Identification and characterization of herbicide tolerant mutant lines using SNP marker(s) in chickpea (*Cicer arietinum* L.)

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B.Sc. (Ag.)

MASTER OF SCIENCE IN AGRICULTURE
(GENETICS AND PLANT BREEDING)

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Identification and characterization of herbicide tolerant mutant lines using SNP marker(s) in Chickpea (*Cicer arietinum* L.)

By

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B.Sc. (Ag.)

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2017

DECLARATION

I**, ASHWINI M. TEGGI,** hereby declare that the thesis entitled **"Identification and characterization of herbicide tolerant mutant lines using SNP marker(s) in chickpea (*Cicer arietinum* L.)"** submitted to the **Professor Jayashankar Telagana State Agricultural University** for the degree of **Master of Science in Agriculture** is the result of original research work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

Place: Hyderabad **(ASHWINI M. TEGGI)**

Date: **I. D. No. RAM/15-043**

CERTIFICATE

This is to certify that the thesis entitled **"Identification and characterization of herbicide tolerant mutant lines using SNP marker(s) in chickpea (*Cicer arietinum* L.)"** submitted in partial fulfilment of the requirements for the degree of 'Master of Science in Agriculture' of the Professor Jayashankar Telangana State Agricultural University, Hyderabad, is a record of the bonafide research work carried out by **Ms. ASHWINI M. TEGGI** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance received during the course of the investigation have been duly acknowledged by the author of the thesis.

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CERTIFICATE

Ms. ASHWINI M. TEGGI has satisfactorily prosecuted the course of research and that thesis entitled **"Identification and characterization of herbicide tolerant mutant lines using SNP marker(s) in chickpea (*Cicer arietinum* L.)"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by her for a degree of any university.

Date: **(Dr. KULDEEP SINGH DANGI)**

Place: Hyderabad **Chairperson**

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LIST OF ABBREVIATIONS AND SYMBOLS

AHAS	: Aceto Hydroxy Acid Synthase
MAS	: Marker Assisted Selection
DNA	: Deoxy Ribo Nucleic Acid
RFLP	: Restiction Fragment Length Polymorphism
RAPD	: Randomly Amplified Polymorphic DNA
SSR	: Simple Sequence Repeats
SNP	: Single Nucleotide Polymorphisms
RFLP	: Restriction Fragment Length Polymorphism
ABLs	: Advanced Back Cross Breeding Lines
PCR	: Polymerase Chain Reaction
CTAB	: Cetyl Trimethyl Ammonium Bromide
EDTA	: Ethylene Diamine Tetra Acetic Acid
TRIS	: Tris (Hydroxyl Methyl) Amino Methane
TBE	: Tris Borate EDTA
dNTP	: Deoxynucleotide Triphosphate
Taq	: *Thermus aquaticus*
KASPar	: Kompetitive Allele Specific PCR
Mbp	: Megabases
M. ha	: Million Hectares
M. t	: Million Tonnes
Sl.no	: Serial Number
et al.,	: And Others
viz.,	: Namely
µl	: Micro Litres
µM	: Micro Molar
mM	: Milli Molar
G	: Gram
Ng	: Nano Grams
UV	: Ultraviolet
H2O	: Water
%	: Percent

/	: Per
°C	:degree Celsius
bp	: base pair
CAPS	:Cleaved Amplified Polymorphic Sequences
DNA	:Deoxyribonucleic Acid
aa	: amino acid

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ABSTRACT

Chickpea is a major food legume and an important source of protein in many countries of south Asia and sub-Saharan African. Globally, chickpea is cultivated over an area of 13.9 million hectares, with the production of 13.7 million tons. Besides a number of biotic and abiotic stresses that lead to significant yield losses in chickpea, weeds are also reported to reduce yield up to 84%, and severe yield losses as high as 98% are reported in autumn-sown chickpea. Hand weeding and mechanical weed control methods traditionally followed are becoming expensive owing to increased cost of human labour. Chickpea cultivars with herbicide tolerance can serve as an alternative to this problem. Hence, it is essential to identify sources of herbicide tolerance and utilize them in developing herbicide tolerant cultivars.

Development of chickpea cultivars with herbicide resistance is considered to be an economic and effective way for weed control. Earlier studies have reported large genetic variation existing in chickpea germplasm for Imidazolinone (IMI) herbicide tolerance.IMI group of herbicides are considered as powerful means of weed control, and have many agronomic advantages. Imidazolinones are protein synthesis inhibitors and act by inhibiting the enzyme *acetohydroxyacid synthase* (AHAS, also known as *acetolactate synthase*, ALS), which is a critical enzyme in the biosynthsis pathway of branched chain amino acids.

A point mutation in the chickpea AHAS gene at Cytocine675 to Thymin675 confers resistance to imidazolinones. Thompson and Taran (2014) developed an allele-specific SNP (KASPar) marker using this point mutation to predict the phenotypic response of the genotypes to IMI herbicides. This KASPar marker was used to genotype set of forty EMS mutant lines (developed in the background of JG 11 and KAK 2) and eighty four breeding lines in this study. In total of 124 genotypes along with check varieties (JG 11 and KAK 2) were phenotyped for herbicide resistance under field conditions at ICRISAT, Patancheru during *Rabi,* 2016. Herbicide tolerance ratings based on plant injury on a 1-5 scale (Gaur *et al.,* 2013) was used for phenotyping. All the genotypes exhibited plant injury symptoms under IMI herbicide treatment. Among 126 genotypes, 8 were highly susceptible, 24 were moderately tolerant and others were susceptible. The highly susceptible lines had 80-100% mortality. The genotypes which survived put forth secondary growth after 20-25 days of herbicide application leading to flowering and pod set. Upon genotyping with the KASPar marker 124 out of 126 genotypes yielded the fluorescent data. Graphical visualization of the

SNP genotyping data (KlusterCaller software) showed all the genotypes forming a single cluster near to allele ‘C’, associated with IMI susceptibility.

In addition to KASPar genotyping, an attempt was made to find the possibility of other allelic variation associated with herbicide tolerance. The AHAS gene sequence was blasted in the chickpea reference genome, and the best hit was used as query sequence to find SNP candidates from the available resequencing data of chickpea genotypes. Among all the variations obtained, one SNP showing consistent variation was selected and converted to CAPS marker. Forty randomly selected genotypes belonging to different phenotypic classes were analysed using the CAPS marker. All the genotypes exhibited similar banding pattern showing no variation at the locus.

The KASPar marker reported by Thompson and Taran does not serve as a diagnostic tool in identification of herbicide tolerance. As none of the genotypes used in the study were highly resistant, further screening of a large set of germplasm lines for herbicide tolerance and amplicon sequencing of the AHAS gene in these lines will help in the identification of alternate alleles and the development of diagnostic marker for herbicide resistance.

Chapter I

INTRODUCTION

Chapter I

INTRODUCTION

Chickpea is a major food legume and an important source of protein in many countries of Asian and African continents (Jukanti *et al.,* 2012). This is the fourth important legume crop after soybean, common bean and common pea (Gupta *et al.,* 2017). Globally, chickpea is cultivated over an area of 13.9 million hectares, with the production of 13.7 million tons (FAO, 2016). India is the top chickpea producing country; which alone produces about 72% of the world's chickpea. In India, chickpea is cultivated over an area of 9.9 million hectares with the total production of 9.88 million tons (FAOSTAT, 2014). In Telangana, chickpea covers an area of 1.08 lakh hectares with a production of 0.81 lakh tonnes and productivity of 1367 kgs ha^{-1} *(Agriculture Action Plan 2015-16, Department of Agriculture, Telangana*).

Besides a number of biotic and abiotic stresses that lead to significant yield losses in chickpea, weeds are also reported to reduce yield up to 84%, and severe yield losses as high as 98% are reported in autumn-sown chickpea (Gaur *et al.,* 2013). They also result in harvesting difficulties (Taran *et al.,* 2013). Chickpea is sensitive to weed competition because of its slow growth rate and limited leaf area development at early stages of growth and establishment (Solh and Pala, 1990). It was reported that initial four to six weeks were most critical for weed competition (Saxena *et al.,* 1976). Weeds compete with chickpea plants for water, nutrients, sunlight, and space and also harbor insect-pests and diseases (Mukherjee, 2007). Thus, weed management is crucial to realize maximum yields and also to maintain high quality of produce.

Hand weeding and mechanical weed control methods traditionally followed in the developing countries are becoming expensive owing to increased cost of human labor (Chaturvedi *et al.,* 2014). Because of the sensitivity of chickpea to herbicides, most effective are the pre-emergence herbicides and the choices for post-emergence herbicides are limited (Solh and Pala, 1990). The pre-emergence herbicides provide limited weed control as they are effective in controlling weeds at early stages of crop growth (Goud *et al.,* 2013). Till date no post-emergence herbicide is recommended for weed control in South Asia where bulk of chickpea is grown. This is mainly because of the sensitivity of available chickpea cultivars to herbicides (Gaur *et al.,* 2013; Chaturvedi *et al.,* 2014). Chickpea cultivars with improved herbicide tolerance are required by the farmers in order to have greater flexibility for use of

post-emergence herbicides. Hence, it is essential to identify sources of herbicide tolerance and utilize them in developing herbicide tolerant cultivars (Gaur *et al.,* 2013).

Imidazolinone herbicides are considered a powerful means of weed management (Shaner, 2003). Imidazolinones control weeds by inhibiting the enzyme *acetohydroxyacid synthase* (AHAS, also known as *acetolactate synthase*, ALS), which is a critical enzyme for the biosynthesis of branched chain amino acids in plants. These herbicides control a wide spectrum of grass and broadleaf weeds, effective at low application rates, have low mammalian toxicity, and possess a favorable environmental profile (Weed Science Society of America, 2007). Furthermore, imidazolinone tolerant plants with altered AHAS genes and enzymes have been discovered in many crops. This makes it possible to develop imidazolinone-tolerant crops based on the resistance mechanism at the site of action for these crops (Tan *et al.,* 2005).

