1.0 Introduction

In vitro techniques have opened new doors in crop improvement. Several tissue culture techniques have been developed in recent years that have commercial application to a wide range of crops. In vitro techniques are used to screen large number of genotypes at their early growth stages for various stresses. This technique is preferred over field experiments because in these experiment -

1. environment is controlled
2. results are more accurate and reproducible
3. require less time and space
4. can be conducted at any time of year

Various types of biotic and abiotic stresses create a hazard to agriculture. These stresses cause huge damage to crops and reduce economic profit all over the world. So to increase crop productivity development of stress tolerant plants and genotypes are of great importance. Traditional breeding methods and proper weed management strategies play an important role in crop improvement. The conventional breeding techniques are being used to incorporate desirable genes by intergeneric and interspecific hybridization into the crops to induce stress tolerance. But little success has been achieved by conventional breeding methods and they are not enough to provide desirable results mainly due to the time and labor requirements (Purohit et al., 1998). Various methods used for increasing and stabilizing production of black gram have helped in developing varieties resistant to disease and pests, with other desirable agronomic traits. However, genetic improvement of black gram by conventional breeding methods has met with limited success due to the lack of sufficient and satisfactory level of genetic variability within germplasm (Jaiwal and Gulati, 1995). Biotechnological techniques can address the serious problems of crop improvement for sustainable agriculture due to short time in which results become obvious.

1.1 In vitro Selection

Abiotic stress factors like drought, salinity, osmotic, heavy metals and pesticides limit plant growth and productivity in agriculture. They cause changes at all levels morphological, physiological, biochemical, and molecular which can negatively affect plant growth and productivity (Wang et al., 2001). Plants have developed mechanisms to withstand and also to adapt in such adverse conditions. Plants can countenance adverse
environmental conditions by regulating specific sets of genes in response to stress signals, whose expression may vary according to severity of stress conditions, other environmental factors, and the plant species (Wang et al., 2003). Among many biotechnological techniques used in plant breeding in vitro selection through the application of selective pressure in culture conditions, for developing stress tolerant plants, have proved to be the most effective approaches (Sakhanokho and Kelley, 2009). It is difficult to investigate the response of plants to different abiotic stresses in the field or in greenhouse conditions, because of complex and variable nature of these stresses. With the help of in vitro techniques we can also better understand the physiology and biochemistry of plants cultured under adverse environmental conditions (Benderradji et al., 2012).

Tissue culture based in vitro selection methods are practical and cost-effective for developing stress-tolerant plants. Plants tolerant to any type of stress can be obtained by adding the selecting agents such as NaCl (for salt tolerance), PEG or mannitol (for drought tolerance), pathogen culture filtrate, phytotoxin or pathogen itself (for disease resistance) and herbicides (for herbicide tolerance) in the culture media. Only the explants capable of tolerating/surviving such modified stressful in vitro environments can express organogenetic phenomenon and can be selected. In vitro selection is based on induction of genetic variation among cells, tissues and/or organs in cultured and regenerated plants (Rai et al., 2011). By in vitro culture of plant cells, tissues and organs on medium containing selective agents, one can select and regenerate plants with desirable characteristics. Tissue culture technique has also been successfully utilized to induce stress tolerance which includes the addition of selection agents that allows the preferential survival and growth of tolerant genotypes (Purohit et al., 1998). There are two types of selection methods: (1) stepwise long-term treatment, in which cultures are exposed to stress with gradual increase in concentrations of selecting agent and (b) shock treatment, in which cultures are directly subjected to a shock of high concentration and only those which would tolerate that level will survive (Purohit et al., 1998). In vitro selection can considerably reduce the time for the selection of desirable characters under selection pressure with least environmental interaction, and can be used in place of field selection (Jain, 2001).

In vitro selection pressure technique is widely used for the selection of genotypes tolerant to abiotic stress which is based on in vitro culture of plant cells, tissues or organs on a medium supplemented with selective agents, allowing selecting and regenerating
plants with desirable characteristics (Pérez-Clemente and Gómez-Cadenas, 2012). The regenerants that survive in the selection pressure under \textit{in vitro} conditions can be characterized at morphological and physiological levels.

Callus culture techniques have a distinct advantage in that tissue cells can be exposed uniformly to a selection pressure incorporated in the medium and many putative mutants can be recovered quickly and efficiently. The variations that have genetic basis can be transmitted through seed in a predictable manner. Callus culture techniques for the development of stress resistant lines in different crops have been utilized by a number of scientists. Many workers reported stress tolerant genotypes /somaclones induced through various types of stresses (Table- 2.2, 2.3).

In the cell culture selection technique, a suitable selection pressure is applied to allow the preferential survival/growth of variant cells. This technique has been successfully utilized to obtain genotypes resistant to various toxins, herbicides, high salt concentration etc. (Zair et al., 2003) (Table-2.3).

Plant tissue culture methods help plant breeders by creating and manipulating genetic variability. \textit{In vitro} methods improve both crop quantity and quality by increasing the plant tolerance against abiotic stresses as they limit crop productivity. Genetic variation in black gram is also required to create cultivars that are highly resistant to these stresses (Abdel-Raheem et al., 2007).

Elevation of the plant antioxidant defense system has been positively correlated with abiotic stress tolerance (Rai et al., 2011) and the same is also true for \textit{in vitro} cultures (Lascano, 2001, Soni et al., 2012). Therefore, by assessing activities of antioxidative enzymes \textit{in vitro}, a speedy preliminary screening of tolerant genotypes could be achieved. It has been reported that the main antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) can help in selection of stress tolerant plants (Hossain et al., 2007).

Lipids play an important role as the structural component of cellular membranes (Parida and Das, 2005). It is well documented that free radical-induced peroxidation of lipid membrane is a symbol of stress-induced damage at cellular level. Therefore, the level of malonyldialdehyde, produced during peroxidation of membrane lipids can be used as an indicator of oxidative damage (Demiral and Turkan, 2005). Selected callus lines of \textit{Solanum tuberosum} subjected to NaCl showed an increase in lipid peroxidation in comparison with salt tolerant lines (Queiros et al., 2007).
Among protective mechanisms used against abiotic stresses, osmotic adjustment is of great importance in which there is accumulation of compatible solutes such as proline, glycine betaine and polyols (Ghoulam et al., 2001). It has been also reported that proline levels increased in response to water stress in tomato calli (Aazami et al., 2010). Increase in proline content has been also reported in response to herbicide stress (Parween et al., 2012, Huang et al., 2012, Fayez et al., 2011, Saladin et al., 2003a and El Tayeb and Zaki, 2009).

Based on review of literature on plants responses to abiotic stress conditions, the determination of antioxidant enzyme activities, and levels of malonyldialdehyde and proline in plants recovered under selective conditions can be used as an important parameter for identifying tolerance among genotypes.

