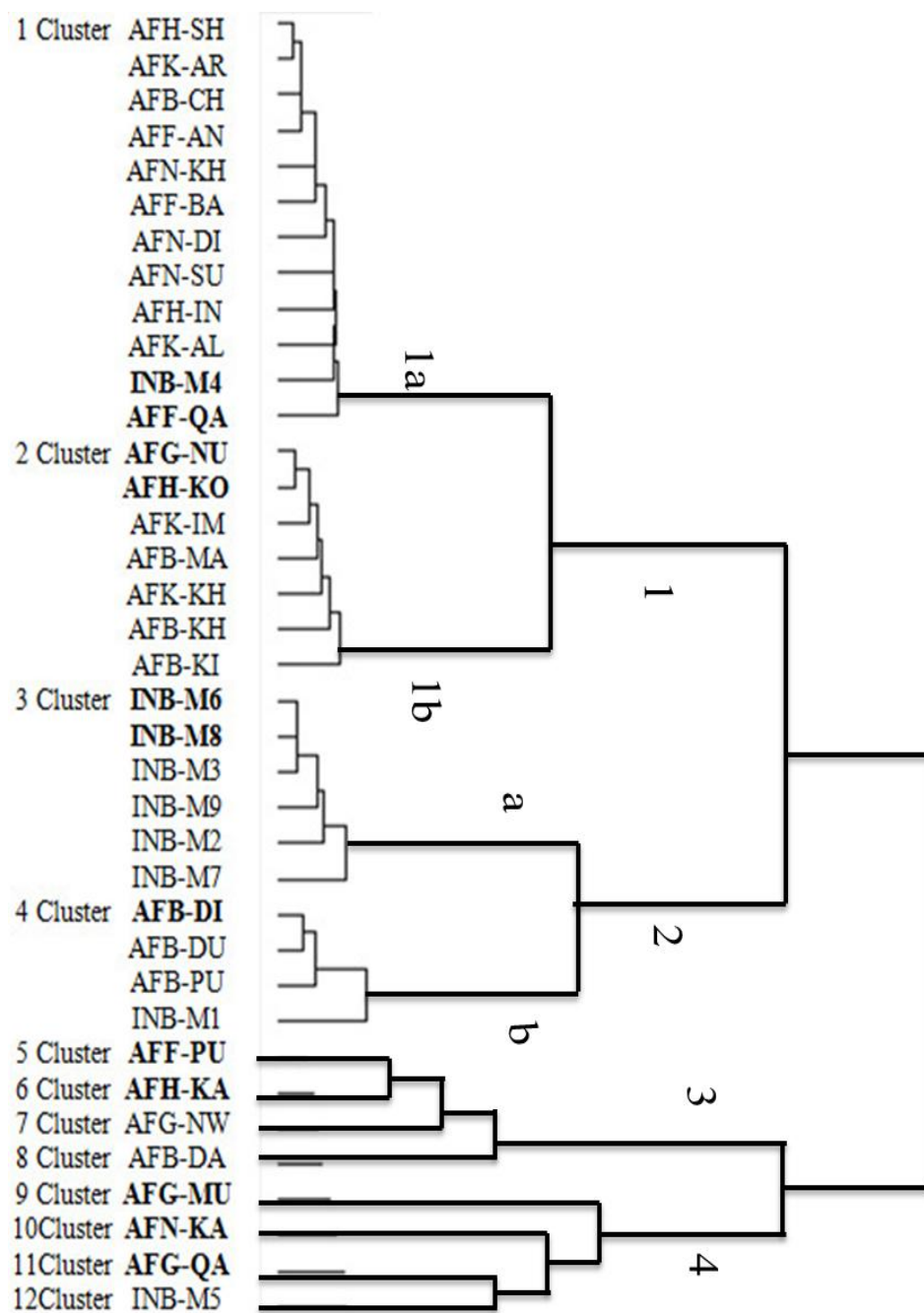


Figure 22: Dendrogram of 37 accessions (Late *Rabi* 2104), grouping in to different clusters.

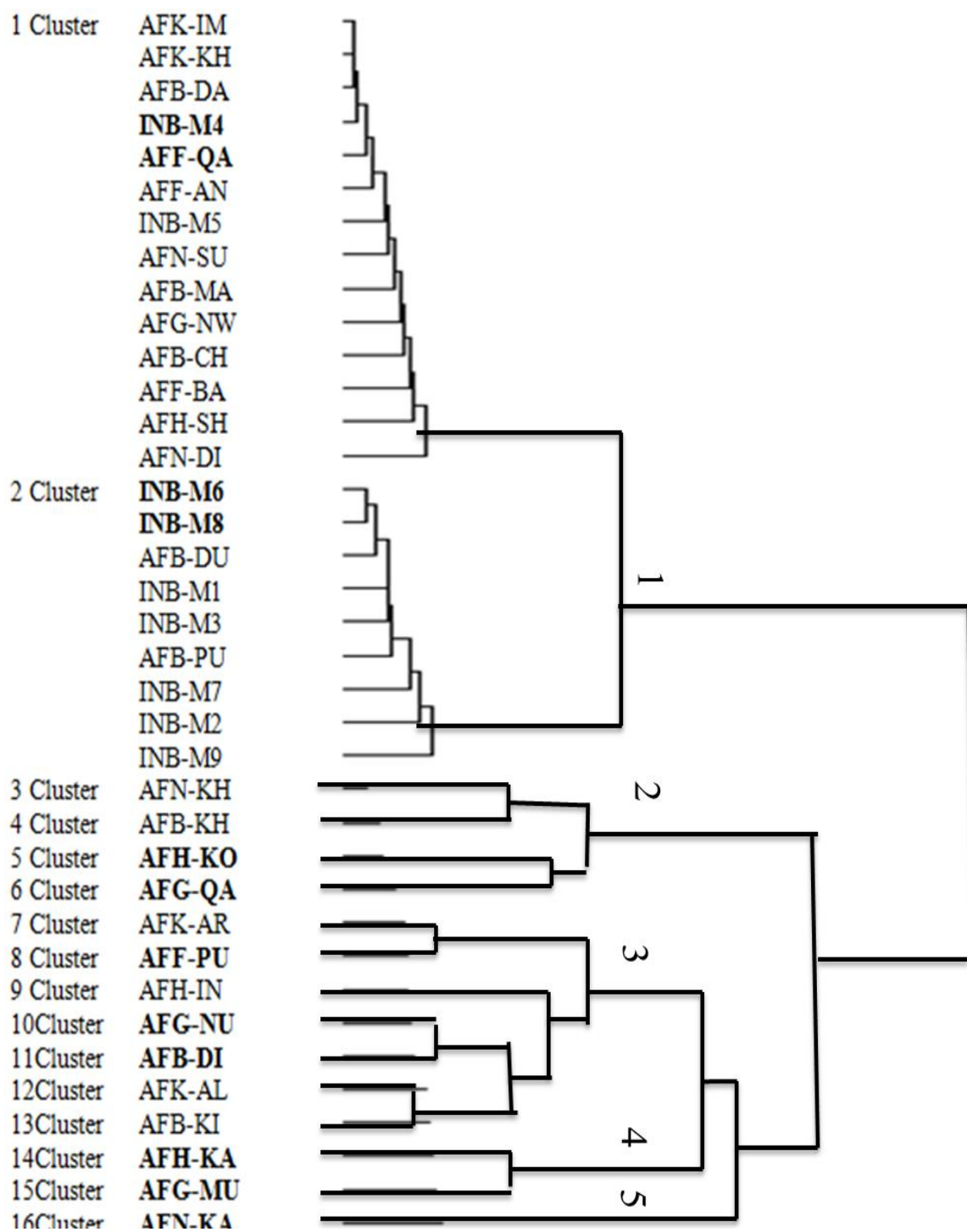


Figure 23: Dendrogram of 37 accessions (Early *Rabi* 2105), grouping in to different clusters.

There was a general agreement between the two seasons in the pattern of clustering of accessions, though not exactly similar. For example, all Indian accessions, except INB M4 were found grouped in cluster- 2 during 2014 while they are grouped in Cluster-1 during 2015. Similarly, Afghan accessions AFN-KA and AFG-MU appeared to be distant from all other afghan lines in both the years. Similarly Indian accession IND-M4 always grouped with Afghan lines in both the years.

Using Mahalanobis D^2 values the 37 accessions were grouped in to seven clusters using Tocher's method during both the years. The accessions included in each of these clusters are listed in the table 6 and table 7. Again as in dendrogram analysis the Indian accessions were grouped in to distinct clusters during both the years: clusters-6 and 7 had only Indian accessions during 2014 ; so also during 2015 except that AFB-PU was also included in cluster 6. AFN-KA and AFN-MU always behaved differently during both the years.

- i) The Indian and Afghan accessions clustered separately to a great extent. The Afghan accessions grouped in to several clusters indicating greater diversity among them.
- ii) The hierarchical clustering of the accessions as seen from the dendrograms also showed that across the clusters also there was an agreement between the two years.