In many plant species, resistance to Group 2 herbicides (ALS inhibitors) is the result of a point mutation in the AHAS gene causing amino acid substitution at various codon positions. Mutations may affect key herbicide binding sites, preventing Group 2 herbicides from binding and inhibiting *acetohydroxyacid synthase* enzyme activity (Tan *et al.,* 2005). In chickpea, genotypes with resistance to imidazolinones have been identified. A point mutation in the chickpea AHAS gene at Cytocine675 to Thymin675, resulting in an amino acid substitution from Ala205 to Val205 confers resistance to imidazolinones, and it is inherited as a single gene in a semi-dominant fashion (Thompson and Taran, 2014).

In recent days, SNP markers are gaining popularity because of automation potential, biallelic variation, high abundance in the genome, low cost, and highly reproducible results (Rafalski, 2002; Ganal *et al.,* 2009). Phenotypic identification of herbicide tolerant genotypes is complicated, time consuming and labor intensive. Hence, the use of diagnostic bio-assays and molecular markers for early screening of herbicide resistance is needed (Bulos *et al.,* 2013).The allele-specific SNP (KASPar) marker developed by Thompson and Taran (2014) targeting the point mutation conferring resistance to IMI herbicides in chickpea is an useful tool in this direction. This KASPar marker can be tested for its potential use in marker assisted selection (MAS) for IMI-resistant chickpea, which will increase the selection efficiency in developing resistant varieties.

The present study entitled “Identification and characterization of herbicide tolerant mutant lines using SNP markers in chickpea (*Cicer arietinum* L.)” was undertaken with the following objectives:

1. Phenotyping of chickpea mutant lines for herbicide tolerance
2. Characterization of mutant lines using allele-specific SNP marker
3. Identification of candidate genes for herbicide tolerance in chickpea

Chapter II

REVIEW OF LITERATURE

Chapter II

REVIEW OF LITERATURE

2.1 Chickpea

Chickpea (*Cicer arietinum* L.) is a diploid (2n = 2x = 16) food legume grown worldwide with the genome size of 738 Mbp (Varshney *et al.,* 2013).It is believed to be originated in an area of present-day south-eastern Turkey and adjoining areas of Syria (Van der Maesen 1987). Chickpeas belong to the family *Fabaceae*, tribe *Cicereae* and the genus *Cicer* (Kupicha 1977). The genus *Cicer* has 43 species, among them 9 are annual, 33 are perennial, and one is unspecified (Van der Maesen, 1987). Morphologically chickpea is divided in two classes: Kabuli and Desi (Auckland and Van der Maesen, 1980). The Kabuli type produces large, cream-colored, round to ram-head shaped seeds with a thick seed coat and white flowers, whereas desi type is characterized by smaller, angular seeds with a thick pigmented seed coat and pink or purple flowers (Maiti and Wesche-Ebeling, 2001).Chickpea seeds contain 20–30% crude protein, 40% carbohydrate, and 3–6% oil (Gil *et al.*, 1996). It is one of the most important food legumes in sustainable agriculture system because of its low production cost, wider adaptation, and ability to fix atmospheric nitrogen, presence of prolific tap root system and flexibility to fit in various crop rotations (Singh *et al.,* 2014).

India is the world's leading chickpea producer and in 2014 produced 72% of the world's chickpea (FAOSTAT, 2014).The top ten chickpea producing countries are: India, Australia, Pakistan, Myanmar, Turkey, Ethiopia, Iran, Mexico, Canada, and United States (FAOSTAT, 2014). In India production of chickpea is concentrated in central and southern parts. Madhya Pradesh, Rajasthan, Maharashtra, Andhra Pradesh and Karnataka are the major chickpea growing states. Madhya Pradesh is the single largest producer in the country, accounting for over 40% of total production. Rajasthan, Maharashtra, Uttar Pradesh and Andhra Pradesh contribute about 14%, 10% and 7%, respectively (www.aicrpchickpea.res.in, 2012-13)

2.2 Weed management in chickpea

2.2.1 Importance of weeds

Weeds have become a serious problem in both autumn and winter grown chickpeas and also under irrigated conditions. Significant yield losses up to 84% have been reported due to weeds; severe yield reduction as high as 98% is reported in autumn-sown chickpea (Gaur *et al.,* 2013). Chickpea is a poor competitor to weeds because of slow growing nature at early growth stages and establishment (Solh and Pala, 1990). Weeds compete with the crop for water, nutrients, sunlight and space and also harbor insect-pests and diseases (Kaushik *et al.,* 2014; Gaur *et al.,* 2013). Many species of weeds have been reported to infest chickpea fields. The common weeds found in Indian chickpea fields are *Chenopodium album, Melilotus indica, Lathyrus aphaca, Medicago denticulata, Trigonella polycerata, Polygonum plebijum, Asphodelus tenuifolius, Euphorbia dracunculoides, Anagallis arvensis,Trichodesma indicum* and *Cuscuta hyaline*, a parasitic weed (Singh and Diwakar, 1995).

In a survey conducted by Singh *et al.* (2014) in Bundelkhand region, weed menace was ranked third among the various production constraints by the farmers. Yield reduction of 87% (Singh and Bajpai, 1996) and 88% (Bhalla *et al.*, 1998) was reported with the elimination of weed control among different crop production inputs. Weeds also tend to contaminate the produce and reduce the quality. Excessive weed competition may adversely affect seed size which is an important quality parameter in Kabuli chickpea (Goud *et al*., 2013). Thus, weed management is crucial to realize maximum yields and also to maintain high quality of produce (Gaur *et al.,* 2013).

2.2.2 Mechanical and cultural weed control

Integrated weed management system with multiple control strategies is needed for optimal weed control. Prevention of weed development and dispersal is the most cost-effective measure (Yenish, 2007). Current chickpea weed control strategies include crop rotations, mechanical practices, manual weeding and chemical control with pre-emergence herbicides (Goud *et al.,* 2013). Mechanical weed control methods like hand pulling, hoeing, or human powered equipment traditionally followed are not effective besides being costly and uneconomical (Chaturvedi *et al.,* 2014). Crop rotation, aggressive cultivars and managing sowing dates and crop geometry are some of the cultural practices employed by farmers as a part of chickpea production system. Though they are cost effective and safe tools for

integrated weed management, limited options are available in chickpea (Jefferies, 2014; Mukherjee, 2007).

2.2.3 Chemical weed control

Herbicides are effective tools in man's everlasting struggle with weeds (Mukherjee, 2007). Chickpea is sensitive to many herbicides and therefore, pre-emergence herbicides are mostly relied upon (Gaur *et al.,* 2013). Pendimethalin is extensively used as pre-emergence herbicide for weed management in chickpea field (Dewangan *et al.,* 2016). Pre-emergence herbicides are effective in controlling the weeds at initial stage of crop growth, and the weeds germinating after crop emergence become dominant in the field and cause substantial yield loses (Gaur *et al.,* 2013). The use of post emergence herbicides for season long weed control is thus, preferred over early use of herbicides as pre-plant incorporation and pre-mergence herbicides (Goud *et al.,* 2013). Quizalofop ethyl, Chlorimuron and Imazethapyr are new generation post-emergence herbicides used in many leguminous crops (Kumar *et al.,* 2015).

A few herbicides have been registered officially for weed control in chickpea in Canada, Turkey and Australia. Herbicides such as sulfentrazone, saflufenacil, and metribuzin are registered in Canada (Jefferies *et al.,* 2016); isoxaflutole is registered in Australia (Datta *et al.,* 2009). Imazethapyr has been registered and authorized in Turkey along with other herbicides, methabenzthiazuron and terbutryne (Kantar *et al.,* 1999). Though bulk of chickpea is grown in South Asia, no post-emergence herbicide is recommended owing to the sensitivity of available chickpea cultivars to herbicides (Gaur *et al.,* 2013).

2.3 Imidazolinone herbicides

Imidazolinone herbicides are considered a powerful tool for weed management. This group includes six different herbicides: imazamethabenz methyl, imazethapyr, imazamox, imazapic, imazapyr, and imazaquin (Shaner, 2003). Imidazolinones are able to control wide spectrum of troublesome grass and broadleaf weeds at very low doses. They are non-corrosive, non-flammable, have low mammalian toxicity and possess favorable environmental profile (Weed Science Society of America, 2007; Cobb and Read, 2010).

Imidazolinones(IMI) along with other Group-2 herbicides *viz.*, sulphonylureas (SU), triazolopyrimidines (TP), sulfonylaminocarbonyltriazolines (SCT) and pyrimidinylthiobenzoates (PTB) share the same site of action, namely acetolactate synthase

(ALS, E.C. 4.1.3.18, also known as acetohydroxyacid synthase, AHAS). AHAS is a nuclear-encoded, chloroplast-localised enzyme which catalyses the biosynthesis of branched chain amino acids; valine, leucine, and isoleucine (Cobb and Read, 2010).Inhibition of ALS impedes with the biosynthesis of these amino acids in the plants and leads to starvation. This is thought to be the primary mechanism by which ALS-inhibiting herbicides cause plant death. But other secondary mechanisms, such as buildup of 2-ketobutyrate, disruption of protein synthesis, and disruption of photosynthate transport, have also been implicated in the mechanism of plant death (Tranel and Wright, 2002).