Plant tolerance to abiotic stress has been linked with changes in proteome composition. Since proteins are directly involved in plant stress response, proteomics studies can help us to understand the possible correlation between protein abundance and plant stress acclimation (Kosová et al., 2011). Metabolome analysis is also can be used to study plant metabolic changes that occur in response to abiotic stresses. With the help of metabolome analysis we can identify those compounds that are accumulated by exposure to stress conditions. An integrated approach involving in vitro plant tissue, proteomics and metabolomics technique, may help to explain the metabolites involved in stress response and select desired genotypes at early stages of plant development or at callus stage (Palama et al., 2010). In vitro cell and tissue-culture based systems provide a significant method for studying physiological, biochemical and molecular aspects of stress responses phenomenon and the results are being used for the development and isolation of stress tolerant genotypes. In vitro selected genotypes should be finally field-tested to confirm the genetic stability of the selected traits under field conditions. In vitro selection associated with molecular approaches will provide a new opportunity to improve stress tolerance in important crops related to food production and environmental sustainability. In vitro selection reduces the time considerably for selection of desirable trait under selection pressure. Somaclonal variation associated with in vitro mutagenesis can be beneficial for screening of stress tolerant lines in a short duration using in vitro selection. Genetically stable somaclones, confirmed in field-testing can be used in crop improvement programmes (Patade et al., 2006).

1.2 Agrochemicals
Agrochemicals are various chemical products used in agriculture and it refers to the broad range of pesticides, including insecticides, herbicides, and fungicides. It may also include synthetic fertilizers, hormones and other chemical growth agents, and concentrated stores of raw animal manure. Herbicides and pesticides mostly contributed to the increase in crop productivity in terms of both yield and quality but their excessive and improper use is creating havoc in environment. Some of these compounds persists in plant-derived foods (Lyndon and Darlington, 1998; Jame et al., 1999) and show a harmful effect on both animal and human health (Castro et al., 2005). Most of the research has been focused on the efficacy of herbicides on weeds and/or the secondary effects on crop yield, the toxicity of such substances on non-target species and the subsequent plant responses but areas like screening of herbicide tolerant genotypes in cultivated crops are poorly investigated including pulse crops. The lack of herbicide tolerance for effective weed control has limited its adoption in broad acre farming systems. In order to breed herbicide-tolerant cultivars, a source of resistance needs to be identified. In present study attempts have been made to screen tolerant genotypes of black gram, an important pulse crop in Indian subcontinent to broad spectrum herbicide glyphosate.

1.3 Pulses

Pulses form the major source of protein in the predominantly vegetarian diet of Indian people. They also occupy distinctive position in Indian agriculture as they enrich the soil with nutrients for succeeding crops. It has been realized that pulses are perhaps the only means of solving the protein malnutrition problem in India, as they are generally rich in lysine and contain 20-30% protein on dry basis, which is nearly three times the value found in cereals.

India is the world’s largest producer, consumer and importer of pulses as they are the major protein source in the largely vegetarian Indian diet. Pulses are grown both in the kharif and rabi seasons. With nearly 84 percent of pulse cultivation in rainfed conditions using subsistence agricultural practices, pulse production depends largely on the monsoon and winter rains. Limited varietal improvements, low resilience to soil moisture stress, poor pest resistance, and low input use have contributed to poor yields. Madhya Pradesh, Uttar Pradesh, Maharashtra, Andhra Pradesh and Karnataka together account for about 70 percent of the country’s pulse production, with Madhya Pradesh contributing more than 25 percent. Pulse production has fallen also due to competition from less risky crops like wheat and rice.
Pulse production has not been attractive to farmers due to low government support of improved production technology and a largely ineffective procurement policy.

India contributes about 40-80% of global production of crops like chickpea, pigeon pea, lentil, cowpea, green gram and black gram. The production of pulses in India has been more or less stagnant fluctuating between 10-12 millions tonnes over the last 3 decades. Pulses have played a very important role in human diet in our country, as a source of protein. Because of their high protein content, which varies from 20 to 30%, almost three times that of the cereals, the pulses are an essential supplement of cereal based diet; besides amino acid composition of pulse protein complements that of cereal proteins. The pulse proteins are rich in lysine and poor in sulphur containing amino acid, a reverse situation exists in the cereal proteins. A mixed diet of cereals and pulses has a greater biological value as compared to of either component alone.

1.4 Constraint in Pulse Production in India

Production of major pulses is constrained by both biotic and abiotic stresses. Most of the pulses in India are grown in low fertility, problematic soils and unpredictable environmental conditions. More than 87% of the area under pulses is rainfed. Drought and heat stress may reduce seed yields by 50%, especially in arid and semi-arid regions. Another major problem is salinity and alkalinity of soils which is high both in semi-arid tropics and in the Indo-Gangetic plains. With recent changes in the global temperatures the grain yield is likely to be drastically affected by temperature extremities. Poor drainage/water logging during the rainy season causes heavy losses to pigeonpea on account of low plant stand and increased incidence of Phytophthora blight disease, particularly in the states of UP, Bihar, West Bengal, Chhattisgarh, MP and Jharkhand (Laxmipathi Gowda et al., 2013).

Pulse crops reported huge losses due to biotic (pests and diseases) and abiotic (drought, high temperature, etc) stresses. Some of the studies estimated the losses in the range from 15% to 20% of normal production (IIPR, 2011). This means, India can increase pulses availability by 15% to 20% with investments in appropriate crop protection research and development. As a strategy to cope with this situation, cultivars having combined resistance to most frequent and major biotic and abiotic stress factors need to be developed and adopted by the farmers. The scope for development of multiple resistant varieties has increased after recent advances in genomics and needs to be exploited further. The past success in management of biotic and abiotic stress is encouraging. Instability in yield decreased from 13.6% to 5.5% due to the adoption of
biotic and abiotic stress resistant varieties and adoption of plant protection technologies (Reddy et al., 2013).

The crop plant chosen for the present investigation, *Vigna mungo* [L.] Hepper, is a member of family Fabaceae, sub-family Papilionaceae. The family includes herbs, shrubs, trees and climbers which are cosmopolitan in distribution. It is second largest family among dicots, and is represented by more than 550 genera and about 1300 species. Hutchinson, (1973) treated Caesalpiniaeae, Mimosaceae and Papilionaceae as families. According to Engler and Prantl, (1897-1915) all these three are sub families of Leguminosae. Lawrence and others, who treated them as sub-families, opine that characters which distinguish one sub-family from the other are more apparent than the characters which bind them together. Sub-family Papilionaceae is the largest and includes 375 genera. Lawrence, (1951) divided it into ten tribes, on the basis of testa topography. This sub-family is characterized by gamosepalous calyx, papilionaceous corolla and diadelphous condition (9+1) of stamens.