4.2 Molecular diversity analyses

In the present study RAPD primers were used to study diverse accessions, the RAPD primers produced totally bands. (Figure 25) genetic similarity measured through analysis of bands among 12 accessions. The similarity indices and consensus dendrogram were developed on the basis of scoreable banding pattern of twelve genotypes (0 for absence and 1 for presence). The similarity coefficients ranged from 0.43 to 0.75 (Table 5). The genotypes AFB-DI and INB-M8 showed the lowest similarity index of 0.43, whereas AFG-QA and AFH-KO showed highest genetic similarity of 0.57. Most of the similarity values were between 0.51 and 0.70 (78.78 %; Figure 24). Indian lines showed higher similarity among themselves (0.50 to 0.73) while the Afghan lines showed a greater range of similarity values (0.43 to 0.75; Table 5).

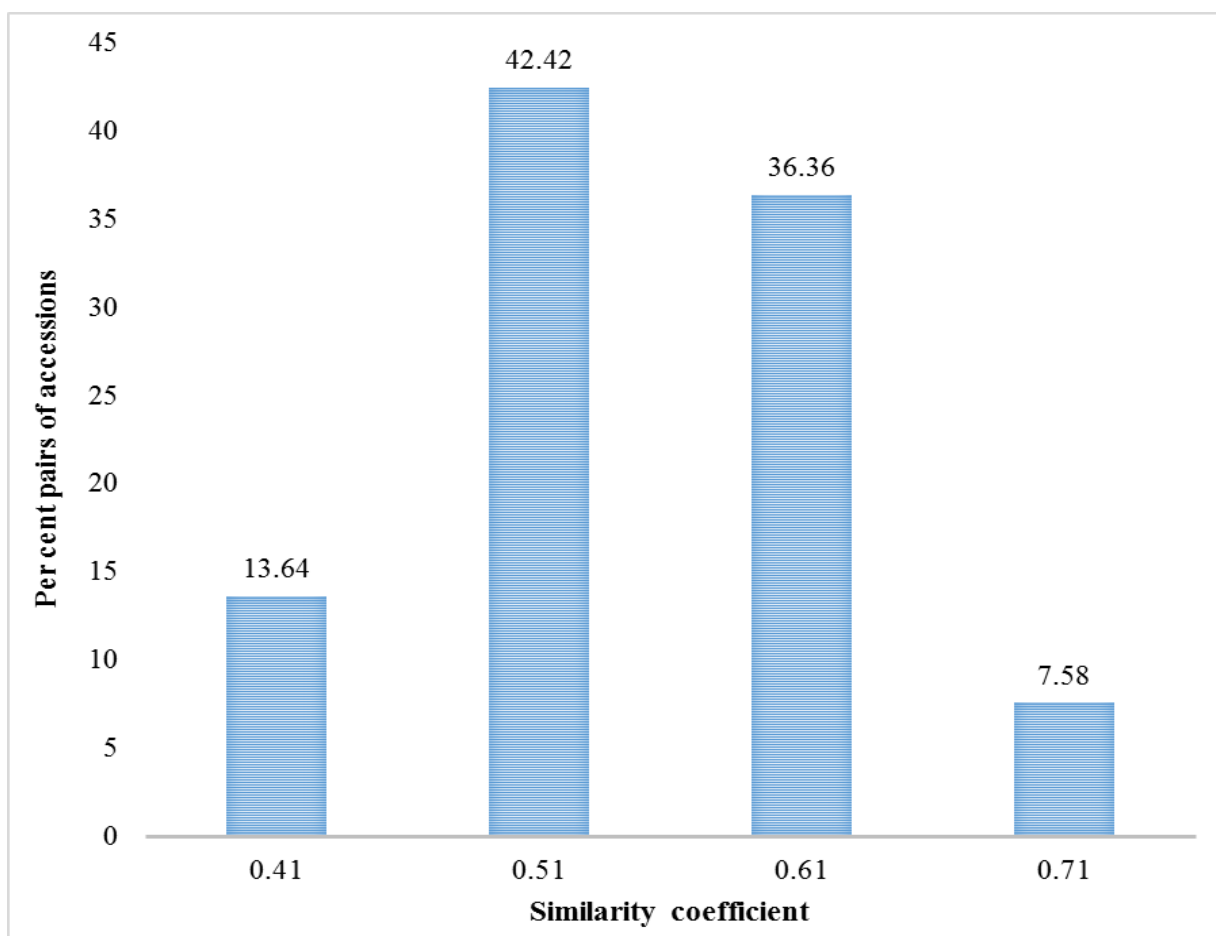


Figure 24: Per cent pairs of accessions with varying similarity coefficient values based on DNA analyses.

Table 5: Similarly coefficient matrix based on DNA analysis among the 12 selected *Nigella sativa* L., accessions

	AFB-DI	AFF-PU	AFF-QA	AFG-MU	AFG-NU	AFG-QA	AFH-KO	AFH-KA	AFN-KA	INB-M4	INB-M6	INB-M8
AFB-DI	1.00											
AFF-PU	0.60	1.00										
AFF-QA	0.66	0.71	1.00									
AFG-MU	0.56	0.56	0.59	1.00								
AFG-NU	0.50	0.44	0.53	0.58	1.00							
AFG-QA	0.47	0.63	0.64	0.60	0.55	1.00						
AFH-KO	0.44	0.65	0.59	0.56	0.52	0.75	1.00					
AFH-KA	0.46	0.67	0.63	0.58	0.50	0.64	0.71	1.00				
AFN-KA	0.58	0.69	0.65	0.60	0.56	0.61	0.60	0.67	1.00			
INB-M4	0.50	0.63	0.62	0.51	0.60	0.70	0.70	0.69	0.71	1.00		
INB-M6	0.52	0.54	0.55	0.52	0.52	0.64	0.61	0.66	0.70	0.73	1.00	
INB-M8	0.43	0.60	0.55	0.54	0.54	0.69	0.58	0.63	0.62	0.69	0.63	1.00

The Polymorphic information content (PIC) values which denote allelic diversity and frequency among the genotypes had an average of 0.22 /marker and ranged from 0.0 to 0.35 with the highest PIC obtained by I.1 primer. The gene diversity was also calculated and ranged from 0.0 to 0.42 for the same primers used in the study and the highest gene diversity was with I.1 primer with an average of 0.27 per marker (Table 6). The percentage polymorphism ranged from 0 to 100 with polymorphic bands ranging from 3 to 10, Zero polymorphism was found with I.14 primer and 100 percent polymorphism was found with AB.10 and AB.13 primers (Table 7).

The dendrogram constructed using SHAN clustering separated the twelve varieties of *Nigella sativa* L. into two major clusters: the first major cluster (cluster-1) contained ten accessions (Viz., AFB-DI, AFF-PU, AFF-QA, AFG-QA, AFH-KO, AFH-KA, AFN-KA, INB-M4, INB-M6 and INB-M8) and other cluster-2 contained two accessions (Viz., AFG-MU and AFG-NU) sharing a common node at a similarity coefficient of 0.55 (Figure 25). The first major cluster was again divided into two sub clusters viz., 1a and 1b at a similarity of 0.578; cluster 1a had seven while 1b had three accessions. Cluster 1a with seven clusters was further subdivided into two clusters, one with a solitary accession and other with the remaining six accessions.

Table 6: Polymorphic information content (PIC) among the 12 selected *Nigella sativa* L., accessions

Sl. No	Primer Name	Gene diversity	PIC
1	AB.4	0.12	0.10
2	AB.10	0.33	0.27
3	AB.13	0.33	0.27
4	I.14	0.00	0.00
5	AK.7	0.28	0.23
6	BH.2	0.22	0.19
7	BH.14	0.32	0.25
8	I.1	0.42	0.35
9	I.7	0.33	0.27
10	I.15	0.29	0.24
11	I.18	0.32	0.26
Mean		0.27	0.22

Table 7: Total bands, polymorphism and polymorphism percentage among the 12 selected *Nigella sativa* accessions

S/N	Primer name	Total bands	Polymorphism	(%) polymorphism
1	AB.4	7	3	42.8
2	AB.10	10	10	100
3	AB.13	9	9	100
4	I.14	7	0	0
5	AK.7	9	8	88.8
6	BH.2	11	9	81.8
7	BH.14	10	9	90
8	I.1	8	7	87.5
9	I.7	8	6	75
10	I.15	11	10	90.9
11	I.18	6	5	83.3