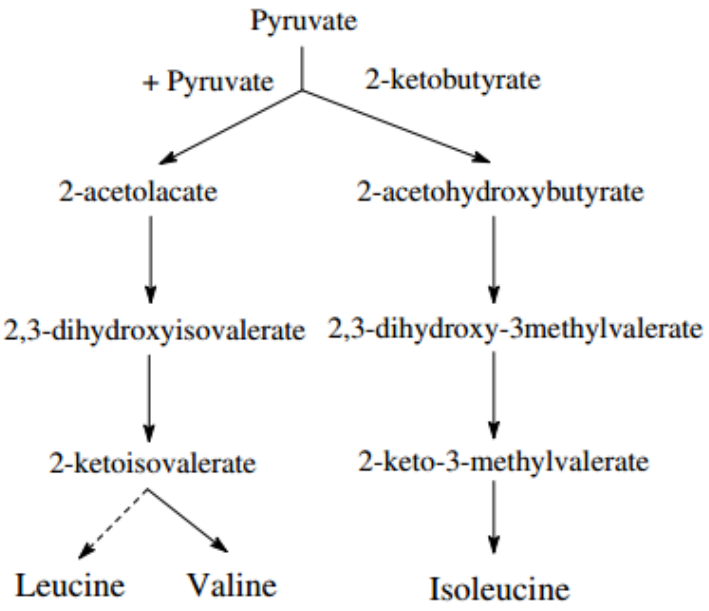

Figure 2.1 Biosynthesis pathway of branched chain amino acids (Zhou *et al.* 2007)

2.4 AHAS gene mutation and Imidazolinone-tolerance trait

Resistance to IMI herbicides is the result of a point mutation in the AHAS gene causing amino acid substitution. Common AHAS amino acid substitutions causing herbicide resistance are: Ala122, Pro197, Ala205, Asp376, Trp574, and Ser653 (amino acid position based on *Arabidopsis* AHAS) (Jain and Taran, 2015). List of AHAS amino acid substitutions causing IMI-resistance in major crops reported is presented in Table 2.1. Amino acid substitutions at Ala122 and Ser653 confer high levels of resistance to imidazolinone herbicides (IMIs), whereas substitutions at Pro197 endow high levels of resistance against sulfonylureas and provide low-level resistance against IMIs and triazolopyrimidine

herbicides. Substitutions at Trp574 endow high levels of resistance to imidazolinones, sulfonylureas, and triazolopyrimidines, whereas substitutions at Ala205 provide resistance against all AHAS-inhibiting herbicides (Lee *et al.,* 2011).

Table 2.1 List of AHAS amino acid substitutions reported in major crops

Sl. No.	**Crop**	**Codon position**	**Reference**
1	Rice	G_{654} and S_{653}	Kadaru *et al.,* 2008
2	Wheat	S_{653}	Ellison *et al.,* 2015
3	Barley	S_{653}	Lee *et al.,* 2011
4	Sunflower	A_{205}, P_{197} and A_{122}	Bulos *et al.,* 2013
5	Soybean	P_{197}	Ghio *et al.,* 2013

2.5 Chickpea AHAS gene

Chickpea AHAS genes are intronless and might have cyanobacterial origin like arabidopsis AHAS and other plastid protein-encoding genes (Jain and Taran, 2015). In chickpea, there are two homologous AHAS genes, AHAS1 and AHAS2. Even though these genes share 80% amino acid similarity, only a mutation in AHAS1 confers IMI resistance, and AHAS2 sequence do not show any mutation consistent with the herbicide resistance across IMI susceptible and resistant chickpea genotypes. They were also shown to cluster independently in chickpea and across other legume genera (Thompson and Taran, 2014).

The consensus AHAS1 sequence is 2,183bp (658 aa) long with no introns. A point mutation in the AHAS1 gene at C675 to T675 resulting in an amino acid substitution from Ala205 to Val205 confers the resistance to IMI in chickpea (Thompson and Taran, 2014). The same substitution (A205V) has been reported to be associated with resistance to IMI herbicide in several other plant species including *Solanum ptychanthum*, *Helianthus annuus*, and *Xanthium strumarium* (Jain and Taran, 2015). The chickpea 1536 Illumina GoldenGate® SNP genotyping platform was used by Thompson and Taran (2014) to develop molecular map of the segregating population, CDC 512-51 (IMI susceptible) x ICCX860047-9 (IMI resistant)

and confirm the location of the locus for IMI resistance. They were able to map *AHAS1* gene on chromosome 5.

2.6. Developing Imidazolinone Herbicide Resistance

2.6.1 Plant Breeding Techniques

Herbicide resistant varieties can be developed through classical breeding using resistant germplasm (e.g. gene banks), tissue culture techniques or mutagenesis. Taran *et al.* (2010) screened diverse chickpea germplasm and cultivars available in Canada and identified four chickpea lines (ICC2242, ICC2580, ICC3325 and ICCX860047–9) resistant to IMI herbicides. Gaur *et al.* (2013) screened 300 diverse chickpea genotypes (278 accessions from the reference set and 22 breeding lines) in order to identify the sources of tolerance to imazethapyr and metribuzin herbicides. They found several genotypes tolerant to imazethapyr (ICC 3239, ICC 7867, ICC 1710, ICC 13441, ICC 13461, ICC 13357, ICC 7668, and ICC 13187) and metribuzin (ICC 1205, ICC 1164, ICC 1161, ICC 8195, ICC 11498, ICC 9586, ICC 14402, and ICC 283). Similar experiment was conducted by Chaturvedi *et al*. (2014). They screened 509 chickpea accessions (reference set and elite breeding lines) for Imazethapyr tolerance and reported 3 accessions (ICC 1164, IPC 2010-81 and IPC 2008-59) to be most tolerant.

Toker *et al*. (2012) used gamma ray irradiation (300 and 400 Gy) for inducing mutation to improve imidazolinone resistance in chickpea. They used nine accessions of chickpea belonging to three species (*C. arietinum*, *C. bijugum*and, *C. reticulatum*) for this purpose and were able to isolate one highly IMI-resistant mutant of *C.reticulatum* and some IMI-tolerant mutants in cultivated chickpea. Other examples of Group 2 herbicide resistance species developed through the use of mutagenesis include: *Arabidopsis thaliana* and *Medicago truncatula* using EMS mutagenesis (Haughn and Somerville, 1986; Heap, 2000); wheat and barley using sodium azide (Newhouse *et al.,* 1992; Li *et al.,* 2008; Lee *et al*., 2011). Tissue culture and somatic cell IMI resistance selection was utilized by Wright and Penner (1998) to develop IMI resistant sugarbeet. Once resistant lines have been developed, conventional breeding methods can be implemented to incorporate the trait from a mutant line into an agronomically adapted variety (Salimath *et al.*, 2007).

2.6.2 Genetics of IMI-Resistance

Herbicide studies on segregating populations can determine the gene action and mode of inheritance of resistance to Group-2 herbicides. Segregation studies in various plant species (*Arabidopsis thaliana*, sugarbeet, *Medicago truncatula*, and barley) demonstrated that the resistance to IMI herbicide is monogenic with semi-dominant to dominant gene action (Haughn and Somerville 1990; Wright and Penner 1998; Oldach *et al.*, 2008; Lee *et al.*, 2011). Sunflower (*Helianthus annuus* L.) showed a unique mode of inheritance, where the *AHAS1–1* allele is codominant to recessive depending on IMI dose and *AHAS1–3* is semi to fully dominant but dominant over *AHAS1–1* (Sala and Bulos, 2012). In chickpea, resistance to IMI-herbicides is inherited as a single gene in a semi-dominant fashion (Thompson and Taran, 2014).

2.7 Identification of herbicide tolerance

Phenotypic identification of herbicide tolerance traits involves the spraying of herbicide onto plants grown in the field or greenhouse at early stages of development, and selection of tolerant genotypes. It is time consuming and requires a large amount of resources and space. And the heterozygous plants are not recovered since they fail to survive the herbicide application. Under these circumstances, the development of efficient and reliable diagnostic bioassays or molecular markers for early screening of HT is needed (Bulos *et al.*, 2013)

In recent days, researchers have devised many diagnostic techniques to confirm AHAS inhibitor resistance. Conventional whole plant-based diagnostic techniques such as seedling bioassays or enzyme-based *in vitro* bioassays provide accurate results, but they tend to be labor and/or space intensive and the experiment has to be repeated with multiple herbicides for the elucidation of a cross-resistance pattern. Some DNA-based diagnostic tests have been developed with the recent advances in DNA technologies coupled with the knowledge of sequence information. Three main techniques used in this regard are: PCR-RFLP (restriction fragment length polymorphism), PASA (PCR amplification of specific alleles) and DHPLC (denaturing high-performance liquid chromatography). These techniques are relatively rapid and provide clear identification of the mutations causing resistance (Corbett and Tardif, 2006).

Ellison *et al.* (2015) demonstrated a novel DNA-based screening protocol using pyrosequencing to screen for the IMI tolerant mutation (S_{653}) in wheat. This presents useful strategy for determining copy number of the target mutation in polyploid species and requires fewer steps than current assays. Bulos *et al*. (2013) developed three types of PCR markers (SSRs, CAPS and SNPs) in sunflower which allow the precise identification of different alleles conferring tolerance to AHAS inhibitor herbicides (A_{205}, P_{197} and A_{122}) at the *Ahasl1* locus. Kadaru *et al.* (2008) developed simple, rapid, relatively high-throughput and precise allele-specific SNP genotyping technique (AS-PCR) to differentiate imazethapyr resistant clearfield rice and susceptible red rice.