1.5 *Vigna mungo* (Black gram)

1.5.1 Origin and Distribution

Black gram is considered to have been domesticated in India from its wild ancestral form (*V.mungo* var. *silvestris*) (Lukoki et al., 1980). Center of genetic diversity is found in India (Zeven and de Wet, 1982). Natural distribution of *Vigna mungo* var. *silvestris* ranges from India to Myanmar (Tateishi, 1996). *Vigna mungo* occupies about 14 % of total area under pulse production in India and ranks fourth in area and production after *Cicer arietinum*, *Cajanus cajan*, and *Vigna radiata*. In India *Vigna mungo* occupies about 3.17 million tones. It is a highly prized pulse and cultivated under a wide range of predominately rainfed farming system in dry and intermediate agro ecological zones on marginal lands with low moisture and fertility conditions. It is grown in several parts of Asia and Africa, mainly in countries like India, Bangladesh, Pakistan, Burma and Ceylon. In India it is grown all over the country, as kharif (summer) crop in northern India and as rabi (winter) crop in southern India with total area of about 24 lakh hectares.

Black gram belongs to the subgenus Ceratotropis in the genus Vigna. The genus Vigna, together with the closely related genus Phaseolus, forms a complex taxonomic group, called Phaseolus-Vigna complex. Verdcourt, (1970) proposed a very restricted concept of Phaseolus, limiting it exclusively to those American species with a tightly coiled style and pollen grains lacking course reticulation, hence, promoting significantly
the concept of *Vigna*. Marechal et al., (1978) followed Verdcourt and presented a monograph on the *Phaseolus-Vigna* complex. Their taxonomic system is generally accepted now. Verdcourt, (1970) proposed that these two species should be treated as a single species. However, Marechal et al., (1978) considered these two as distinct species and his proposal was supported by many taxonomists. Two botanical varieties were recognized in *Vigna mungo*. *Vigna mungo* var. *mungo* is the cultivated form (black gram); var. *silvestris* is the wild ancestral form of black gram (Lukoki et al., 1980). The somatic chromosome number of *Vigna* is 2n=22.

### 1.5.2 Plant Characteristics

*Vigna mungo* is climbing, prostrate or erect herb or sub shrub, rarely small shrubs. Leaves are pinnately trifoliolate; stipules bilobed or spurred at the base or not spurred, stipels generally present. Inflorescence axillary or terminal, falsely racemose or flowers in dense 1-many flowered subumbellate clusters; rachis usually thickened and glandular at the point of insertion of pedicels; bracts and bracteoles deciduous. Calyx 5-lobed, 2-lipped; upper lip of 2 lobes completely or partly united, lower lip 3-lobed. Vexillum with inflexed auricles and 2-4 appendages or rarely appendages absent; keel truncate, obtuse or beaked, sometimes the beak incurved through up to 360°. Stamens diadelphous 9+1, anthers uniform, ovary 3-many ovuled; style with tenuous lower part obsolete or quite long, filiform or flattened, upper part thickened and cartilaginous, straight or curved, upper portion barbate or hirsute on inner side, sometimes produced beyond the stigma to form a short to long subulate or rarely flattened or capitate beak; stigma completely lateral, oblique or rarely terminal. Pod linear or linear oblong, cylindrical or flattened, straight or curved. Seeds mostly reniform or quadrate; aril obsolete to well developed, often 3-pronged. Black gram is an annual food legume. It shows both erect and crawling growth habit. There are several distinct characters between black gram and mungbean. Flower color of black gram is bright yellow, while that of mungbean is pale yellow. Pocket on the keel, which is a characteristic of the subgenus *Ceratotropis*, of black gram is longer than that of mungbean. Pod of black gram is shorter than that of mungbean. Pod of black gram attaches upright to the peduncle, while mungbean pod attaches sideward or downward to the peduncle. In most cases, seed color is dull black. However, shiny black and shiny green seeded black gram is also cultivated in Nepal. The area of traditional cultivation of black gram is confined to the South Asia and adjacent regions (India, Pakistan, Afghanistan, Bangladesh and Myanmar). Black gram is cooked as "dhal soup" (split dehusked bean soup) in South
Asia and adjacent regions. Black gram is used in making delicious curry, sweets, idlis, etc.

1.5.3 Environment and Ecology

Black gram requires a warm and humid climate. Its growth and development is proper when atmospheric humidity is 50 -70 % with a temperature of 20 -25°C. A region with annual rainfall above 500 mm is quite suitable for its cultivation. It can be grown in a wide range of climate. It grows successfully from sea level to an elevation of 1800 metres or more. Black gram comes up in areas receiving annual rainfall of 600-1000 mm. It is a short-day plant and most cultivars flower in 12-13 hrs photoperiod. Flowering gets delayed with extended photoperiod. As elevation increases, flowering is delayed due to low ambient temperature. It is grown on a variety of soils ranging from sandy to heavy black cotton soils. It prefers water retentive heavy soils. Well drained heavy soils with a pH ranging from 5.0 to 7.5 appear to be ideal. It can't withstand saline and alkaline conditions. Black gram can be cultivated through out the year both as rainfed and irrigated crop. It is predominantly a rainfed crop during rainy and post rainy seasons.

1.5.4 Natural Benefits and Curative Properties of Black Gram

Black gram is demulcent or soothing and cooling agents. It is an aphrodisiac and nerve tonic. However, excessive use of black gram causes flatulence which can, however, be prevented by adding little asafoetida, pepper and ginger in the culinary preparations. It should not be taken by those who are easily predisposed to rheumatic diseases and urinary calculi as it contains oxalic acid in high concentration.

- **Diabetes** - Germinated black gram, taken with half a cupful of fresh bitter gourd juice and a teaspoonful of honey is highly beneficial in the treatment of milder type of diabetes. It should be used once daily for three to four months with restriction of carbohydrates. Even in severe cases, regular use of this combination, with other precautions, is useful as a health giving food for the prevention of various complications that may arise due to malnutrition in diabetic patients.

- **Sexual Dysfunction** - Black gram dhal soaked in water for about six hours and then fried in pure cow's ghee. After draining the water is an excellent sex tonic. It can be used with wheat bread and honey with highly beneficial results in functional impotency, premature ejaculation and thinness of the semen.
• **Nervous Disorders** - The above preparation eaten with half boiled egg is an excellent tonic in nervous disorders such as nervous weakness, weakness of memory, schizophrenia and hysteria.

• **Hair Disorders** - Washing the hair with a paste of cooked black gram dhal and fenugreek lengthens the hair, keeps them black and cures dandruff.

• **Digestive System Disorders** - Black gram is valuable in digestive system disorders. In the form of decoction, it is useful in dyspepsia, gastric catarrh, dysentery and diarrhea.

• **Rheumatic Afflictions** - A liniment made from black gram is useful as an external application in rheumatism, contracted knee and stiff shoulder. It is prepared by boiling about 4 kg of black gram pulse in 38.4 litres of water. It should be boiled down to about 9.6 litres and strained. The strained decoction should be boiled with about 2.5 litres of sesame oil and 1/2 kg of rock salt till the water has been evaporated. Paste of the fresh root is also useful in rheumatic pains.

1.6 **Effect of Pesticides on Non-Target Plants**

   Plant species that are by mistake exposed to pesticides are treated as non-target plants. Effect on a non-target plant may be direct or indirect causing significant change in the survival, health or reproduction. Pesticides cause damage to crops and vegetation growing near agricultural lands. In agricultural area, herbicide drift-related problems have been reported for many years. Non-target plant may be variously affected including vegetative growth changes, plant death, altered reproductive capability that can generally result in reduced fitness, and detrimental economic or ecological impacts.