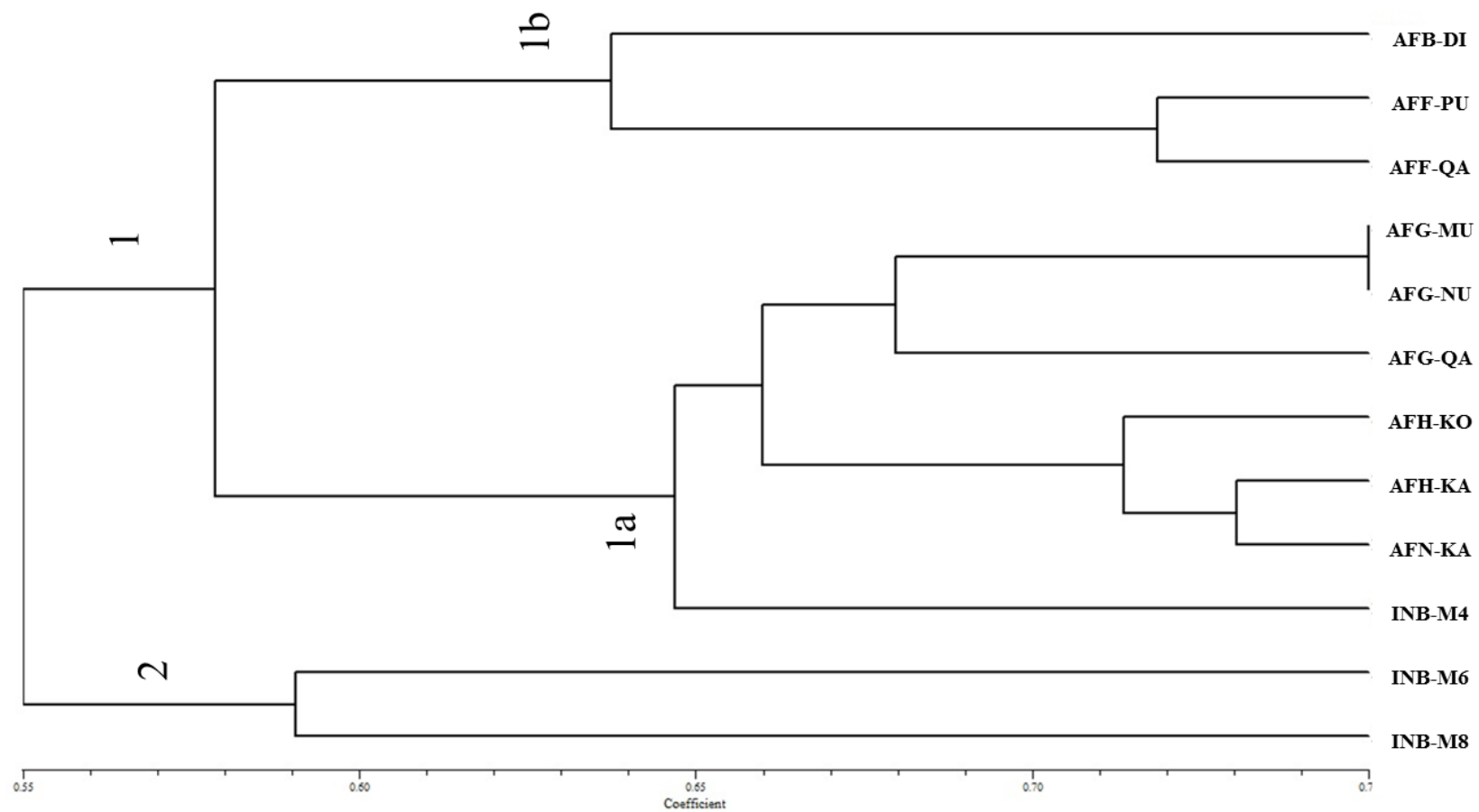


Figure 25: UPGMA Dendrogram representing relationship among 12 selected accessions of *Nigella sativa* L.

V. DISCUSSION

Black cumin (*Nigella sativa* L.) is an important medicinal plant with high commercial value in Afghanistan and parts of Asia (Chakraborty and Chakraborty 1964; <http://www.tlo-afghanistan.org>). The raw material and processed products such as oil, seed and seeds' residue for poultry uses are exported from Asian countries to several European and western countries (Gilani *et al.*, 2001). However the crop has not been subjected to systematic genetic improvement program for the following three reasons. Firstly, this crop has still not been brought under systematic and intensive cultivation in parts of Asia such as Afghanistan. In fact it is still being harvested from the wild and marketed in Afghanistan, Pakistan etc., (Ahmad *et al.*, 2004). Secondly, the complete genetic diversity available among the global population, of *Nigella Sativa* is yet to be studied; this is because the accessions are not yet collected from areas of its occurrence in Afghanistan, Pakistan etc., owing to poor information and other political problems. Consequently, the full genetic potential of the crop is not yet understood. Thirdly, owing to lack of attention by the breeders and other concerned scientists in the areas of its abundant occurrence, especially in North West part of Asia, no systematic genetic improvement has been attempted in this crop. Thus as in several under exploited medicinal plants, *Nigella sativa* remains unexplored and poorly understood making it a good case for initiating preliminary studies on the performance of accessions, the genetic potentiality and genetic diversity. Therefore 37 accessions of *Nigella sativa* from Afghanistan (28) and India (9) were collected and evaluated for their performance, seasonal stability and genetic diversity using morphological traits and DNA markers. The results are discussed here under:

5.1 Performance of accessions

The 37 accessions showed significant differences for all the characters including seed yield per plant and oil content during late *Rabi* 2014. During early *Rabi* 2015 the accessions did differ significantly for most characters except plant height, number of locules per capsule and seeds per capsule. Such difference in the performance of crops in different seasons has been reported in other medicinal plants such as *Adhatoda vasica* (Pandita *et al.*, 1983), *Mentha pulegium* (Stengele *et al.*, 1993), *Sargassum wightii* (Reeta, 1993), *Mentha spicata* (Kofidis *et al.*, 2004), *Toona sinensis* (Wang *et al.*, 2007), *Adiantum capillus-veneris* (Ahmad *et al.*, 2008), *Ocimum basilicum* (Hussain *et al.*, 2008) and *Ulva reticulata* (Shannrugam & Palpandi, 2008).

In general accessions performed better during late *Rabi* 2014 than during early *Rabi* 2015. All the accessions gave higher seed yield per plant (2.31 g) and higher oil content (23.25%) during late *Rabi* 2014 compared to early *Rabi* 2015 (1.93 g of yield per plant and 21.33% oil). The highest yielding accession (INB-M7; 3.35 g) was 2.33 times more than least yielding accession (AFH-KA; 1.44 g) during *Rabi* 2014. However this range was only 1.74 times the least yielding accession during early *Rabi* 2015. Similarly the range of oil percentage was also higher (2.18 times than lowest oil percentage) during late *Rabi* 2014 compared to early *Rabi* 2015 (1.95 times). Thus the full genetic diversity of this crop seems possible to be expressed during late *Rabi* season than in early *Rabi* season. In fact the crop is generally harvested in Afghanistan after the end of *Rabi* season and thus it is likely the crop is well adapted for *Rabi* or late *Rabi*. Such differences in the expression of the genetic potential of germplasm have been reported in other crops as well (Geetha and Prabhakaran 1987, Lal *et al.*, 1986 and Singh *et al.*, 2008).

5.2 Genetic potential for improvement:

The highest genetic advance expressed as percent over mean was found for seed yield per plant (36.74 during late *Rabi* 2014 and 32.29 during early *Rabi* 2015) and for oil content (37.75% during late *Rabi* 2014 and 30.88% during early *Rabi* 2015) compared to any other character (Table 2). Thus immediate improvement in yield can be attained by selecting for yield per plant and oil percentage than focussing on other characters. In fact the broad sense heritability (H^2) was also the highest for these two characters: for example while oil content had broad sense heritability (H^2) of 0.96 during both years, seed yield per plant had broad sense heritability (H^2) of 0.7 and 0.91 during late *Rabi* 2014 and early *Rabi* 2015.

respectively. In other words, purely by focussing on yield and oil percentage it would be possible to attain substantial improvement in yield of the crop. Accordingly it appears profitable to suggest the cultivation of the lines INB-M7 and AFF-PU that have yielded highest seed per plant and oil percentage.

5.2.1 Seasonal stability

The collected accessions showed stable performance for almost all traits. There were three kinds of responses to the season.

- A. Linear response: For traits such as oil content, capsule weight, number of branches per plant and plant height, accessions showed linear response between the two seasons, i.e., the performance of the accessions during early *Rabi* 2015 was merely a linear function of their performance during late *Rabi* 2014. In other words, for these traits there was no differential seasonal effect.
- B. Nonlinear response: For traits such as seed yield per plant and number of seed per capsule, a set of accessions showed a linear response and the rest did not respond at all during early *Rabi* 2015. For example, five of the Indian lines and a few Afghan lines though showed wide variation in seed yield per plant during late *Rabi* 2014 (2.5 g to 3.5 g, Figure 15; INB-M1, INB-M2, INB-M3, INB-M5, INB-M7, AFG-NW, AFH-IN, AFF-AN, AFK-IM and AFK-A) they showed very little variation during early *Rabi* 2015 (between 2.0 g and 2.3 g per plant); AFK-IM, AFB-DA and AFH-IN did not show much variation during late *Rabi* 2014 but varied considerably during early *Rabi* 2015 (Figure 13).
- C. Absolute seasonal effect: Days to first flowering and number of locules per capsule showed no relation (slope = 0) between the two seasons suggesting that there is a clear effect of season on the performance of accessions. For example, the lines that flowered early during late *Rabi* 2014 were not essentially early to flower during early *Rabi* 2015 and vice versa. This differential response was not consistent for all lines suggesting that there is an G X E interaction for these traits. It is likely that the moisture level and photoperiodism may play a significant role in shaping these traits during the two seasons. Differential response of the accession to the photoperiod and to the moisture is well known in crop plants (Jordan *et al*, 2003; Jaleel *et al*, 2008; Yu and Yao 1962; Vergara and Lilis 1967; Wada and Nojima 1954; Pollet *et al*, 2009; Jeong *et al*, 1996; Mortensen and Moe, 1992; Carvalho *et al.*, 2002). The consequence of such differential response on the yield and oil percentage in 37 lines is depicted in as the ratio of their performance during late *Rabi* 2014 to early *Rabi* 2015 (Figure 26 and figure 27). Accordingly accessions AFG-NW and INB-M7 have given relatively higher yield during late *Rabi* 2014 compared to early *Rabi* 2015 whereas accessions AFH-KO and AFK-AL have given the lowest yield. Accession AFG-NW has also given high oil percentage during late *Rabi* 2014 along with AFK-KH and AFN-SU whereas the line INB-M9 has given relatively low oil percentage during late *Rabi* 2014.