In chickpea, an allele-specific SNP marker (KASPar) was developed by Thompson and Taran (2014) in order to increase the selection efficiency in developing resistant varieties. The main benefits of using of SNP markers include good distribution throughout the genome, low cost, reproducible results, and automation potential. The KASPar marker was used to screen a chickpea RIL population (CDC 512-51x ICCX860047-9) segregating for herbicide resistance and it accurately predicted the phenotypic response to IMI herbicides.

2.7.1 KASPar (Kompetitive Allele Specific PCR) marker

Single nucleotide polymorphism (SNPs) designates a nucleotide site of a given sequence for which substitution polymorphism has been observed in 1% or more of the population. KASPar SNP genotyping assay developed by LGC Genomics (Teddington, UK; www.lgcgenomics.com) is an efficient and cost-effective approach, which utilizes a unique form of competitive allele-specific PCR combined with a novel, homogeneous, fluorescence based reporting system (Semagn *et al.*, 2014).In chickpea, availability of KASPar and other SNP genotyping assays greatly accelerate the large-scale validation and high-throughput genotyping of previously discovered SNPs in diverse accessions, specifically for genetic diversity studies, phylogenetics and genetic linkage map construction (Bajaj *et al.*, 2015).

KASPar reaction uses three components: test DNA with the SNP of interest; KASPar Assay Mix (containing two different, allele specific, competing forward primers with unique tail sequences and common reverse primer); KASPar Master mix (containing FRET cassette and Taq polymerase in an optimised buffer solution). In the initial stage of PCR, the appropriate allele-specific primer matches the target SNP and amplifies the target region with the common reverse primer. As PCR proceeds further, the fluor labelled part of the FRET

cassette complementary to amplified tail sequences binds and releases the fluor from the quencher to generate a fluorescent signal. If the genotype at a given SNP is homozygous, only one or the other of the possible fluorescent signals will be generated. If the individual is heterozygous, the result will be a mixed fluorescent signal. After the completion of KASPar PCR, reaction plates are read and the data analysed using any cluster analysis viewing software. Detected signals are plotted as a graph, with samples of the same genotype clustering together (www.lgcgenomics.com; Chunlin He *et al.*, 2014).

2.8 SNP2CAPS computer program

Most of the SNP genotyping assays require expensive and specialized equipment and chemicals for analysis. As a solution to this problem SNPs are converted to CAPS (Cleaved Amplified Polymorphic Sequence) markers. SNP2CAPS is a computer program that facilitates the computational conversion of SNP markers into CAPS markers. It involves a simple algorithm which screens multiple aligned sequences for restriction sites followed by a selection pipeline that allows the deduction of CAPS candidates by the identification of putative alternative restriction patterns (Thiel *et al.*, 2004).

Chapter III

MATERIAL AND METHODS

Chapter III

MATERIAL AND METHODS

The present study was conducted to identify and characterize the herbicide tolerant chickpea genotypes using a SNP marker. The work involved phenotypic evaluation of a set of chickpea genotypes for herbicide tolerance, genotyping of these lines using a SNP marker and identification of candidate gene(s) responsible for herbicide tolerance. In addition the genotypes were also screened using CAPS marker(s) developed by converting the SNP reportedly conferring herbicide resistance in chickpea. The materials and methods followed to conduct the present study are described in this chapter.

3.1 Experimental material

3.1.1 Experimental Location

The present study was conducted at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India (located at 18° N, 78° E and 545 m above sea level). The field experiment for phenotyping was conducted during post rainy season (November to February) of 2016-17. The weather data (total rainfall during the crop season, minimum and maximum temperature, relative humidity, total evaporation, solar radiation and bright sunshine hours) for the cropping season at the experimental site is given in APPENDIX-I.

3.1.2 Plant material

A set of 40 EMS mutant lines generated in the background of JG11 and KAK2 were screened for herbicide tolerance along with JG11 and KAK2 as susceptible checks. In addition, 84 breeding lines were also included in the field trial. List of the genotypes used in this study is given in the Table 3.1.

3.1.3 Experimental design

The genotypes were evaluated using alpha lattice design with three replications. Each replication contained seven blocks and each block had eighteen treatments allotted randomly. Each plot was divided into two parts (2meter each); one part was sprayed with the herbicide and other part was maintained as control (Figure 3.1 and Figure 3.2).

Table 3.1. Panel of genotypes used in the study

S. No	Genotypes	S. No	Genotypes
1	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P2	26	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P108
2	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P10	27	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P111
3	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P15	28	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P112
4	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P18	29	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P113
5	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P20	30	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P114
6	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P23	31	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P115
7	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P35	32	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P118
8	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P51	33	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P120
9	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P54	34	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P121
10	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P59	35	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P124
11	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P61	36	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P126
12	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P62	37	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P128
13	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P63	38	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P131
14	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P64	39	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P132
15	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P65	40	Mutation Breeding - KAK 2 - M2(P)-Bulk-BP-P42
16	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P72	41	AGBL 110
17	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P73	42	AGBL 122
18	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P75	43	AGBL 134
19	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P76	44	AGBL 146
20	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P78	45	AGBL 158
21	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P79	46	AGBL 160
22	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P82	47	AGBL 172
23	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P91	48	AGBL 184
24	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P95	49	GJG 0814
25	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P103	50	GJG 0904

Table 3.1 (cont.)

S. No	Genotypes	S. No	Genotypes
51	GJG 0919	76	24043-4-1
52	GAG 1107	77	IPC 2010-25
53	GAG 1111	78	IPC 2010-37
54	GJG 1211	79	IPC 2008-89
55	GJG 1304	80	IPC 2010-219
56	GJG 1311	81	IPC 2011-69
57	24001-4-1	82	IPC 2011-141
58	24002-4-3	83	IPC 2011-70
59	24003-1-1	84	IPC 2011-64
60	24003-2-1	85	IPC 2011-123
61	24004-3-1	86	IPC 2010-94
62	24005-3-1	87	FLIP01-29C
63	24006-2-1	88	ICC 7441
64	24007-5-1	89	ICC 8621
65	24015-2-1	90	ICC 14402
66	24015-4-1	91	ICC 15618
67	24017-1-1	92	ICC 16207
68	24017-2-1	93	ICC 3325
69	24018-2-1	94	ICC 15868
70	24031-1-1	95	ICC 1098
71	24031-3-1	96	ICCV 13101
72	24032-2-1	97	ICCV 13102
73	24034-4-1	98	ICCV 13103
74	24042-1-1	99	ICCV 13104
75	24042-5-1	100	ICCV 13105

Table 3.1 (cont.)

S. No	Genotypes	S. No	Genotypes
101	ICCV 13106	114	ICCV 13312
102	ICCV 13107	115	ICCV 13314
103	ICCV 13109	116	ICCV 13316
104	ICCV 13111	117	ICCV 13317
105	ICCV 13116	118	ICCV 13318
106	ICCV 13117	119	ICCV 14103
107	ICCV 13118	120	ICCV 14106
108	ICCV 13305	121	ICCV 14107
109	ICCV 13306	122	ICCV 14108
110	ICCV 13307	123	ICCV 14112
111	ICCV 13308	124	ICCV 14118
112	ICCV 13309	125	JG 11
113	ICCV 13311	126	KAK 2

Figure 3.1 Treating plots with herbicide by maintaining 2 meter as control

Figure 3.2 Field view at three weeks after herbicide treatment

3.1.4 Herbicide

Imazethapyr (Pursuit: BASF) was used at the rate of 4ml/liter spray solution (double the recommended dose). In addition, Cyboost (2 g/liter) and Cyspread (1.5 ml/liter) were used for uniform spread and absorption.

3.1.5 Markers

3.1.5.1 SNP marker

An allele specific marker (KASPar) developed by Thompson and Taran (2014) was used in this study.

3.1.5.2 CAPS markers

Two CAPS markers developed using SNP2CAPS computer program were used in this study (Table 3.2).

Table 3.2 Details of CAPS markers used in the study

S no.	Oligo name	Sequence (5'→ 3')	
1	CpAHAS-*Sfa*NI	F	CGAACAAGGCGGTATATTCG
		R	CGTCTTTGGGTACGTCAATG
2	Ca1_AHAS1-*Hpy*AV	F	GGTAACTAGGTCAATTACAAAGCACA
		R	ATCCCTTTACTCTCCAAAATCCTATT

3.1.6 Chemicals and buffers

The lists of chemicals used for this study are given in Appendix-II.

3.1.7 Preparation of stocks and buffer solutions

Procedure for the preparation of stocks and buffer solutions used in this study are given in Appendix-III.

3.2 Methods

3.2.1 Phenotyping for herbicide tolerance

To determine IMI resistance, treatment plots were uniformly sprayed with imazethapyr, 30-days after sowing using a shoulder-mounted hand operated knapsack sprayer. The plots were subjected to three visual injury ratings at 10-days interval. Herbicide tolerance ratings based on plant injury on a 1-5 scale (Gaur *et al.*, 2013) was used for this purpose.

Table 3.3 Scale used for visual scoring of herbicide reaction as per Gaur *et al.* (2013)

Score	Plant state	Type of reaction
1	Excellent plant appearance, no chlorosis	Highly tolerant
2	Good plant appearance, minor chlorosis	Tolerant
3	Fair plant appearance, moderate chlorosis	Moderately tolerant
4	Poor plant appearance, severe chlorosis	Sensitive
5	Complete chlorosis leading to plant mortality	Highly sensitive

Apart from herbicide scoring the following observations were also taken independently from control and treated plots -

1. Days to 50% flowering (DF) :
2. Days to maturity (DM)
3. Number of primary branches
4. Number of secondary branches
5. Plant height (cm)
6. Biomass (g)
7. Seed yield (g)
8. 100 seed weight (g)

3.2.2 DNA extraction

DNA isolation was carried out for all the genotypes, using high throughput mini-DNA extraction method as described by Cuc *et al.* (2008). The steps involved in DNA extraction protocol are explained below.