   Increased impacts to non-target vegetation may result from increased herbicide usage for weed control in crops, roadsides, railroads, and industrial sites, and because of drift and run-off from non-point sources. Sensitivity to pesticides may differ among species. The major basis for the tolerance of crops to most herbicides is differential rates and routes of herbicide metabolism. Due to the selection pressure caused by pesticides many plant species led to the natural evolutionary development of tolerant plant genotypes in response to a wide range of air- and soil-borne agrochemical pollutants. Many plant species are known to either possess or lack the genetic variability for tolerance of agrochemicals. Differential susceptibility to the nonselective herbicides can allow the establishment of resistant species and the elimination of susceptible ones along with their competitive effects, altering the species composition drastically (Dickinson et al., 1991). Tolerant species and
tolerant biotypes of herbaceous plants are favored by natural selection in pesticide-contaminated environments, due to their ability to survive or else to competitively exclude non-tolerant plants. In many herbaceous plants, the selection pressure of pollution has led to the natural evolutionary changes (Karthikeyan et al., 2003).

Tolerant plant genotypes develop in response to wide range of herbicides. Sensitive species exhibit marked changes in the vegetative growth and reproductive performance when exposed to pesticides. The seedling stage is the most sensitive period for most species, although surviving plants exposed to pesticides at later stages may show considerable effects on reproduction. Many annual or colonizing plants show rapid selection for tolerance. However, the scenario for trees and other long-lived perennials is different. They may respond to agrochemical pollution in the same way they would respond to other temporary or fluctuating stresses such as drought.

1.7 Weeds

Weeds are the plants, which grow where they are not wanted. They grow in the fields where they compete with crops for water, soil nutrients, light and space and thus reduce crop yields (ICAR, 1997). Weeds are divided into terrestrial and aquatic types on the basis of their habitats. Nature has bestowed upon weeds a good many characters that make them a better competitor in the crop-weed competition for water, soil, nutrients, space, etc.; weed seeds germinate earlier; their seedlings grows faster; they flower earlier; form many seeds; mature ahead of the crop they infest; and weed seeds can germinate under varied conditions. Besides, seeds of a weed are easily dispersed along with the produce of crop. Weed seeds have very long dormancy period, which is an intrinsic physiological ability of the seed to resist germination even under favorable conditions, and also the seeds do not lose their viability for years even under adverse conditions. Weeds are, therefore, a major reason of huge crop losses.

1.8 Weed Control Methods

1.8.1 Manual and Mechanical Control Techniques

Manual and mechanical techniques such as pulling, cutting, and otherwise damaging plants, may be used to control some invasive plants, particularly if the population is relatively small. These techniques can be extremely specific, minimizing damage to desirable plants and animals, but they are generally labor and time intensive. They are often used in combination with other techniques, for example, when shrubs are pulled and cut, and re-sprouts and seedlings are treated with herbicides or fire several weeks or months later (Tu et al., 2001).
1.8.2 Cultural or Cropping Methods

Many farming practices are capable of changing the condition in such a way as to enable the crop plants to compete with weeds successfully or to reduce their interference to the minimum and thus preventing them from acting as impediments to increased crop production. Seeds with good germination give the crop a vigorous and close stand and enable it to outgrow weeds. The crop varieties which are well adapted to a region compete better with the weeds than varieties poorly adapted to it. Plant breeders try to evolve quick-growing and short-duration varieties of crop plants with a large leaf area and good branching, and agronomists work out the proper seed-rate, depth, time and method of sowing, applying the most effective methods of irrigation and fertilizers and adopt a proper system of rotation. Some crops can compete better with weeds than others. For instance crops like Sudan grass, sorghum and cowpea are good competitors while crops, such as linseed, groundnut and lentil are poor competitors. The raising of highly competitive crops (also known as smother crops) is useful in reducing weed infestations. One disadvantage from which many smother crops suffer is that their seedlings grow slowly. The cultural cropping methods are probably the most sensible control methods available.

1.8.3 Chemical Methods

The use of herbicides or weedicides to selectively remove weeds is the efficient chemical control method. Chemical weed control is suitable in situations and under conditions where manual or mechanical weeding is difficult. A great advantage of this method lies in killing weeds in the crop row or in the immediate vicinity of crop plants. The chemical method is easier, less time-consuming and less costly than weeding by hired labourers.

1.8.4 Biological Methods

It involves the use of living agents as a method of control, and is exercised through the use of natural pests or pathogens. The living agents could be insects, bacteria, fungi, or animals such as sheep, goats, cattle or horses. Biological weed control plays an important role in managing parasitic weeds (Sauerborn et al., 2007).

1.9 Herbicide

The chemical control of weeds is accomplished by the use of herbicides. Herbicides belong to a group of chemicals known as pesticides. Herbicides are chemicals that specifically kill or inhibit plant growth by altering the normal plant metabolism.
1.9.1 Classification of Herbicides

Herbicides can be classified on the basis of their selectivity, time of application or mode/mechanism of action (Table-1.1) (Streibig, 2003).

Table-1.1: Classification of herbicides with reference to their use and action on plants

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of selectivity</td>
<td>Selective</td>
</tr>
<tr>
<td></td>
<td>Non-selective</td>
</tr>
<tr>
<td>Time of application</td>
<td>Pre-sowing</td>
</tr>
<tr>
<td></td>
<td>Pre-emergence</td>
</tr>
<tr>
<td></td>
<td>Post-emergence</td>
</tr>
<tr>
<td>Method of application</td>
<td>Foliage</td>
</tr>
<tr>
<td></td>
<td>Soil</td>
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<tr>
<td>Translocation in plants</td>
<td>Systemic</td>
</tr>
<tr>
<td></td>
<td>Contact</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Photosynthesis</td>
</tr>
<tr>
<td></td>
<td>Auxin action</td>
</tr>
<tr>
<td></td>
<td>Amino acid metabolism</td>
</tr>
<tr>
<td></td>
<td>Microtubules</td>
</tr>
<tr>
<td></td>
<td>Lipid metabolism</td>
</tr>
</tbody>
</table>

Herbicides can be categorized into **selective** or **non-selective** types on the basis of selectivity. Selective herbicides are those that kill various weed species without causing significant damage to crop species (Marrs, 1996), while non-selective herbicides remove a wide range of vegetation. On the basis of time of application herbicides can be **pre-emergent** or **post-emergent** types: Post-emergent herbicides are applied to the foliage of the plant, where as pre-emergent herbicides are sprayed on the soil before sowing and before the weed germinates. Pre-emergent herbicides either disrupt germination or kill the germinating seedling. The herbicides applied to the foliage can be **contact herbicides** or **translocated herbicides**. Contact herbicides (e.g. dicryl, potassium cyanate, Solan, Propanil) kill only the plant or portions of the plant that comes into contact with the chemical. Translocated herbicides or systemic herbicides (e.g. Caebine, 2, 4-DB, MCPB, 2, 4-5-T) move within the plant, and therefore, effective in
destroying the roots of perennial weeds. Herbicides are unique in that they are designed to kill plants. Sufficiently high doses will kill both crop and weed, while small doses have no effect upon crop and weed. The action of a herbicide is usually determined by its chemical and physical properties, its effect on plant metabolism, the plant and the environment.