5.3 Correlation studies

Correlation studies indicated three distinct patterns:

First, there was no similarity between the two seasons in the strength of association among traits (Table 3). Most of the associations were high during late *Rabi* 2014 (below the diagonal; table 3) compared to early *Rabi* 2015 (above the diagonal).

Second, there were also differences in the patterns of associations among the traits. For example, the association between number of branches per plant and number capsules was high during both early *Rabi* 2015 and late *Rabi* 2014, (0.96 and 0.94), while that between number of locules per capsule and number of capsules was the highest during early *Rabi* 2015 (0.96) and only modest during late *Rabi* 2014 (0.27). In fact the association between days to 5% flower was highly significant during late

Rabi 2014 (0.39) while during early *Rabi* 2015 it was negative but not significant (-0.16). There were several such differences between the two seasons.

Third, some of the most predictable correlations were not showing up in this study. For example, plant height and number of branches are generally correlated in crop plants (Diepenbrock, 2000). But in this study, during early *Rabi* 2015 it was not significant (but negative); similarly traits that are expected to be directly contributing to yield such as number of capsules and seeds per capsule did not show significant relation with yield during early *Rabi* 2015 (-0.10) and late *Rabi* 2014 (-0.18).

Thus *Nigella sativa* plants seem to have very distinct ideotypic relations and needs to be carefully understood. Such unusual relations are occasionally reported in other crops as well (Balko and Seddig 1994).

Perhaps for these same reasons, that the ideotypic relations among traits are different in *Nigella sativa*, and that they respond variedly in different seasons, the direct contributions of the traits to yield were also different between the two seasons. Number of branches per plant, plant height and number capsules emerged as the most important determinants of yield during late *Rabi* 2014 while along with number of branches per plant, 1000 seed weight and ten capsule weight contributed to yield during early *Rabi* 2015. However it may be important to take note of the fact that the residual component of the path effects was very high during both seasons (0.63 during late *Rabi* 2014 and 0.72 during early *Rabi* 2015; Figure 28 and Figure 29). In summary, it appears that the results of the association and path studies are to be carefully examined before they can be directly imported to be applied in the crop improvement and selection programmes. There are a number of studies that indicate similar impact of season on the inter-trait associations (Dayal *et al.*, 1983, Ram *et al.*, 2005, Panesar and Jadeja 2008; Jotshi *et al.*, 2008, Chitra *et al.*, 2010 and Shanmugasundaram 1998).

5.4 Diversity analyses

Genetic diversity among the accessions is an important determinant of the extent of direct selection possible, and the possibility of hybridization and crossing in genetic improvement programmes. Higher the genetic diversity greater the opportunity for selection (Bessey 1906 and Harlan 1972). Similarly while choosing the parent lines for the crossing program or for hybridization, it is generally proven that genetically distant lines result in widely segregating population and also in high hybrid vigour (Burbank 1914). Therefore genetic improvement program of any crop requires assessment of genetic diversity among the accessions. There are several techniques of assessing genetic diversity and they are borrowed from other fields. For example Mahalanobi's D^2 analysis that was first used for assessing the morphological diversity among Asian races (Mahalanobis 1936) has been used to assess the same among germplasm lines in crop plants (Murthy and Arunachalam 1960). Principle Component Analysis (PCA) is equally useful tool to identify the relative distances among lines on a two or three dimensional space. While D^2 analysis provides a summed distance between accessions based on the differences in all the characters, PCA attaches weightages to the variables (characters) based on their covariance or correlations and from those weights, it is possible to display the accessions in a space defined by the weighted PC axes. Such relation between accessions can be obtained, in D^2 analysis by clustering them based on the inter accession D^2 distances. The final result can be seen as either clusters, with inter cluster distances or as a dendrogram. The present study utilized both the tools separately for the data collected during the two years.

- a. PCA: there were differences in the weightages assigned for different characters during two seasons. This may be because of the differences in association between the traits during the two years (Figure 18 and Figure 19). Nevertheless, plant height, locules per capsule, 1000 seed weight, seed yield per plant, oil content and number of capsules per plant were found to be most important in discriminating the accession.

During both the years the first two axes explained little above of 50% of the total variability among the lines; even with this, there was clear separation of the Indian lines from the Afghan lines during both seasons (marked as dotted area in Figure 20 and Figure 21). Thus clearly except for one or two Afghan lines that mixed with Indian lines, collections from India isolated distinctly. Such geographical based genetic diversity has been reported in several studies (Ganeshaiah *et al.*, 2003; Cote 2003; Wang *et al.*, 2010). PCA analysis also identified AFN-KA line from Nangarhar province of Afghanistan, AFB-KI and AFBI-BU from Baghlan, AFH-KA from Herat and INB-M7 from India as the most distinct types among the 37 accessions. Within Afghanistan there was no geographical distance based separation among the accessions.

- b. D^2 analysis: The patterns observed in PC analysis were substantiated by the D^2 analysis also. For example during early *Rabi* 2015 Indian lines grouped together in to sub cluster 1b and similarly during late *Rabi* 2014 seven of them grouped in cluster 2. During both seasons AFN-KA, AFH-KA, AFG-MU and AFB-KI clustered separately just as in PCA. Among those AFN-KA appeared to be the most distinct type. The distinctness of these types did not appear to be reflected in the yield and oil percentage (Appendix 2). All these showed varied levels, of yield and oil percentage suggesting that their distinctness is a result of differences among all the traits.

The study clearly brought out the wide genetic diversity existing among the lines of black cumin and that this diversity could be explained by the geographic isolation. Several studies have shown such relation between geographical distance and genetic diversity in several crops (Singh, and Chaudhary 1985; More and Seshadri, 2002; Choudhary and Ram, 2003; Krishnaprasad *et al.*, 2004; Musmade *et al.*, 2008; Rad *et al.*, 2010) and this relation can be attributed to genetic adaptation of the lines to specific local conditions.

5.5 Molecular diversity analyses

The development in molecular biology prompted genetic divergence to be evaluated by means of molecular markers. The advantage of this procedure is that a large number of polymorphic markers leading to a number of allelic variants can be obtained, which allows genetic variability to be sampled in a more efficient way and consequently to obtain a more precise estimate of genetic divergence (Bruel *et al.*, 2007). Studies of molecular diversity across individuals have been stimulated by the amplification of DNA sequences using polymerase chain reaction (PCR; Saiki *et al.*, 1988). Several methods have been applied in this regard. RAPD is widely used in many plant species owing to its low cost, multi loci analysis and rapid detection of polymorphisms (Carvalho *et al.*, 2004; Bruel *et al.*, 2007). It has been suggested that the use of the RAPD seem more appropriate when the objective was to cluster genotypes in many other plant species; it showed similarities between the individuals (Ristic *et al.*, 2013). RAPD marker have been extensively used for DNA fingerprinting (Moreno *et al.*, 1998 and Gilbert and Feigon, 1999), genetic diversity studies (Hoz *et al.*, 1996). Population genetic studies (Wolfe *et al.*, 1998 and Wu, 2005). The molecular characterization of the genetic diversity in germplasm can provide breeders with useful information for the identification and selection of parents. These markers are important tools since they assess different DNA sequences in the genome of organisms and can complement the results during the selection process in the breeding programs. Further, the molecular diversity analysis is more relevant in this study as the genetic source and geographic origin of the genotypes used in the study are clearly distinct but the extent of diversity are not known as they were selected from some open pollinated populations.

In the present study, RAPD primers were used for molecular diversity analysis of 12 accessions representing diverse morphological clusters. The number of bands produced by each primer ranged from 1 to 11. The highest number of loci was amplified in I.15 and BH.2 primers and the lowest was in I.18 (Table 6). Such a variation in the number of fragments produced by these arbitrary primers may be attributed to the differences in the binding sites throughout genome of the genotypes included.