1. Sample preparation
   - Leaves were collected from 30 days old seedlings.
   - 70-100 mg of leaf tissue was placed in 12 × 8-well strip tube with strip cap (Marsh Biomarket, USA) in a 96 deep-well plate together with two 4 mm stainless steel grinding balls (SpexCertiPrep, USA).
2. CTAB extraction
   - For each sample 450 µl of preheated (at 65^0C for half an hour) CTAB buffer (100Mm Tris-HCl (pH-8), 1.4 M NaCl, 20mM EDTA, CTAB (2-3% w/v), mercaptoethanol) was added and secured with strip caps.
   - Samples were homogenized in a Genogrinder 2000 (SpexCertiPrep, USA), following the manufacturer's instructions, at 500 strokes/min for 3 times at 2 min interval.
   - Plate was fitted into locking device and incubated in hot water bath at 65^0C for 30 Min with intermittent shaking.
3. Solvent extraction
   - For each sample, 450µl of chloroform-isoamyl alcohol (24:1) was added and gently inverted to mix.
   - Plate was centrifuged at 5,500 rpm for 10 min. The aqueous layer was then transferred to fresh tubes.
4. Initial DNA precipitation
   - 0.7 vol (approximately 210µl) of cold isopropanol was added to the collected aqueous layer. The solutions were mixed thoroughly by inverting and kept undisturbed in freezer (-20^0C) for 1hour.
   - Plate was centrifuged at 5,000 rpm for 15 min.
   - Supernatant was discarded from each sample and pellet was air dried for 20 min using Speedvac.

5. RNase treatment

- In order to remove co-isolated RNA, 200µl low salt $T_1E_{0.1}$ (10mM Tris EDTA (pH-8)) and 3µl RNase (10mg/ml) was added to each sample and incubated at 37^0C for 30 min.

6. Solvent extraction

- 200µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample and mixed thoroughly by inverting.
- Plate was centrifuged at 5,000 rpm for 5 min.
- Aqueous layer from each sample was transferred to a fresh 96 deep-well plate.
- 200µl of chloroform-isoamyl alcohol (24:1) was added to the aqueous layer and carefully mixed by inverting.
- Plate was centrifuged at 5,000 rpm for 5 minutes. Aqueous layer was transferred to fresh 96 deep-well plate.
- A total of 315µl ethanol-acetate solution (30µl ethanol, 1.5µl 3M Sodium Acetate (pH-5.2)) was then added to each sample and placed in 20^0C for 1 hour.
- Plate was again centrifuged at 5,000 rpm for 5 min.
- Supernatant was discarded from each sample and pellet was washed with 70% ethanol.
- Plate was centrifuged at 6,000 rpm for 10 min.
- Supernatant was again decanted from each sample. Samples were air dried for 1 hour.
- Pellet was re-suspended in 100µl low-salt $T_{10}E_1$ and stored at 4^0C.

3.2.3 DNA quantification

The extracted DNA was quantified by loading the samples on 0.8 % agarose gel pretreated with 0.5µl/10ml Ethidium Bromide (10mg/ml). Submarine electrophoretic gel was run at 100V for about an hour and DNA was visualized using UV transmitted gel documentation system (SYNGENE). After quantification, the DNA plates were labelled and stored at -20^0C as stocks. DNA was normalized to 10ng/µl concentration for PCR amplification and quantified following the above mentioned procedure.

3.2.4 KASPar SNP genotyping assay

KBiosciences protocol was followed for KASPar SNP genotyping. 2.5µl of genomic DNA (10ng/µl), 2.5µl 2X KASPar master mix and 0.055µl KASPar assay mix (12µM each allele-specific forward primer and 30µM reverse primer) were mixed in each well of a PCR plate and following PCR conditions were used.

Table 3.4 Thermal cycling conditions for KASPar

94 ^{0}C for 15 min	Hot-start activation
94 ^{0}C for 20 s 65 ^{0}C for 60 s (dropping 0.8 ^{0}C per cycle)	10 cycles – touchdown
94 ^{0}C for 20 s 57 ^{0}C for 60 s	26 cycles
10 ^{0}C	Hold

3.2.5 Analysis of KASPar genotypic data

The fluorescence endpoint reading of reactions was done using TECAN microplate reader (Infinite F200 Pro, Austria). Further details on principle, procedure and chemistry of the KASPar assay are available at http://dna.uga.edu/wpcontent/uploads/2013/12/KASPar-SNP-Genotyping-Manual-KBioscience.pdf. Genotyping data obtained based on the florescence detected from the KASPar assay was graphically viewed and analyzed through KlusterCaller Version 3.4 software (http://results.lgcgenomics.com/software/klustercaller/).

3.2.6 Developing CAPS markers

SNP2CAPS computer program was used for the conversion of SNP sites into CAPS markers. It requires two input files that contain data about the sequence alignments and the restriction enzymes. The first input file is a modified FASTA formatted file that stores one or more multiple alignments of sequences of different accessions. The second input file contains

data on the restriction enzymes that can be downloaded in different formats from the restriction enzyme database REBASE (http://rebase.neb.com/).

3.2.6.1 CpAHAS - *Sfa*NI

The resistant and susceptible AHAS1 gene sequences (reported by Thompson and Taran, 2014) were aligned using ClustalW online program and screened for restriction enzyme showing alternative restriction patterns.Primer3 Input (version 0.4.0) was used to design the primers for putative CAPS candidates.

3.2.6.2 Ca1_AHAS1-*Hpy*AV

The resistant and susceptible sequences of AHAS1 gene (reported by Thompson and Taran, 2014) were blasted on to CDC Frontier reference genome (Varshney *et al.,* 2013), the best hits were selected based on e-value and the corresponding coordinates were retrieved. These coordinates were used as query in chickpea resequencing data of 429 lines to identify SNP candidates. Among all the variations obtained, one SNP was selected for mining the CAPS candidates. For this purpose, 1000bp flanking sequences were extracted for the selected SNP candidate and SNP2CAPS analysis was carried out as stated above. Primer3 Input (version 0.4.0) was used to design the primers for putative CAPS candidate.

3.2.7 CAPS genotyping assay

PCR amplification was performed in Eppendorf mastercycler using the PCR program mentioned in Table 3.4. Reaction mix was prepared for 10µl volume with components of 2µlDNA (10ng/µl), 1µlTaqbuffer with $MgCl_2$, 1µl of 10pM primers, 1µl of dNTPs, 0.05µl of Taq (500U/ml) and volume make up with MiliQ water. Amplification was checked by loading the PCR product on 1.5% gel pretreated with 0.5µl/10ml Ethidium Bromide (10mg/ml).

PCR product was subjected to restriction digestion with the corresponding restriction enzyme following the NEB protocol. 6µl of PCR product, 1µl of enzyme specific buffer (provided by the manufacturer), 1 unit of restriction enzyme (*Sfa*NI and *Hpy*AV - 0.5µl) were used per 10µl reaction. The reaction mixture was then incubated in Eppendorf mastercycler at 37 ^{0}C for one hour (enzyme specific) followed by enzyme inactivation at 65^{0}C for 5 minutes. Restriction digested product was then loaded on 3% agarose gel and banding pattern was visualized using the UV-transmitted Gel-doc system.

Table 3.5 PCR conditions for CAPS assay

94^0C for 5 min	Initial denaturation	
94^0C for 20 sec	Denaturation	25 cycles
60^0C for 30 sec	Annealing	
72^0C for 30 sec	Extension	
72^0C for 20 min	Final Extension	
10^0C	Hold	

3.2.7 Identification of candidate gene(s)

Sequence for the genes reported to be associated with herbicide tolerance were taken from the NCBI website. The sequences were selected on the basis of review status of their protein product from Uniprot. These sequences were searched against chickpea genome using BLAST standalone program. Upon the basis of blast results, sequences with high similarity were searched for any known gene annotation. From the data of a recent report by Iquebal *et al.* (2017) the genes were checked for differential expression in herbicide susceptible and tolerant chickpea genotypes.

Chapter IV

RESULTS AND DISCUSSION

Chapter IV

RESULTS AND DISCUSSION

Weeds are one of the major biotic stresses affecting chickpea production. The increasing labor cost and inefficiency of pre-emergence herbicides to control the weeds throughout the cropping period has resulted in a constant search for resistance sources to post-emergence herbicides. With such a goal in mind, present study was aimed at identifying the IMI-tolerant mutant lines by phenotyping under field conditions, and genotyping using a SNP marker.

4.1 Phenotypic characterization for herbicide tolerance

Forty mutant lines generated in the background of JG 11 and KAK 2 were evaluated for herbicide tolerance along with eighty four breeding lines. JG11 and KAK 2 were included as susceptible checks. Mean herbicide scores for different genotypes are presented in the Table 4.1. Out of 124 genotypes, eight genotypes were found to be highly susceptible with phenotypic scoring of five, twenty four genotypes were moderately tolerant with phenotypic scoring of three, and rest of the genotypes were found susceptible with the phenotypic score of four. Imazethapyr was showed to affect both crop growth and reproduction. Genotypes exhibited various phytotoxicity symptoms after the herbicide treatment. The first symptoms were seen in the meristematic tissue (growing tips and young leaves) (Figure 4.1). Death of the plants was observed in highly sensitive genotypes (Figure 4**.**2) with the meristemic tissues dying first followed by slow necrosis of the mature tissues. In moderately tolerant genotypes, the death of epical meristem induced branching, similar to the effects of nipping practice followed in chickpea (Figure 4.3 and Figure 4.4). Other abnormalities observed were – stunted growth, elongation of branches similar to tendrils with very small or needle shaped leaves (Figure 4.5), delaying of flowering, reddish leaves (Figure 4. 6), deformed flowers (Figure 4.7), and poor pod setting. Secondary growth was observed in several lines 20–25 days after herbicidal application leading to flowering and pod set.