1.9.2 Mode of Action of Herbicides

Herbicides target key enzymes in the plant metabolic pathway, which disrupt plant food production and eventually kill it. The time and method of application of herbicide is many a time determined by its mode of action (Tu et al., 2001). On the basis of which they are categorized into following types:

**Auxin Mimics:** They mimic plant growth hormone auxin, and cause uncontrolled and disorganized growth in susceptible plant species. The susceptible plants exhibit symptoms called ‘auxin overdose’. The application of this class of herbicides leads to a rapid loss of H+ ions (protons) from the cell wall, which makes the wall more elastic, and results in measurable cell growth. Grasses and other monocots are generally not susceptible to auxin-mimic herbicides. This difference may be due to differences in vascular tissue structure or differences in ability to translocate or metabolize the herbicide (Di Tomaso, 1999). Some of the commonly used auxin mimics are 2,4-D, clopyralid, picloram, and triclopyr.

**Mitosis Inhibitors:** They inhibit mitosis through direct interaction with tubulin (Duke, 1990). They are also known as dormancy enforcers. They prevent new growth in summers. Fosamine is the most commonly used mitotic inhibitor.

**Photosynthesis Inhibitors:** There are two types of photosynthesis inhibitors. One class of inhibitors blocks the transfer of electrons in photosystem II. They block electron transport from QA to QB in the chloroplast thylakoid membrane by binding to the D-1 protein. These compounds cause chloroplast swelling, membrane leakage, ultimately leading to cellular destruction. Hexazinone belongs to this class of inhibitors. It is absorbed from soil solution by plant roots and transported to the site of action in the chloroplasts of the plant. The second type of photosynthesis inhibitors accepts electrons from photosystem I and after several cycles generate toxic hydroxyl radicals. The hydroxyl radicals destroy unsaturated lipids and chlorophyll. This leads to the destruction of cell membrane integrity, cell leakage, leaf wilting and desiccation, and eventually to plant death (WSSA, 1994). Paraquat and diquat are the prime examples of this class of herbicides.
Amino Acid Synthesis Inhibitors: Three enzymes of amino acid biosynthesis constitute important sites of herbicide action: 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP synthase), acetolactate synthase (ALS), and glutamine synthetase (GS). They are firmly established as the primary sites of action of several important herbicide classes (Duke, 1990). Glyphosate [N-(phosphonomethyl) glycine] inhibits EPSP synthase, an enzyme in the shikimic acid pathway for aromatic amino acid biosynthesis. Blockage of this enzyme leads to massive accumulation of shikimate, and scarcity of the three aromatic amino acids required for protein synthesis. This leads to the killing of plants. Three different herbicide groups, the sulfonylureas, imidazolinones (Kishore and Shah, 1988), and the 1,2,4-triazol [1, 5A] pyrimidines (Kishore and Shah, 1988), inhibit the ALS enzyme. ALS is a key enzyme in the branched chain amino acid pathway that produces leucine, isoleucine, and valine. All of these compounds inhibit ALS by slow, tight-binding kinetics (Duke, 1990). GS is the first enzyme involved in assimilating inorganic nitrogen to produce an amino acid. It converts L-glutamic acid to L-glutamine in the presence of ammonia and ATP. Bialophos is a potent GS inhibitor.

Lipid Biosynthesis Inhibitors: Aryloxyphenoxy alkanoic acids and cyclohexanediones inhibit acetyl-CoA carboxylase, the enzyme responsible for catalyzing early step in fatty acid synthesis. The inhibition of acetyl-CoA carboxylase, and the subsequent lack of lipid production leads to losses in cell membrane integrity, especially in meristems. Fluazifop-p-butyl and sethoxydim are the two commonly used herbicides, which block acetyl-CoA carboxylase.

1.9.3 Effect of Herbicide Overdose on Plants

Biochemically, herbicide overdose could lead to redox imbalance in the cell by the production of reactive oxygen species (ROS), leading to oxidative stress, DNA damage, lipid peroxidation in plasma membranes, etc, ultimately leading to cell death (Rutherford and Krieger-Liszkay, 2001; Beligni and Lamattina, 1999; De Prado et al., 1999; Matters and Scandalios, 1986). Nemat Alla and Hassan, (2006) reported enhanced accumulation of H2O2, lipid peroxides and carbonyl groups in the shoots of 10-day-old maize line Giza2 indicating an induced oxidative stress in maize following atrazine treatments. The treatment with chlorotoluron induced the accumulation of O2· and H2O2 in leaves and resulted in the peroxidation of plasma membrane lipids in the wheat plants (Song et al., 2007).

Plants have evolved several mechanisms through which they can tolerate lethal doses of herbicides. Plants have different mechanisms of herbicide uptake and herbicide
detoxification. Different enzyme systems have evolved in plants to protect them from herbicide-induced oxidative stress, or to conjugate herbicides and subsequent sequestration in the vacuoles. Besides these enzyme systems, some small carbohydrate molecules like sucrose and glucose (Sulmon et al., 2004; Sulmon et al., 2007) also confer herbicide tolerance in plants. Sulmon et al., (2004 and 2007), reported that sucrose and, to a lesser extent, glucose conferred a high level of tolerance to atrazine in *Arabidopsis thaliana* seedlings, with maintenance of chlorophylls, carotenoids, and D1 protein, and protection of photosystem II. Sulmon et al., (2007) showed that sugar-induced atrazine tolerance in *A. thaliana* seedlings also involved the activation of ethylene signalling pathways. The study also showed that sugar and atrazine activated the sugar signaling pathways, resulting in derepression of hexokinase-mediated glucose repression and induction of protection mechanisms against atrazine injury.

### 1.9.4 Herbicides and Oxidative Stress

Oxidative stress is induced by a wide range of environmental factors including UV stress, pathogen attack (hypersensitive reaction), herbicide exposure, and oxygen shortage. Oxygen deprivation stress in plant cells is distinguished by three physiologically different states: transient hypoxia, anoxia and reoxygenation (Blokhina et al., 2002). Generation of reactive oxygen species (ROS) is characteristic for hypoxia and especially for reoxygenation. Of the ROS, hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) are both produced in a number of cellular reactions, including the iron-catalysed Fenton reaction, and by various enzymes such as lipoxygenases, peroxidases, NADPH oxidase and xanthine oxidase. The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids (Blokhina et al., 2002). The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols) (Janiszowska and Korczak, 1980) and enzymes regenerating the reduced forms of antioxidants. In plant tissues many phenolic compounds (in addition to tocopherols) are potential antioxidants: flavonoids, tannins and lignin precursors may work as ROS-scavenging compounds. Plants also have evolved ROS-interacting enzymes viz. superoxide dismutases (SOD), peroxidases (POX) and catalases (CAT). All these help plants tolerate herbicide-induced oxidative stress. These enzymes are involved in normal plant growth and metabolism, and also protect plants from endogenously produced oxidative stress during the plant metabolism. Other enzymes involved in oxidative stress metabolism are glutathione peroxidase (GPOX),
ascorbate peroxidase (APOX), polyphenol oxidase (PPO), glutathione transferase (GST) etc.