Different primers showed the variation in their ability to detect polymorphism. The gene diversity observed ranged from 0.00 in primer I.14 to 0.42 in primer I.1 with a mean gene diversity of 0.27. The mean gene diversity was moderate with a limited number of primers used in this study suggesting that there is a lot of variation in the genotypes studied. It is expected as the genotypes were collected from geographically different locations of India and Afghanistan

The Polymorphic information contents (PIC) of the 11 RAPD primers that were used varied between 0.00 and 0.35. Average PIC was 0.22. The selected primers displayed moderate frequencies of polymorphic bands. When an overall evaluation is made, it was observed that PIC values of the primers used in the study were quite low. Normally if the diverse genotypes are used the PIC values are expected to be more. The low PIC could be due to the ploidy level of the species. It has also been reported in other crops with higher ploidy levels that the SSR and cytochrome P450 gene based markers would be a better tool than RAPD markers for phylogenetic studies (Panwar *et al.*, 2010). The present study based on the RAPD technique varied from those obtained in previous studies on different plant species, like barley (77.06%), rice bean (70.30%), Vigna species (48%) and maize (76.14%) where the PIC values were more.

Clustering of genotypes tended to follow pedigree relatedness, geographical isolation, and origin of genotypes in many plant species. Ability of these markers to discriminate closely related lines is evident from the dendrogram. The similarity coefficient matrix based on DNA markers suggested that the genotype AF-GOA and AF-HKO are very similar with a similarity coefficient value of 0.75, while the genotype AF-BDI and IN-BM8 are highly diverse with a similarity value of 0.43. Thus the similarity values seem to represent the geographical origin of the genotypes. The genotypes from Afghanistan and India had similar similarity index values within each group (or country) than between them suggesting that short geographical isolation has low influence on the genetic diversity of the genotypes. However the Indian and Afghan lines tended to separate in the dendrogram suggesting that long geographic distances have influenced the genetic structure of black cumin. The tendency for genetic diversity to increase with geographic distance has been reported in many earlier studies in different crop plants (Inoue and Kawahara, 1990).

The UPGMA phenograms suggested that the genotypes from different geographical regions, especially from India and Afghanistan showed tendency to cluster separately suggesting that there is restricted movement of genes across countries (between India and Afghanistan). However the dendrograms showed that samples collected from Afghanistan did not separate based on their origin within the country. This may be because we have sampled only one or two population(s) from each cluster and also we have used the population pools for marker analysis, we might have underestimated the genetic distance between two populations within the country. Further high intra population variability reduces the ability of RAPD markers to differentiate between populations. We recognize the limitations of RAPD, particularly in reproducibility, and that techniques such as RFLP, AFLP and next-generation sequencing are available for more accurate and precise population genetic studies. However, in this study we were able to clearly determine the genetic variability in our isolates with RAPD and arrive at our conclusion that there is certain recognizable pattern of isolation of lines from India and Afghanistan.

5.6 Dendrogram and subspecies Black Cumin

For long there is some confusion about the exact taxonomic name of black cumin (Anon., https://en.wikipedia.org/wiki/Bunium_bulbocastanum). While most people refer *Nigella sativa* as black cumin, a few also refer to the long seeded *Bunium bulbocastanum*, as black cumin. However our collections from the areas of cultivation of the material from the two countries where it was referred as 'black cumin' indicated that at least among these accessions there does not seem to be any confusion as all the seeds collected from 37 sources were small in size; none belong to the size of *Bunium bulbocastanum*. Further, the populations of *N sativa* from across the geographic distribution of the crop in Asia appear to be having only minor taxonomic distinctness, perhaps at sub species or ecotype level. In the morphology based analysis, there was a clear separation of collections from the two countries but

some accessions from both countries did mix with each other. DNA analysis of the twelve selected accessions showed that there were two distinct clusters formed at a higher level of genetic dissimilarity (or at lower levels of similarity). And this clustering was also associated with the broad geographic separation of Indian and Afghan accessions. However this distinctness did not appear to be sufficient enough to segregate the collections in to two separate species.

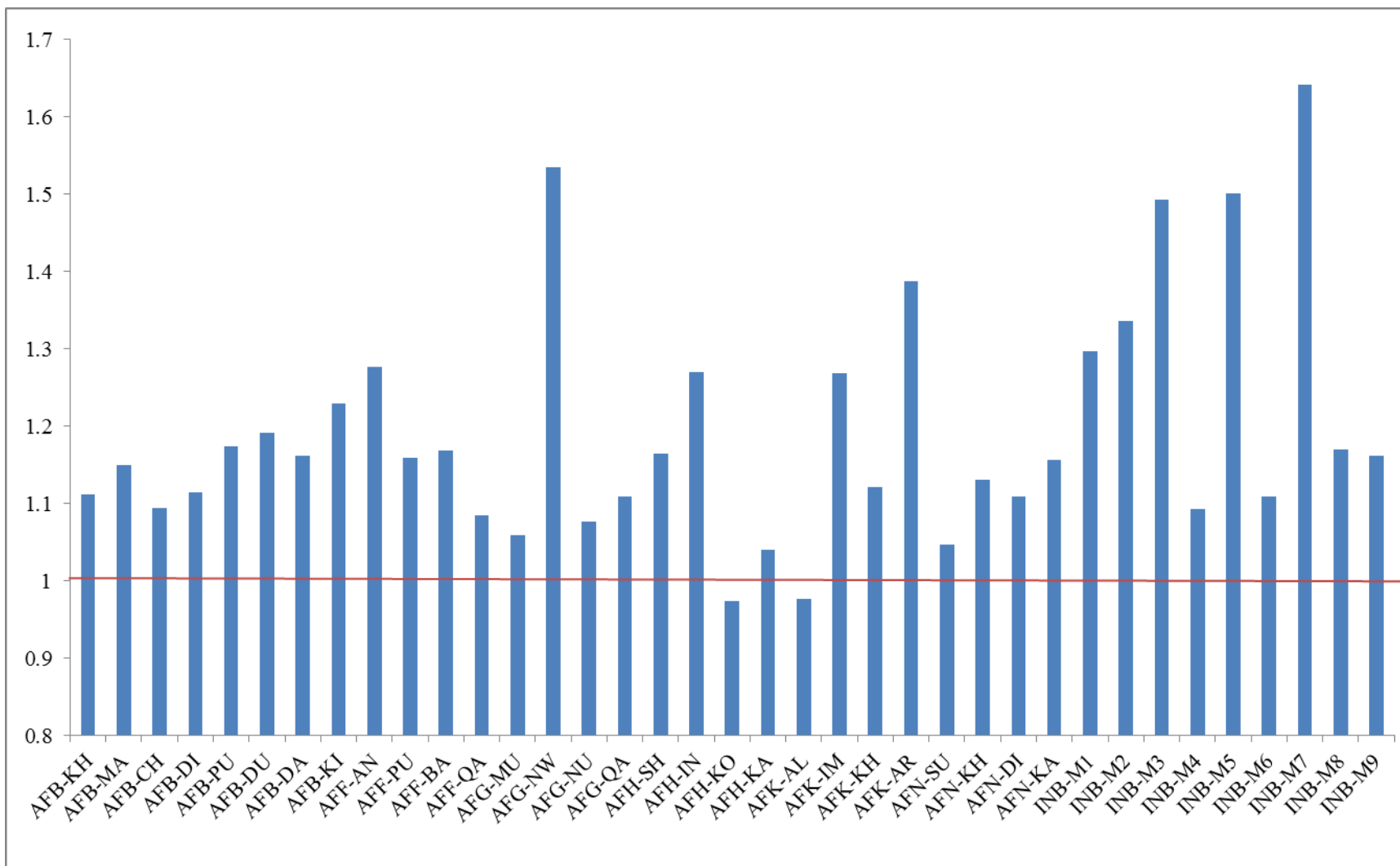


Figure 26: Ratio of seed yield between two seasons (late *Rabi* 2014: early *Rabi* 2015)