As imidazolinones inhibit the synthesis of branched chain amino acids valine, leucine, and isoleucine, there will be rapid decrease in the pool sizes of these amino acids. This leads to decrease in protein synthesis. A lower rate of protein synthesis, in turn causes slowdown in the rate of cell division, and eventually death of the cells. Since mature tissues contain larger pools of amino acids as well as protein reserves which can be catabolized to amino acids upon

protein starvation. Thus, mature leaves take longer to express the phytotoxic effects of the imidazolinones. Apart from decrease in protein, factors such as accumulation of the cytotoxic AHAS substrate 2-ketobutyrate or derivatives , amino acid content imbalance, inhibition of DNA synthesis and cell division, and reduction of assimilate translocation will result in herbicide-induced growth (Duggleby and Pang, 2000).

Table 4.1. Mean herbicide scores for the panel of genotypes.

Entry No	Mean score	Entry No	Mean score	Entry No	Mean score	Entry No	Mean score
1	4	33	4	65	4	97	4
2	4	34	4	66	4	98	4
3	3	35	4	67	4	99	4
4	3	36	4	68	4	100	4
5	4	37	4	69	4	101	4
6	4	38	3	70	4	102	4
7	4	39	3	71	4	103	4
8	4	40	3	72	4	104	4
9	4	41	4	73	4	105	4
10	4	42	4	74	4	106	4
11	4	43	4	75	4	107	4
12	4	44	4	76	4	108	4
13	4	45	5	77	4	109	4
14	3	46	4	78	3	110	5
15	4	47	3	79	3	111	4
16	4	48	3	80	3	112	5
17	4	49	4	81	4	113	4
18	4	50	4	82	4	114	4
19	3	51	4	83	4	115	3
20	4	52	4	84	3	116	4
21	3	53	3	85	3	117	3
22	3	54	3	86	4	118	4
23	4	55	5	87	3	119	4
24	4	56	4	88	4	120	4
25	4	57	3	89	4	121	3
26	4	58	5	90	4	122	4
27	4	59	4	91	5	123	4
28	4	60	4	92	4	124	3
29	4	61	4	93	4	125	4
30	4	62	4	94	4	126	4
31	4	63	4	95	5		
32	4	64	4	96	4		

Figure 4.1 Initial damage occured at the growing tips after herbicide treatment.

Figure 4.2 Highly susceptible line showing complete mortality.

Figure 4.3 Moderately tolerant line with good plant stand and secondary growth.

Figure 4.4 Injured plant exhibiting secondary growth

Figure 4.5 Injured plants showing elongation of branches similar to tendrils with very small, needle shaped leaves.

Figure 4.6 Herbicide treated plants showing reddish leaves.

Figure 4.7 Herbicide treated plants showing deformed flowers.

4.2 KASPar SNP Genotyping

All the genotypes used for the field trial were included for KASPar SNP Genotyping. In total, 124 out of 126 genotypes yielded the fluorescent data. Graphical visualization of the SNP genotyping data using KlusterCaller software showed all the genotypes forming a single cluster near Y-axis corresponding to allele 1 (Allele 'C'), associated with IMI susceptibility (Figure 4.8). None of the genotypes were found to have resistant allele (Allele 'T').

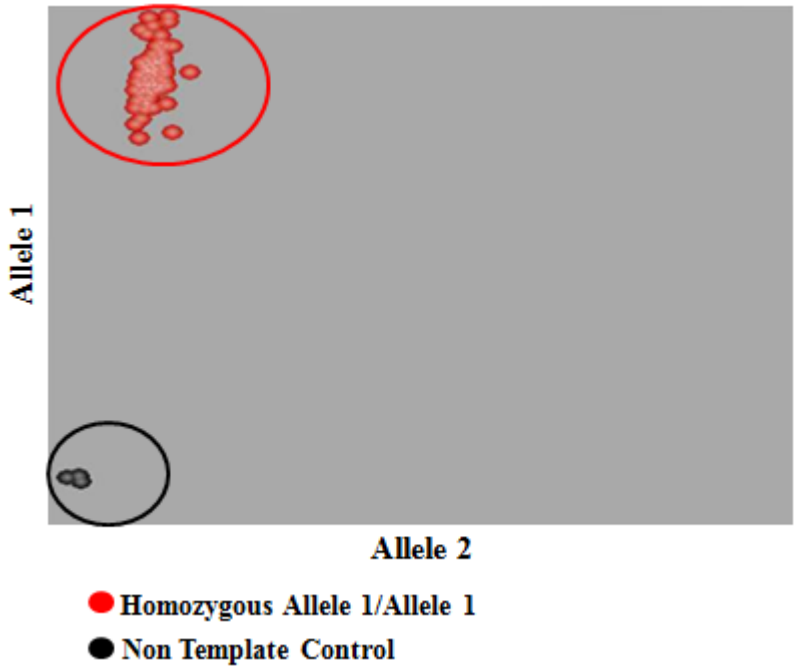

Figure 4.8 Genotyping data plotted using KlusterCaller software showing a single cluster near the X-axis corresponding to susceptible allele 'C'.

4.3 CAPS genotyping assay

4.3.1 Genotyping with CpAHAS-*Sfa*NI

Chickpea AHAS resistant and susceptible genes were aligned (Figure 4.9) and the SNP candidate was converted to CAPS. The SNP2CAPS analysis showed different endonucleases restricting the AHAS sequence and yielding alternate restriction patterns in resistant and susceptible sequence. Based on the availability of the enzymes, *Sfa*NI was selected. The PCR amplification with the respective primers yielded 343bp product. The resistant allele had no restriction sites, and the susceptible allele was restricted at 175bp giving two fragments with sizes 175bp and 168bp. All the genotypes tested for the CAPS yielded similar results as KASPar, showing the presence of susceptible allele 'C'. Since the restricted fragments had small base difference (7bp), they were unresolved in the gel picture forming a single band (Figure 4.10)

```
Ca_AHAS1_S      ATGGATAGTATTCCAATAATCGCTATCACCGGTCAAGTTCCCCGGAGAATGATCGGAACC
Ca_AHAS1_T      ATGGATAGTATTCCAATAATCGCTATCACCGGTCAAGTTCCCCGGAGAATGATCGGAACC
                ************************************************************

Ca_AHAS1_S      GATGCTTTTCAAGAAACCCCCATCGTTGAAGTAACGAGATCCATCACAAAGCATAACTAC
Ca_AHAS1_T      GATGTTTTTCAAGAAACCCCCATCGTTGAAGTAACGAGATCCATCACAAAGCATAACTAC
                **** ********************************************************

Ca_AHAS1_S      CTCATTCTTGAGGTTGATGATATTCCTAGGGTTGTTAGAGAGGCTTTTTTCGTTGCTAAT
Ca_AHAS1_T      CTCATTCTTGAGGTTGATGATATTCCTAGGGTTGTTAGAGAGGCTTTTTTCGTTGCTAAT
                ************************************************************
```

Figure 4.9 Sequence alignment of susceptible and tolerant AHAS genes exhibiting point mutation (C to T).

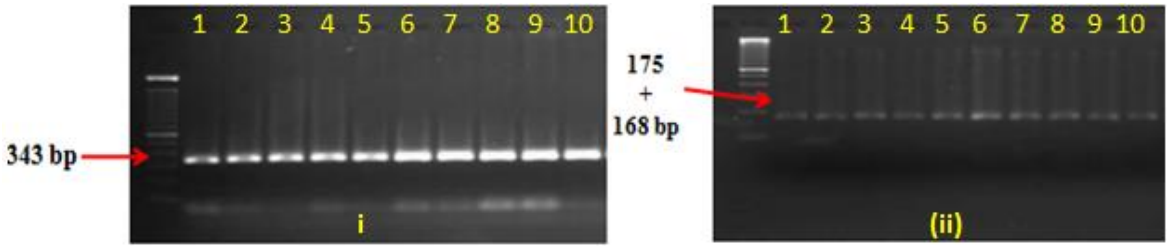

Figure 4.10 Representative Gel picture of genotyping with CpAHAS-*Sfa*NI. (i) PCR amplification (ii) Restriction Digestion

4.3.2 Genotyping with Ca1_AHAS1-*Hpy*AV

Chickpea IMI-Susceptible (IMI-S) consensus and IMI-Resistant (IMI-R) consensus were blasted against CDC Frontier reference genome to identify homologous AHAS1sequences. Two best hits were found on first and fifth chromosomes. The homologous sequence on chromosome-5 was 100% similar to IMI-S consensus and 99.9% similar to IMI-R consensus. The other hit showed 80.6% similarity with both the consensus. These sequences when used as query to find SNP candidates from the available resequencing data of chickpea genotypes, a total of 20 variations were obtained. These variations were compared among the panel of genotypes whose resequencing was done (Table 4.2). Ca1_2268128 SNP candidate was selected for CAPS marker development. *Hpy*AV enzyme was found to restrict the PCR amplicon (654 bp) at 222 bp resulting in two fragments with sizes 432bp and 222bp.