**Catalase (H₂O₂: H₂O₂ oxidoreductase; EC 1.11.1.6)**

Catalase is a tetrameric iron porphyrin that catalyzes the dismutation of H₂O₂ to water and oxygen. CAT, together with SOD and hydroperoxidases, make up a defense system for the scavenging of superoxide radicals and hydroperoxides (ROS) (Beyer and Fridovich, 1987).

**Peroxidase (EC 1.11.1.7)**

Peroxidases are a large family of enzymes that typically catalyze kind of

\[ \text{ROOR'} + \text{electron donor (2 e^-) + 2H}^+ \rightarrow \text{ROH + R'}\text{OH} \]

reactions. Most of the peroxidases utilize H₂O₂ as a substrate; however, some are more active with organic hydroperoxides such as lipid peroxides. Peroxidases are now known to be closely associated with general oxidative stress metabolism in plants, and so also in xenobiotics metabolism.

**Superoxide Dismutase (EC 1.15.1.1)**

Superoxide dismutases (SOD; EC 1.15.1.1) react with superoxide radicals to produce hydrogen peroxide. The three known types of SOD are classified by their metal cofactor: the copper/zinc (Cu/Zn SOD), manganese (Mn SOD), and iron (Fe SOD) forms (Bowler et al., 1992). SOD has often been correlated with herbicide, particularly paraquat, tolerance. The effects of paraquat on SOD enzyme activity in plants have been studied in several cases. The treatment of *Phaseolus vulgaris* (Chia et al., 1982) and *Lemna* (Srivastave and Tel-Or, 1991) leaves with paraquat caused a general increase in SOD activity.

### 1.10 Lipid Peroxidation /MDA Content

Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under various stresses. During lipid peroxidation, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, MDA, etc and compounds related to them (Garg and Manchanda, 2009). Some of these compounds react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS) (Heath and Packer, 1968). LPO, in both cellular and organelle membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals (Montillet et al., 2005). However, O₂⁻ and H₂O₂ are capable of initiating the
reactions but as OH⁻ is sufficiently reactive, the initiation of LPO in a membrane is initiated by the abstraction of a hydrogen atom, in an unsaturated fatty acyl chain of a polyunsaturated fatty acid (PUFA) residue, mainly by OH⁻ In an aerobic environment, oxygen will add to the fatty acid at the carbon-centered lipid radical to give rise to a ROO⁻. The resulting lipid hydroperoxide can easily decompose into several reactive species including: lipid alkoxyl radicals, aldehydes (malonyldialdehyde), alkanes, lipid epoxides, and alcohols (Davies, 2001; Fam and Morrow, 2003). The overall effects of LPO are to decrease membrane fluidity; make it easier for phospholipids to exchange between the two halves of the bilayer; increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Gill and Tuteja, 2010).

1.11 Proline Content

Proline is considered as a osmolyte, potent antioxidant and potential inhibitor of programmed cell death. It is a nonenzymatic antioxidant that organisms use to mitigate the adverse effects of ROS (Chen and Dickman, 2005). Free proline acts as an osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of LPO, and OH⁻ and ¹⁰₂ scavenger (Ashraf and Foolad, 2007; Trovato et al., 2008). Thus proline is not only an important molecule in redox signaling, but also an effective quencher of ROS formed under abiotic stress conditions in all plants (Gill and Tuteja, 2010).

1.12 Herbicide Tolerance

Herbicide tolerance can be defined as the inherent ability of plant to survive and reproduce with a herbicide treatment at a normal use rate (Vargas and Wright, 2004). In other words tolerance is the ability to compensate the damaging affects of the herbicide with no physiological mechanisms involved (Menalled and Dyer, 2006). According to WSSA, (1998) herbicide tolerance is defined as “the inherent ability of a species to survive and reproduce after herbicide treatment”, without being selected or genetically engineered.

Researchers postulated that weed management could be simplified by spraying a single broad-spectrum herbicide over the field anytime during the growing season. Herbicide-tolerant (HT) crops offer farmers a vital tool in fighting weeds and are compatible with no-till methods, which help preserve topsoil. They give farmers the flexibility to apply herbicides only when needed, to control total input of herbicides and to use herbicides with preferred environmental characteristics.
1.13 Glyphosate

**Common Name:** Glyphosate

**Chemical Name:** N-(phosphonomethyl) glycine

**Common Product Names:** Accord®, Rodeo®, Roundup®, Roundup Pro®

Trade names for products containing glyphosate include Gallup, Landmaster, Pondmaster, Ranger, Roundup, Rodeo, and Touchdown. It may be used in formulations with other herbicides.

**Formulations:** Commercial glyphosate products generally contain one or more inert ingredients. An inert ingredient is anything added to the product other than the active plant-killing ingredient. The names of inert ingredients are not usually listed on the label. The contents of roundup formulation, used in the present investigation, are listed below:

- **Roundup®-glyphosate (41.0%);** related organic acids of glyphosate (1.5%); isopropylamine (0.5%); polyethoxylated tallow amine surfactant (15.4%) and water (41.6%) (Monsanto, 2005).

Glyphosate is a post-emergent, systemic and non-selective (or broad-spectrum) herbicide used in both agricultural and non-agricultural areas. Recommended application rates do not exceed 5.8 kg active ingredient per hectare (a.i./ha) (WHO, 1994). It is used to kill all plant types including grasses, perennials, and woody plants. It is mainly absorbed into the plant through the leaves and then transported throughout the plant where it acts on the plant’s enzyme system. It acts as a potent inhibitor of shikimic acid pathway for biosynthesis of aromatic amino acids. It is a competitive inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase with respect to phosphoenolpyruvate (PEP) and noncompetitive with respect to shikimate-3-phosphate (S3P) (Coruzzi and Last, 2002). The inhibition of EPSP synthase leads to killing of plants due to scarcity of the three aromatic amino acids viz. phenylalanine, tyrosine and tryptophan. This pathway exists in higher plants and micro-organisms but not in animals.

Glyphosate is a weak organic acid. Its chemical name is N-(phosphonomethyl) glycine. It is usually formulated as the isopropylamine or trimethylsulfonium salt of glyphosate. Other ingredients known as inerts or additives are also added to the formulation. A surfactant (wetting agent) known as polyoxyethylene amine (or POEA), which helps the active ingredient penetrate the plant surface, is usually added to glyphosate formulations. Other additives include sulphuric and phosphoric acids. The main breakdown product or metabolite of glyphosate is aminomethyl phosphonic acid (AMPA).
1.13.1 Target Plants

Glyphosate is used to control grasses, herbaceous plants, including deep rooted perennial weeds, brush, some broadleaf trees and shrubs, and some conifers. Glyphosate does not control all broadleaf woody plants. Timing is critical for effectiveness on some broadleaf woody plants and conifers.