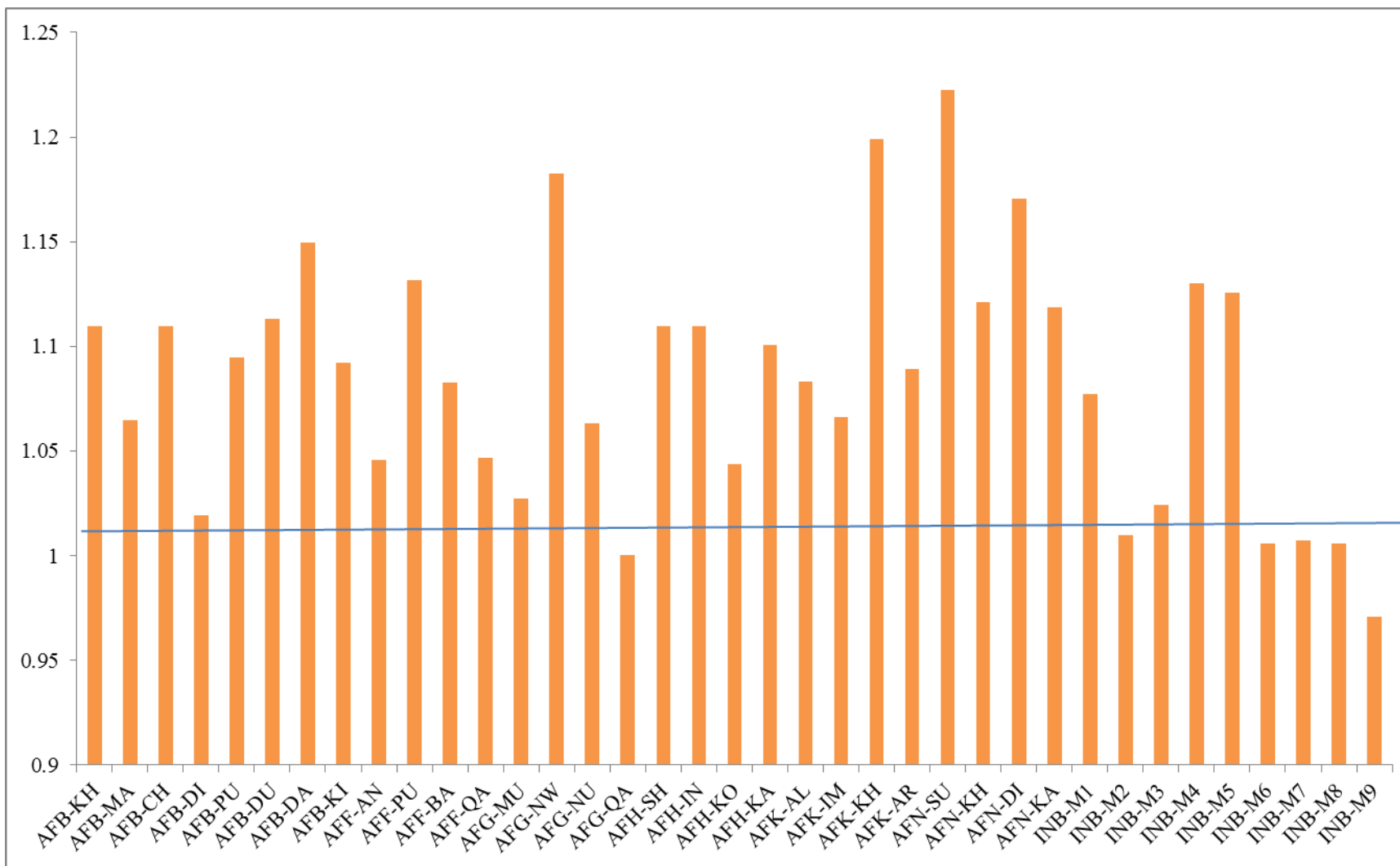


Figure 27: Ratio of oil content % between two seasons (late *Rabi* 2014: early *Rabi* 2015)

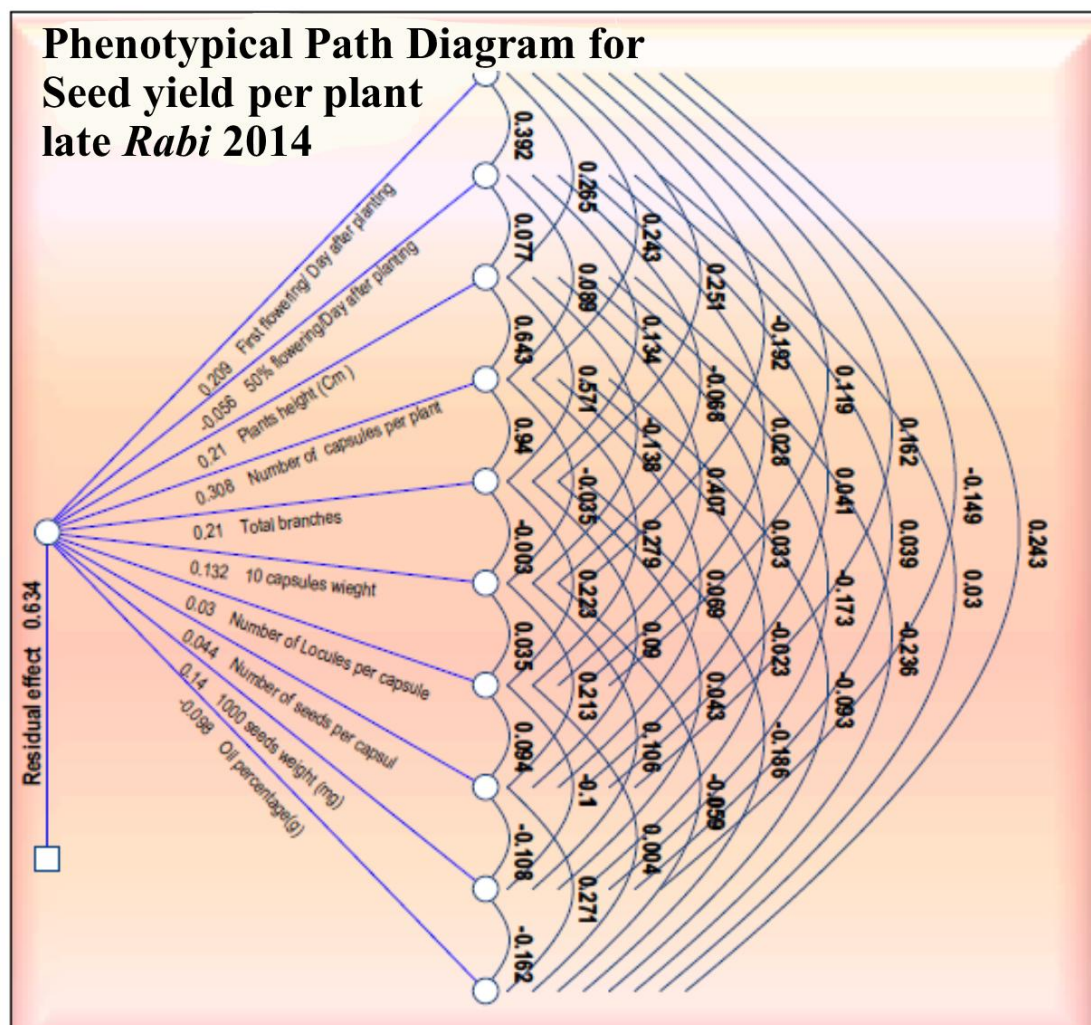


Figure 28: Phenotypical path diagram for seed yield per plant (late *Rabi* 2014).

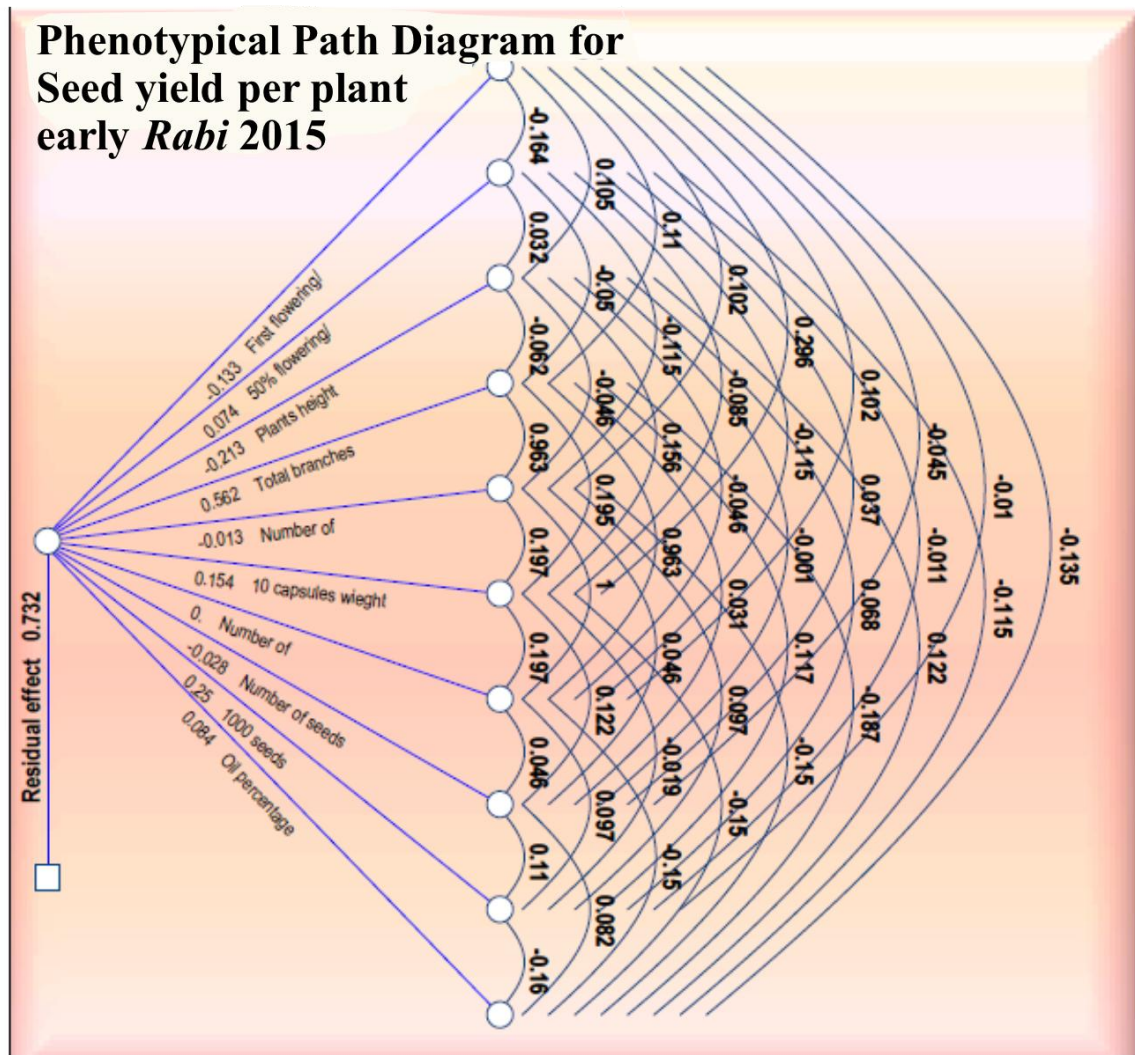


Figure 29: Phenotypical path diagram for seed yield per plant (early *Rabi* 2015).