A set of 40 genotypes were chosen for CAPS genotyping using Ca1_2268128-*Hpy*AV marker. This set included checks (JG 11 and KAK 2), all highly susceptible (eight) and moderately tolerant (twenty four) genotypes and six randomly selected susceptible genotypes from the field trial (Table 4.3). PCR amplification and restriction

Table 4.2. Genotypes used for CAPS analysis

Sl. No	Genotype	Sl. No	Genotype
1	AGBL 172	21	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P132
2	IPC 2011-64	22	FLIP01-29C
3	24001-4-1	23	ICCV 14118
4	IPC 2008-89	24	Mutation Breeding - KAK 2 - M2(P)-Bulk-BP-P42
5	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P115	25	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P76
6	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P121	26	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P79
7	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P51	27	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P18
8	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P59	28	AGBL 158
9	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P15	29	ICC 1098
10	GJG 1211	30	ICC 15868
11	24002-4-3	31	ICCV 13309
12	ICC 15618	32	GJG 1304
13	JG 11	33	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P64
14	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P82	34	ICCV 13314
15	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P63	35	IPC 2010-219
16	ICCV 13307	36	KAK 2
17	ICCV 13317	37	IPC 2011-123
18	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P131	38	Mutation Breeding - JG 11 (1) - M2(P)-Bulk-BP-P112
19	AGBL 184	39	IPC 2010-37
20	ICCV 14107	40	GAG 1111

Table 4.3. Allelic variations obtained in resequenced genotypes used in this study for AHAS gene

rs#	Ca1-2268108	Ca1-2268128	Ca1-2268511	Ca1-2268551	Ca1-2268953	Ca1-2269065	Ca1-2269489	Ca5-3788 6311	Ca5-3788 6371	Ca5-3788 6410	Ca5-3788 6416	Ca5-3788 6446	Ca5-3788 6733	Ca5-3788 6995	Ca5-3788 7316	Ca5-3788 7435	Ca5-3788 7628	Ca5-3788 7670	Ca5-3788 7812	Ca5-3788 7849
Alleles	A/G	T/C	A/G	A/T	A/G	T/A	A/C	T/A	G/A	G/A	C/A	A/G	T/C	A/G	C/T	T/C	G/A	A/C	C/T	A/G
Chr	1	1	1	1	1	1	1	5	5	5	5	5	5	5	5	5	5	5	5	5
ICCV92311	N	C	A	A	A	T	A	T	G	G	C	A	T	A	C	T	N	A	C	A
ICCV93954	N	N	A	A	A	T	N	T	N	N	N	A	T	A	C	T	N	A	C	A
ICC1098	N	N	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A
ICC14402	A	T	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A
ICC15618	A	T	A	A	A	T	A	T	G	A	N	A	T	A	C	T	G	A	C	A
ICC15868	A	T	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A
ICC8621	N	A	A	A	A	T	A	N	G	G	C	A	T	A	C	T	G	A	C	A
ICC16207	A	C	A	A	A	T	A	T	G	R	C	A	T	A	C	T	N	A	C	A
ICC3325	A	T	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A
ICC7441	A	T	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A
CDC Frontier	A	T	A	A	A	T	A	T	G	G	C	A	T	A	C	T	G	A	C	A

digestion by the enzyme *Hpy*AV is shown in Figures 4.11 and 4.12 respectively. There was no variation among the genotypes at this locus.

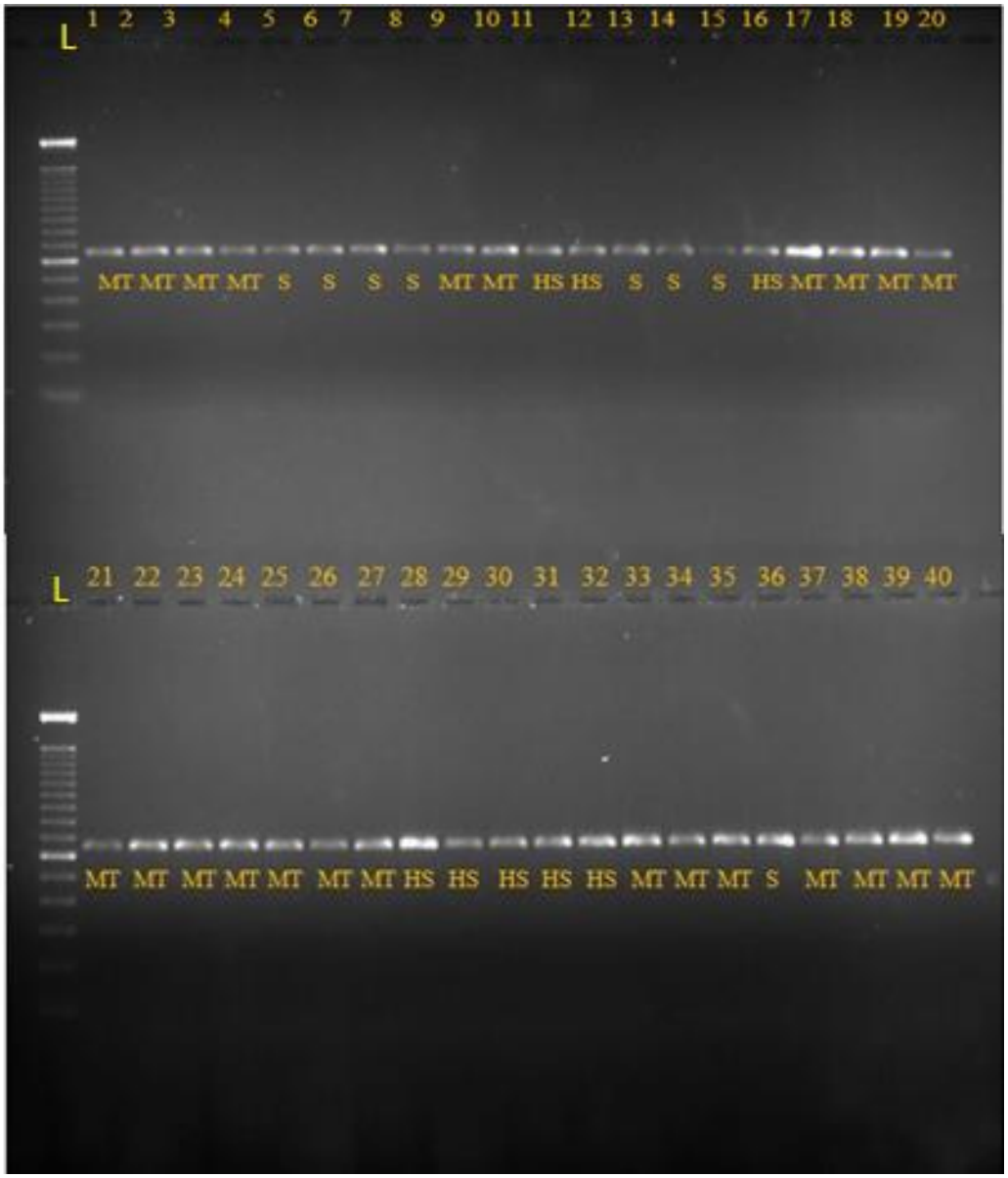

Figure 4.11 PCR amplification with Ca1_AHAS1-*Hpy*AV.
L- 100bp ladder, HS- Highly Susceptible, S- Susceptible, MT- Moderately Tolerant

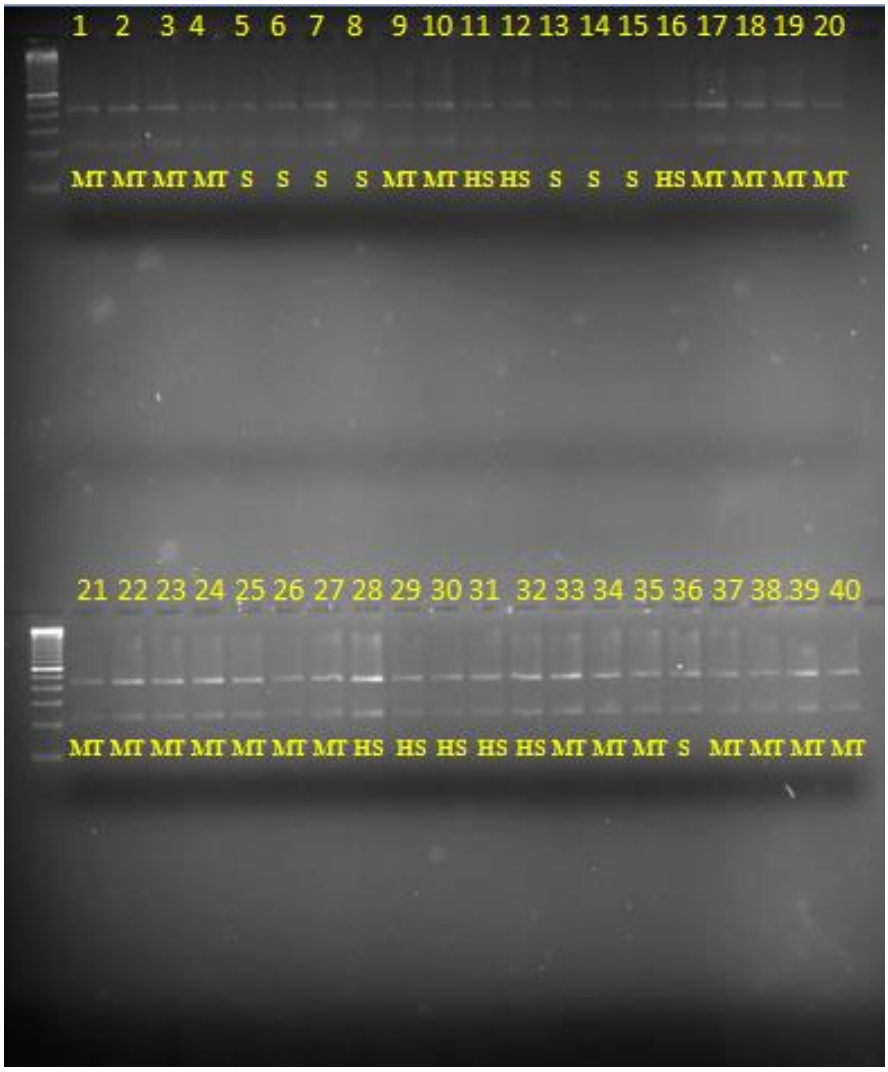

Figure 4.12 Restriction digestion of the PCR product with *Hpy*AV enzyme at 222bp.