1.13.2 Breakdown in Soil and Groundwater

Glyphosate is moderately persistent in soil, with an estimated average half-life of 47 days (Wauchope et al., 1992; WSSA, 1994). Reported field half-lives range from 1 to 174 days. It is strongly adsorbed to most soils, even those with lower organic and clay content. Thus, even though it is highly soluble in water, field and laboratory studies show it does not leach appreciably, and has low potential for runoff (except as adsorbed to colloidal matter) (Wauchope et al., 1992). One estimate indicated that less than 2% of the applied chemical is lost to runoff. Microbes are primarily responsible for the breakdown of the product, and volatilization or photodegradation losses will be negligible (WSSA, 1994).

1.13.3 Breakdown in Water

In water, glyphosate is strongly adsorbed to suspended organic and mineral matter and is broken down primarily by microorganisms. Its half-life in pond water ranges from 12 days to 10 weeks (USEPA, 1992).

1.13.4 Breakdown in Vegetation

Glyphosate may be translocated throughout the plant, including to the roots. It is extensively metabolized by some plants, while remaining intact in others (Kidd et al., 1991).

1.13.5 Mode of Action

Glyphosate is a broad-spectrum, systemic, post-emergence herbicide that is phloem mobile and is readily translocated throughout the plant (Franz et al., 1997). From the leaf surface, glyphosate molecules are absorbed into the plant cells where they are translocated to meristematic tissues (Laerke, 1995). Glyphosate’s primary action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a chloroplast-localized enzyme in the shikimic acid pathway of plants (Della-Cioppa et al., 1986). This prevents the production of chorismate which is required for the biosynthesis of essential aromatic amino acids. These acids are used by plants in protein synthesis and to produce many secondary plant products such as growth promoters, growth inhibitors, phenolics, and lignin (Franz et al., 1997). In forestry, glyphosate is frequently applied to
release small coniferous plants from the harmful effects of competition with undesirable vegetation such as grasses, broadleaf weeds, and woody shrubs. Unlike many contact herbicides, phytotoxic symptoms of glyphosate injury often develop slowly. Visible effects on most annual weeds occur within two to four days and may not occur for 7 days or more on most perennial weeds. Extremely cool or cloudy weather following treatment may slow activity of glyphosate and delay development of visual symptoms. Visible effects are a gradual wilting and yellowing of the plant which advances to complete browning of above-ground growth and deterioration of underground plant parts.

The effect of glyphosate on plants, its toxicity and tolerance, has been studied mainly in relation to EPSP synthase (Sandermann, 2006; Lorraine-Colwill et al., 2002; Forlani et al., 1992; Dyer et al., 1988, Yuan et al., 2002; Shyr et al., 1992; Pline-Srnic, 2006). Glyphosate-resistant transgenic plants harboring EPSP synthase have also been made (Arnaud et al., 1998; Zhou et al., 2006).

1.14 Objectives of the Present Study

The research conducted on in vitro screening for tolerant genotypes in Vigna mungo has following objectives:
1. Screen black gram genotypes for herbicide tolerance using in vitro methods.
2. To study the effect of glyphosate on the morphological parameters of six genotypes of Vigna mungo like germination and survival percentage, seedling length, fresh weight to monitor the response of genotypes of Vigna mungo to herbicide stress.
3. Identify parameters that could be used as criterion/index for selection of herbicide tolerance.
4. To establish efficient protocol for callogenesis and regeneration of Vigna mungo testing different media and hormones.
5. To screen tolerant genotypes by various in vitro methods.
6. To find out if glyphosate also induces the activity of antioxidant enzymes, like catalase, peroxidase and SOD, and effect of same on total protein, proline content, chlorophyll content MDA content/(lipid peroxidation) in genotypes of Vigna mungo.
7. Selection of most suitable/appropriate method for the screening of tolerant genotypes.
2.0 Review of Literature

There is paucity of reports on the screening of herbicide tolerant genotypes in *Vigna mungo* and other pulses while there is ample work done on screening of tolerant genotypes for various stresses in other crops (Table- 2.2). But there is no report to the best of my knowledge for selection /screening of herbicide tolerant genotype in pulse crops.

2.1 *Vigna mungo*: An Introduction

Black gram (*Vigna mungo* L. Hepper), popularly known as urd bean, urid or mash is an important self-pollinating diploid grain legume and belongs to the family Leguminosae and subfamily Papilionaceae. An important food legume crop of the Indian subcontinent comprising of India, Burma, Bangladesh, and Sri Lanka (Kumar et al., 2009) and it is rich in protein content (Green and Layman, 1972). Black gram is considered to have been domesticated in India from its wild ancestral form *V. mungo* var. *silvestris*. Center of genetic diversity is found in India (Zeven and de Wet, 1982). The chromosome number of this crop is 2n=2x=22.

2.1.1 Origin and Distribution

Black gram is supposed to have originated in India as evidenced from vedic literature and from India it spread to other countries. In Sikkim two forms of urd are present, (i) Large seeded, early maturing black seeded types (kalodal) which belongs to *Vigna mungo* var. *mungo* (ii) small seeded late-maturing types with colours varying from brown, olive green grey (panhelo dal) to mottled which belongs to *Vigna mungo* var. *viridis*. Black gram is considered to have been domesticated in India from its wild ancestral form *V. mungo* var. *silvestris*, (Lukoki et al., 1980). Center of genetic diversity is found in India (Zeven and de Wet, 1982) whose distribution ranges from India to Myanmar (Tateishi, 1996).

Black gram is distributed mainly in tropical to sub-tropical countries where it is grown mainly in summer season. It is grown in India, Pakistan, Sri-Lanka, Burma, and some countries of South East Asia. In India black gram is very popularly grown in Andhra Pradesh, Bihar Madhya Pradesh, Maharashtra, Uttar Pradesh, West Bengal, Punjab, Haryana, Tamil Nadu and Karnataka with an area of about 3.29 million ha and a total production of 1.60 million tones, with an average productivity of 485 kg/ha. Andhra Pradesh leads with the highest productivity followed by Orissa. ‘Kalodal’ or ‘Panhelo
dal’ as it is known in Sikkim, is extensively cultivated in all the dry belts of South and West districts of Sikkim. In Sikkim total area under urd cultivation is 3.55 thousand hectare production is 2.78 thousand Tonnes and productivity is 783.10 kg/ha.

2.1.2 Uses

Its seeds are highly nutritious with protein (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins. Seeds are used in the preparation of many popular dishes. It is one of the most important components in the preparation of famous south Indian dishes, e.g. dosa, idli, vada etc., it adds about 42 kg Nitrogen per hectare in soil. Protein content in black gram and its fractions range from 12 to 42%, while fat content range from 0.9 to 3.4%. Germ had the highest content of fat and protein, while seed coat and plumule fractions had the lowest (0.9%). Seed coat had the highest dietary fiber content (78.5%) while cotyledon had the lowest (24.4%). Seed coat, plumule and aleurone layer enriched in seed coat extracts show antioxidant potential and good α-glucosidase inhibitory activity (Girisha et al., 2012).