VI. SUMMARY

For centuries medicinal plants are being used in different indigenous systems of health care system for curing range of diseases; they are also used in the preparation of herbal medicines as they are considered to be safe compared to modern allopathic medicines. Consequently, in the recent past, medicinal plants have come under special focus for a thorough investigation of their medicinal value, chemical composition, mechanism of action, and toxicity. Black cumin (*N. sativa*) is one such crop of great medicinal value, but less studied for the extent of variability in the available genetic resources and, for the influence of location and season on crop growth performance. The present study was undertaken to assess 37 accessions collected from Afghanistan and India for their performance, genetic diversity, potential for improvement and for understanding the traits contributing to yield.

The accessions were sown during late *Rabi* 2014 and early *Rabi* 2015 and evaluated for 11 traits including yield and seed oil content. The 37 accessions showed significant differences for all the characters including total yield and oil percentage during 2014. During 2015 the accessions did differ significantly for most characters except plant height, number of locules per capsule and seeds per capsule. In general accessions performed better during late *Rabi* 2014 than early *Rabi* 2015. INB-M7 yielded the highest seed weight during both the years while AFH-KA and AFN-KA yielded highest seed oil per cent during both the years. The response of the accessions to season was assessed by plotting the performance of one season over the other and found that there were three kinds of responses viz., (a) linear response, where the performance of any line was a mere linear function of its performance in the other season; (b) Absolute seasonal effect, where the accessions showed differential response (slope = 0) over their performance in another season; and (c) nonlinear response, where some lines showed linear response while others did not. Perhaps owing to this, the correlations between the two seasons did not correspond much; there was a clear season effect on the relationship of yield and other traits. This was also evident from the different levels of direct and indirect contributions of traits to yield in the path analysis.

The broad sense heritability (H^2) and per cent Genetic advance over mean were higher for yield and seed oil content during both the years suggesting that immediate improvement can be attained purely through phenotype selection in the crop.

Morphometric based PCA and Mahalanobis's D^2 analysis revealed three distinct patterns: (a) there was broad-scale geographic genetic differentiation of the populations of *N sativa*. The Afghan and Indian accessions clearly segregated in the clustering and dendrogram analysis. (b) Gene pools seem to be mixing well at a small geographic scale either because of human mediated commercial exploitation or because of natural outcrossing. The accessions from within Afghanistan, for example, were not showing any geographic based segregation. (c) Populations of *N sativa* from across the geographic distribution of the crop in Asia appear to be having taxonomic distinctness at sub species or ecotype level. Though in the morphology based analysis, some accessions from both countries did mix with those from others, DNA analysis of the twelve selected accessions showed that there were two distinct clusters formed at a higher level of genetic dissimilarity (or at lower levels of similarity), and this clustering was also associated with the broad geographic separation of Indian and Afghan accessions.

Different primers showed the variation in their ability to detect polymorphism. The gene diversity ranged from 0.00 in primer I.14 to 0.42 in primer I.1 with a mean gene diversity of 0.27. The similarity coefficient matrix based on DNA markers suggested that the genotype AF-GOA and AF-HKO are very similar while the genotype AF-BDI and IN-BM8 are highly diverse.

The study clearly brought out the wide genetic diversity existing among the lines of black cumin and that this diversity could be explained by the geographic isolation. Plant breeders can take a cue from the variability existing in the germplasm and effective breeding strategies can be developed to improve crop productivity. Accordingly, it appears profitable to suggest INB-M7 and AFF-PU accessions that yielded highest seed per plant and oil percentage for further cultivation.

VII. REFERENCES

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Appendix I: List of geographical locations of all the accessions collected from Afghanistan

Province	District name	Abbreviation
Balkh	Khulm	AFB-KH
	Marmul	AFB-MA
	Chahar Kint	AFB-CH
	Dihdadi	AFB-DI
Baghlan	Puli Khumri	AFB-PU
	Dushi	AFB-DU
	Dahana i Ghuri	AFB-DA
	Khinjan	AFB-KI
Farah	Anar Dara	AFF-AN
	Pusht Rod	AFF-PU
	Bakwa	AFF-BA
	Qala i Kah	AFF-QA
Ghazni	Muqur	AFG-MU
	Nawa	AFG-NW
	Nawur	AFG-NU
	Qarabagh	AFG-QA
Herat	Shindand	AFH-SH
	Injil	AFH-IN
	Kohsan	AFH-KO
	Karukh	AFH-KA
Kundoz	Ali Abad	AFK-AL
	Imam Sahib	AFK-IM
	Khan Abad	AFK-KH
	Archi	AFK-AR
Nangarhar	Surkh Rod	AFN-SU
	Khogyani	AFN-KH
	Dih Bala	AFN-DI
	Kama	AFN-KA
Abbreviation of Indian material that were procured from different sources at Bangalore city		
(INB-M1), (INB-M2), (INB-M3), (INB-M4), (INB-M5), (INB-M6), (INB-M7), (INB-M8) and (INB-M9).		

Appendix IIa. Mean of days to first flower, days to 50% flower, plant height (cm) and total branches

S/N	Varieties	Days to first flower		Days to 50% flower		Plant height (cm)		Total branches	
		2014	2015	2014	2015	2014	2015	2014	2015
1	AFB-KH	54.67	60.00	67.67	73.50	34.83	24.08	19.22	21.10
2	AFB-MA	54.13	60.85	73.67	72.50	35.53	26.13	21.00	24.35
3	AFB-CH	55.00	59.40	75.00	73.50	42.00	32.17	21.73	22.75
4	AFB-DI	53.87	61.25	75.00	75.50	38.36	28.29	18.67	19.85
5	AFB-PU	53.87	61.90	70.00	72.50	46.44	37.33	25.47	27.25
6	AFB-DU	53.93	59.45	75.00	74.00	41.61	31.42	24.43	24.60
7	AFB-DA	54.53	62.35	71.33	70.50	42.11	32.17	22.12	24.10
8	AFB-KI	53.20	61.45	66.67	70.00	31.50	21.08	16.86	20.15
9	AFF-AN	55.60	60.05	73.33	73.00	42.15	31.83	25.67	25.80
10	AFF-PU	56.07	62.00	73.67	71.00	40.62	31.05	25.37	26.85
11	AFF-BA	55.40	59.30	71.67	73.00	40.08	30.63	23.95	26.05
12	AFF-QA	54.53	61.50	73.00	72.50	38.25	28.38	22.53	23.50
13	AFG-MU	54.40	61.85	72.00	70.50	33.89	23.33	19.84	22.15
14	AFG-NW	55.73	61.10	75.00	73.00	40.69	31.33	28.27	24.80
15	AFG-NU	53.67	61.90	73.33	73.00	35.80	25.70	22.20	24.90
16	AFG-QA	55.40	61.00	77.33	74.00	38.29	27.60	20.87	22.00
17	AFH-SH	55.33	61.75	73.33	74.00	42.47	33.38	22.60	24.35
18	AFH-IN	55.67	60.45	73.00	73.50	37.00	26.83	22.73	23.65
19	AFH-KO	53.67	61.35	70.33	69.50	37.75	27.38	21.73	23.65
20	AFH-KA	53.87	62.38	70.00	70.50	34.94	24.75	18.53	20.60
21	AFK-AL	55.40	60.90	74.00	73.50	34.19	23.79	18.53	20.80
22	AFK-IM	53.47	60.95	72.33	72.50	38.03	28.38	25.27	25.75
23	AFK-KH	53.27	62.25	72.67	72.00	38.62	33.43	24.27	26.30
24	AFK-AR	55.00	61.15	71.33	70.50	44.72	33.83	26.80	28.55
25	AFN-SU	55.87	61.45	77.00	76.50	37.33	27.30	22.53	25.00
26	AFN-KH	55.20	61.30	75.00	74.50	41.40	26.60	23.33	25.90
27	AFN-DI	55.20	60.20	78.33	75.50	36.92	26.50	21.07	22.80
28	AFN-KA	54.00	62.20	73.67	74.00	28.82	1278.13	20.80	22.80
29	INB-M1	54.47	62.70	72.33	71.00	44.59	35.08	28.20	27.85
30	INB-M2	54.07	61.35	70.00	71.00	40.12	34.80	24.73	25.55
31	INB-M3	54.33	63.20	71.67	72.00	41.17	30.00	26.20	27.50
32	INB-M4	53.87	60.25	73.33	72.00	34.34	24.68	22.93	24.70
33	INB-M5	55.13	62.75	73.67	74.50	37.22	27.95	24.20	25.95
34	INB-M6	53.87	61.00	74.33	74.00	38.39	30.83	22.67	24.85
35	INB-M7	55.27	61.80	78.00	76.00	43.41	32.45	23.20	25.70
36	INB-M8	53.67	62.10	70.33	71.00	37.47	28.40	22.60	24.80
37	INB-M9	54.80	61.30	77.00	77.00	36.18	27.67	23.53	24.60

Accessions shown in bold were found to be distinct in diversity analysis

b. Mean of number of capsules per plant, ten capsules weight, number of locules per capsule and number of seed per capsule.