L- 100bp ladder, HS- Highly Susceptible, S- Susceptible, MT- Moderately Tolerant

Though all the genotypes had the susceptible allele (allele 'T') for herbicide tolerance, they exhibited differences in the recovery after IMI treatment. This suggests the influence of other factors like ability to metabolize IMI to nontoxic forms, relative expression of the imidazolinone-resistant genes, rate of enzyme turnover, and other fitness traits linked to the ALS gene in plant's ability to recover after IMI treatment (Hanson *et al*., 2006; Hanson *et al.*, 2007).

4.4 Identification of candidate genes

The sequences for the genes enlisted were retrieved from NCBI database. Two gene sequences from tomato, soybean and cotton were selected. The BLAST results revealed the presence of similar sequences in chromosome 1 and 6 in chickpea genome with considerable amount of identity for two genes *viz.,* trans-resveratrol di-O-methyltransferase-like and peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like

respectively, both belonging to soybean genome (Table 4.4). These highlighted regions from the BLAST results were then searched in chickpea genome for known gene annotation. The results showed the presence of similar genes in the highlighted regions *viz.,* trans-resveratrol di-O-methyltransferase-like (XP_012572656) and peroxisomal (S)-2-hydroxy-acid oxidase (XP_004488699). Further these genes showed differential expression pattern between the herbicide tolerant and susceptible chickpea lines (Iquebal *et al.,* 2017)

Table 4.4. Blast results for the other possible candidate genes for herbicide tolerance

Target gene	**Organism**	**Genomic region (Chickpea genome)**			**Identity / Gaps**	**E-value**
		Chr	Start	End		
trans-resveratrol di-O-methyltransferase-like	Soybean	Ca1	41311772	41311448	83%/5%	1e-77
peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	Soybean	Ca6	21618985	21618257	77%/3%	6e-113

Chapter V

SUMMARY AND CONCLUSION

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Since weed management is becoming a major problem in chickpea production due to increased cost of human labor and inefficacy of pre-emergent herbicides, as a step towards identifying tolerant sources for IMI group of herbicides, the present study involving phenotyping, genotyping of a set of mutant lines and breeding lines was taken up. An attempt was also made to identify the putative genes for herbicide tolerance using in-silico approach.

The salient features of the present investigation are summarized here under:

- Forty mutant lines and eighty four breeding lines along with JG 11 and KAK 2 as checks were screened for Imazethapyr tolerance under field conditions at ICRISAT, Patancheru during *Rabi,* 2016-17.
- Phenotyping data for Imazethapyr tolerance was taken as per scale used by Gaur *et al.* (2013).
- Among the 124 genotypes screened, 24 lines showed moderate tolerance, 8 genotypes showed high susceptibility and the rest were found to be susceptible.
- Genotyping of the lines was carried out with a KASPar marker, which was developed to target a point mutation found to confer resistance for IMI-herbicides.
- Genotyping data generated by fluorescence endpoint reading using TECAN microplate reader was graphically viewed and analyzed through KlusterCaller Version 3.4 software.
- Except for two genotypes, fluorescence data was obtained for all other genotypes. These lines were found to possess susceptible allele ‘C’ in homozygous state.
- A CAPS marker was developed by converting this mutation site using SNP2CAPS computer program. Similar results were obtained upon genotyping with this CAPS marker.
- An effort was made to find other possible allelic variation that might be associated with IMI-tolerance. The SNP candidates from the homologous sequences obtained from blast results of AHAS gene were identified and converted to CAPS marker. Genotyping analysis with this marker did not yield conclusive result regarding its association with herbicide tolerance.
- Using in-silico approach of candidate gene identification we found two genes differentially expressing in herbicide tolerant and susceptible genotypes *viz.,* peroxisomal

(S)-2-hydroxy-acid oxidase (XP_004488699) and trans-resveratrol di-O-methyltransferase-like (XP_012572656).

- All the genotypes screened were found to have susceptible allele for the herbicide tolerance. Since they showed varying degrees of susceptibility under herbicide treatment possible influence of other factors like: ability to metabolize the herbicide to nontoxic forms, relative expression of the imidazolinone-resistant genes, rate of enzyme turnover, and other fitness traits linked to the AHAS gene.
- As none of the genotypes used in the study were highly resistant, further screening of a large set of germplasm lines for herbicide tolerance and amplicon sequencing of the AHAS gene in these lines will help in the identification of alternate alleles and the development of diagnostic marker for herbicide resistance.

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APPENDICES

APPENDIX I

Weekly average weather data from October 2013 to March 2014 at ICRISAT, Patancheru

Year	Std Week	Rain (mm)	Evap (mm)	Max Temp (ºC)	Min Temp (ºC)	Rel Humidity1 at 07:17 (%)	Rel Humidity2 at 14:17 (%)	Wind Velocity (kmph)	Solar Radiation (mj/ m2)	Bright Sunshine (Hrs)
2016	45	0	25.3	29.77	14.2	84.28	33.85	2.88	18.09	8.74
2016	46	0	25.8	29.91	16.57	86.14	42.71	3.88	15.54	6.7
2016	47	0	27.69	29.77	11.28	88	30.85	2.84	17.42	8.35
2016	48	0	26.5	30.94	10.72	87.71	28.57	2.29	17.14	8.41
2016	49	0	22.2	29.11	14.61	89.57	47.28	4.18	14.24	6.72
2016	50	1	22.09	28.05	14.18	85.42	46.28	4.67	13.28	6.42
2016	51	0	27.39	29.22	9.88	90	31.42	2.87	17.17	9.11
2016	52	0	28.69	29.3	9.8	88.87	29.5	3.16	16.72	9.22
2017	1	0	26.39	28.71	10.81	88.14	29.42	3.57	16.77	9.28
2017	2	0	27	28.82	13.82	85.42	37.14	5.11	15.31	7.87
2017	3	0	31.1	28.22	12.92	83.85	36	6.05	16.44	7.77
2017	4	0	34.5	29.88	15.95	82.85	36.71	5.79	16.09	7.79
2017	5	0	35.6	31.45	13.11	85.42	31.42	4.75	18.24	8.97
2017	6	0	37.79	32.29	15.19	77	29.14	5.11	18.5	9.14
2017	7	0	43.29	30.94	14.44	79.71	30	7.11	18.8	8.92
2017	8	0	45.3	34.98	14.75	73	19.85	4.04	21.02	10.31

APPENDIX II

Chemical Used:

1. Tris (1M)

Mol. wt. 121.4g; pH- 8

pH adjustment is done with HCl

121.4g in 1000ml

So, 60.57g in 500ml

2. EDTA (0.5M)

Mol. wt. 292.25g pH- 7

292.25g in 1000ml is 1M

So, 146.125g in 1000ml is 0.5M

3. NaCl (5M)

Mol. wt. 58.44g

No pH adjustment

58.44g in 1000ml is 1M

So, 292.20g in 1000ml is 5M

4. CTAB Buffer

Tris(100mM)- 50ml

CTAB (2%)- 5g

NaCl- 1.4mM-140ml

EDTA(2mM)-20ml

β-mercapto ethanol- 200µl

5. Chloroform:isoamyl alcohol(24:1)

Chloroform- 96 ml

Isoamyl alcohol- 4 ml

6. 70% of ethanol (100 ml)

Absolute alcohol- 70 ml and Distilled water- 30 ml

7. 10X TBE(5 ltrs)

Tris- 540g- pH- 7

Boric acid – 275g

EDTA- 37.5g

8. 6X orange loading dye

10 ml 0.5M EDTA (pH 8.0)

1ml 5M NACL

50ml Glycerol

Orange dye powder

APPENDIX III

Preparation of Stock Solutions:

A. 1M TrisHCl (pH- 8.0):

121.14g of 1M Tris base (Tris hydroxyl methyl amino methane; Mol. Wt.- 121.14)was dissolved in about 500 ml distilled water and pH was adjusted to 8.0 using 1N HCl and finally the volume was made to 1000 ml. The solution was filtered and autoclaved. The stock solution was then stored at room temperature.

B. 0.5M EDTA (pH- 8.0):

186.11g of Ethyl Diamine Tetra acetic Acid (EDTA) of mol.wt.372.22 was dissolved in about 500ml of distilled water and the pH of the solution was adjusted to 8.0 with NaOH. Then, the final volume was made up to 1000 ml. This stock solution was then autoclaved and stored at room temperature.

C. TE Buffer (pH 8.0):

121.14g of Tris (1M) and 372.22g EDTA (1M) were weighed and dissolved in 1000 ml distilled water. Then, the required concentration of 1M Tris and 1M EDTA was diluted to prepare 1X TE buffer.

D. 5M NaCl:

292.2g of NaCl (Mol.wt.58.44) was dissolved in 500 ml of distilled water using magnetic stirrer. Next, the final volume was made up to 1000ml by adding sterile distilled water and stored at room temperature.

E. 10X TBE

37.5g of EDTA firstly dissolved in 3.5 litres of distilled water, 540g of Tris and 275g of Boric acid dissolved in EDTA solution and makeup the volume to 5 litres. Buffer is stored in 4°C for further use.

F. 6X orange loading dye:

10ml of 0.5M EDTA (pH 8.0), 1ml of 5M NaCl and 50ml of Glycerol were mixed in 39 ml of distilled water and add the orange powder dye until the colour is sufficient dark.