S/N	Varieties	Number of capsules per plant		10 capsules weight (g)		Number of Locules per capsule		Number of Seeds per capsule	
		2014	2015	2014	2015	2014	2015	2014	2015
1	AFB-KH	21.70	17.94	1.80	1.39	5.53	5.39	75.92	73.14
2	AFB-MA	22.97	22.10	2.16	2.05	5.43	4.83	86.38	63.89
3	AFB-CH	23.97	20.77	1.68	1.64	5.75	5.74	81.00	76.54
4	AFB-DI	20.77	17.85	1.73	1.60	6.01	6.09	74.53	71.54
5	AFB-PU	26.73	25.00	1.91	2.05	6.00	6.01	73.61	71.02
6	AFB-DU	25.47	22.40	1.77	1.81	5.89	5.74	77.40	71.93
7	AFB-DA	23.37	22.35	2.14	1.99	5.97	6.06	87.07	85.25
8	AFB-KI	18.77	17.96	1.76	1.70	5.54	5.29	74.71	73.00
9	AFF-AN	26.97	23.67	1.73	1.69	5.96	5.78	80.64	79.17
10	AFF-PU	26.83	25.20	1.87	1.77	5.87	5.75	77.60	75.40
11	AFF-BA	26.77	23.80	1.32	1.48	5.89	6.02	75.89	77.63
12	AFF-QA	25.87	21.55	1.87	1.99	5.95	5.99	74.25	72.42
13	AFG-MU	23.37	19.80	1.93	1.87	5.59	5.69	73.56	69.92
14	AFG-NW	29.97	21.56	1.65	1.74	6.16	5.74	78.99	77.96
15	AFG-NU	24.13	22.86	1.74	1.66	6.17	6.13	75.89	72.50
16	AFG-QA	23.17	19.15	1.70	1.74	6.08	5.96	82.00	76.83
17	AFH-SH	25.63	21.67	1.81	1.93	5.99	6.09	88.71	80.00
18	AFH-IN	25.70	21.90	2.20	2.24	6.21	6.20	90.30	85.42
19	AFH-KO	23.90	21.51	2.02	2.05	6.39	6.33	83.72	76.67
20	AFH-KA	22.43	17.45	1.92	1.79	5.88	6.11	85.78	78.50
21	AFK-AL	21.77	17.38	1.97	1.98	5.63	5.53	80.50	74.60
22	AFK-IM	27.30	23.60	2.06	1.99	6.24	5.68	89.67	82.17
23	AFK-KH	26.87	23.89	2.04	1.97	5.71	5.85	82.00	74.96
24	AFK-AR	29.07	26.55	1.89	1.98	6.07	5.74	86.69	78.50
25	AFN-SU	26.07	21.95	1.81	1.78	5.80	5.76	85.78	72.17
26	AFN-KH	26.27	22.55	1.69	1.58	6.02	5.88	84.44	80.00
27	AFN-DI	24.27	20.12	1.53	1.59	5.73	5.96	86.92	73.50
28	AFN-KA	23.93	20.25	1.97	2.00	5.75	5.66	78.42	72.00
29	INB-M1	31.93	25.42	1.75	1.77	5.89	5.90	77.01	76.01
30	INB-M2	28.23	23.16	1.83	1.72	5.53	5.86	89.36	81.46
31	INB-M3	28.97	24.38	1.77	1.76	6.11	5.72	74.31	71.67
32	INB-M4	26.07	22.00	2.07	1.98	5.45	5.74	82.08	75.67
33	INB-M5	28.30	22.72	2.11	2.14	5.83	5.74	84.78	76.96
34	INB-M6	25.73	20.95	1.94	2.02	5.74	5.80	71.28	71.15
35	INB-M7	27.47	22.68	2.07	2.08	6.21	5.78	76.83	74.87
36	INB-M8	26.63	22.08	1.96	1.97	5.94	5.89	79.17	74.92
37	INB-M9	27.70	22.00	1.86	1.86	5.55	5.74	79.44	74.50

Accessions shown in bold were found to be distinct in diversity analysis

c. Mean of for 1000 seeds weight, total yield per plant and oil percentage

S/N	Varieties	1000 seeds weight (mg)		Total yield per plant (g)		Oil percentage	
		2014	2015	2014	2015	2014	2015
1	AFB-KH	1612.00	1604.75	2.23	2.00	25.25	22.75
2	AFB-MA	1607.08	1606.85	2.41	2.09	24.78	23.28
3	AFB-CH	1605.42	1610.45	2.31	2.11	25.28	22.78
4	AFB-DI	1534.58	1547.50	1.71	1.53	19.65	19.28
5	AFB-PU	1568.33	1582.73	2.40	2.04	17.38	15.88
6	AFB-DU	1540.33	1580.38	2.40	2.02	20.46	18.38
7	AFB-DA	1551.50	1589.25	2.37	2.04	26.88	23.38
8	AFB-KI	1572.00	1582.88	1.58	1.29	23.67	21.67
9	AFF-AN	1583.17	1597.25	2.61	2.05	22.82	21.82
10	AFF-PU	1548.58	1588.00	2.29	1.97	30.12	26.62
11	AFF-BA	1566.58	1590.00	2.38	2.04	26.22	24.22
12	AFF-QA	1570.58	1570.38	2.26	2.08	22.30	21.30
13	AFG-MU	1680.42	1639.00	1.84	1.73	18.93	18.43
14	AFG-NW	1570.08	1566.88	3.25	2.12	29.13	24.63
15	AFG-NU	1572.83	1563.75	1.68	1.56	25.20	23.70
16	AFG-QA	1679.42	1651.00	2.30	2.07	22.62	22.62
17	AFH-SH	1544.50	1542.00	2.36	2.03	23.79	21.44
18	AFH-IN	1542.08	1520.63	2.59	2.04	25.32	22.82
19	AFH-KO	1590.92	1568.75	1.75	1.79	23.98	22.98
20	AFH-KA	1536.50	1517.63	1.44	1.39	27.32	24.82
21	AFK-AL	1570.83	1562.88	1.57	1.61	25.98	23.98
22	AFK-IM	1611.50	1599.13	2.62	2.06	24.14	22.64
23	AFK-KH	1655.92	1610.25	2.33	2.07	27.14	22.64
24	AFK-AR	1552.17	1555.25	2.79	2.01	24.48	22.48
25	AFN-SU	1603.67	1602.25	2.14	2.04	27.47	22.47
26	AFN-KH	1562.75	1541.88	2.34	2.07	27.80	24.80
27	AFN-DI	1567.00	1566.75	2.01	1.81	27.48	23.48
28	AFN-KA	1625.00	1609.75	1.71	1.48	28.24	25.24
29	INB-M1	1602.58	1597.63	2.66	2.05	20.94	19.44
30	INB-M2	1593.33	1582.88	2.73	2.04	13.76	13.63
31	INB-M3	1605.25	1589.63	3.17	2.12	16.88	16.48
32	INB-M4	1580.75	1582.50	2.22	2.03	26.07	23.07
33	INB-M5	1614.08	1606.50	3.35	2.23	25.32	22.49
34	INB-M6	1608.33	1601.00	2.19	1.97	17.50	17.40
35	INB-M7	1610.33	1602.00	3.35	2.04	14.20	14.10
36	INB-M8	1617.00	1606.00	2.41	2.06	17.01	16.91
37	INB-M9	1566.50	1567.38	1.89	1.63	14.92	15.37

Accessions shown in bold were found to be distinct in diversity analysis

Appendix III: List of RAPD primers used in the present investigation for genetic diversity analysis in 12 selected accessions of *Nigella sativa* L.

S/N	Primers that were used for PCR analysis	Sequence
1	AB4	CTACGGAGGA
2	AB10	GGCACTGAGG
3	AB13	GAGCCCTCCA
4	I14	AGCGTGTCTG
5	AK7	CTGAGACGGA
6	BH2	GTGCCTAACC
7	BH14	GAACCTGCGG
8	I1	TCACGTCCAC
9	I7	CTGACGTCAC
10	I15	AGGGCCGTCT
11	I18	TCATCCGAGG
Primers those did not function properly for PCR analysis		
12	AK9	ACCTGGACAC
13	AK11	GGAGGAGAGG
14	N8	CAGAAGCCCA
15	AB20	CCGCCTAGTC
16	H18	TGTTCCACGG
17	H19	AAGGCGGCAG
18	AK14	CAGCGACAAG
19	N19	TTTGCCCGGT
20	AK13	TGGAGAGCAG