Table 2.1: Composition of black gram

<table>
<thead>
<tr>
<th>Minerals and Vitamins</th>
<th>Food Value* (%</th>
<th>Minerals and Vitamins</th>
<th>Food Value* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>10.9</td>
<td>Calcium</td>
<td>154 mg</td>
</tr>
<tr>
<td>Protein</td>
<td>24.0</td>
<td>Phosphorus</td>
<td>385 mg</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>59.6</td>
<td>Iron</td>
<td>9.1 mg</td>
</tr>
<tr>
<td>Fat</td>
<td>1.4</td>
<td>Vitamin B-complex</td>
<td>Little</td>
</tr>
<tr>
<td>Minerals</td>
<td>3.2</td>
<td>Calorific value:</td>
<td>374</td>
</tr>
<tr>
<td>Fibre</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Value per 100 g edible portion

2.1.3 Taxonomy

Black gram belongs to the subgenus Ceratotropis in the genus Vigna. The genus Vigna, together with the closely related genus Phaseolus, forms a complex taxonomic group, called Phaseolus-Vigna complex (Verdcourt, 1970). Verdcourt proposed a very restricted concept of Phaseolus, limiting it exclusively to those American species with a tightly coiled style and pollen grains lacking course reticulation, hence, promoting significantly the concept of Vigna. According to his proposal, black gram and its
relatives (which is now recognized as the subgenus Ceratotropis) were transferred to the genus Vigna from the genus Phaseolus. Marechal et al., (1978) followed Verdcourt and presented a monograph on the Phaseolus-Vigna complex. Their taxonomic system is generally accepted now. Taxonomic treatment of black gram and mungbean (V. radiata) has been confused. Verdcourt, (1970) proposed that these two species should be treated as a single species. However, Marechal et al., (1978) considered these two as distinct species and his proposal was supported by many taxonomists. Two botanical varieties were recognized in V. mungo. V. mungo var. mungo is the cultivated form (black gram), var. silvestris is the wild ancestral form of black gram (Lukoki et al., 1980).

### 2.1.4 Plant Characteristics

Black gram is an annual food legume. It shows both erect and crawling growth habit. There are several distinct characters between black gram and mungbean. Flower color of black gram is bright yellow, while that of mungbean is pale yellow. Pocket on the keel, which is a characteristic of the subgenus Ceratotropis, of black gram is longer than that of mungbean. Pod of black gram is shorter than that of mungbean. Pod of black gram attaches upright to the peduncle, while mungbean pod attaches sideward or downward to the peduncle. In most cases, seed color is dull black. However, shiny black and shiny green seeded black gram is also cultivated in Nepal.

### 2.1.5 Botany

Like green gram, black gram is an annual, semi-erect to spreading herb growing to a height of 25-90 cm. Stems are diffuse, branching sometimes procumbent, and covered with long dense brown or black hairs. It possesses strong tap root system with many laterals. Leaves are pinnately trifoliolate, hairy with large ovate to lanceolate and entire leaflets. Flowers are pale yellow, small with a yellow spirally coiled keel. The flowers are borne in clusters of 5-6 on a short hairy peduncle in axillary racemes. Pods are short, erect to sub erect, 4-7 cm long and 0.6 cm wide, brown to black in colour, hairy and with stout hooked beak, containing about 6-10 seeds. Seeds are small, oblong slightly truncated at ends measuring 4-5.2 mm long and 3.5-4.1 mm wide; thousand seeds weight is around 40 g with varying colour from black, dark brown to green. The testa is smooth and hilum white and concave. Pods do not shatter readily. Flowers are self-fertile and self pollinated. Flowering is indeterminate.

### 2.1.6 Environment Requirement

As tropical crop black gram tolerates high temperature, it is cultivated both in kharif and summer seasons. It needs warm weather and due to the same reason the crop
is grown during rainy season and summer season in North India. However in central parts it is grown during rainy as well as winter season. It is a short-day plant, but day neutral cultivars are available for cultivation in the long-days of summer. The optimum temperature for better growth ranges between 25 to 35°C but it can tolerate up to 42 °C. It is a hardy and drought resistant plant, and can be grown in areas receiving moderate to low rainfall. It is sensitive to cloudy weather and cannot tolerate frost. It requires heavier and water retentive soil in places where rainfall in scanty, but in humid regions it is mainly grown in uplands. It is grown mostly as rainfed crop and relayed with maize in the drier areas in Sikkim. It is also grown on paddy field bunds. The crop is grown in the lower and mid-hill up to an elevation of 1400 meters. Though it is grown on a variety of soils ranging from sandy to black cotton soils but the most ideal one is the well drained loam soil. It can also be grown on saline and alkali soils as it tolerates slight alkalinity.

2.1.7 Cultivation

2.1.7.1 Planting Time

Black gram is basically a hot season crop exhibiting tolerance to higher temperature and susceptibility to cold and frost. In northern parts of the county, where the temperatures during winter are quite low, it is cultivated only during the rainy season. i.e. from middle of June to middle of July. However, in the eastern states it is also grown during winter. In the central and southern states where there is not much variation in climate, it is cultivated both during summer and rainy season. Despite the slight variation in optimum date of sowing during different seasons depending on agro-climatic zone, variety and soil conditions, sowing between mid-June to mid-July is found to be optimum time for kharif season. Early planting in first week of July results in higher yield and any delay in sowing beyond this date causes reduction in yield. The optimum time for sowing the summer crop is during March. In Sikkim the optimum time of sowing is during the months of July to August.

2.1.7.2 Weed Management

The first 4-5 weeks after sowing is critical for crop weed competition i.e. 30 days after sowing. The extent of damage due to weeds can be as high as 50-60%. Good seedbed preparation, one or two manual weeding within days after sowing may effectively control the weeds. For years farmers have been fighting against their farm pests: rodents, insects, micro-pathogen and weeds that tolled heavy crop yield losses dwelling a segment of world population from malnutrition to starvation. As research based knowledge accumulated, concepts of modern farm technologies shaped up and
new choices replaced the older cost inefficient farm practices. Attempts were made to kill the pests by spraying pesticides and herbicides.

Glyphosate became a prominent herbicide in agriculture about 18 years ago when it was discovered that glyphosate resistance genes could be inserted into crops using biotechnology (Clark, 2012). Now, glyphosate resistant corn, cotton, soybeans, canola and sugar beets are common. Glyphosate being broad spectrum can kill most or all unwanted weeds while crops remain unharmed.

2.2 *Vigna mungo*: Weeds and Herbicides

Adoption of proper and timely weed management practices plays a pivotal role in increasing crop yields as weed causes severe loss due to competition with crop for essential growth factors like nutrient, moisture, space, light etc. Weeds act as host and thereby they intensify the problems of disease, insect and other pest. Weed management influences production, eco-environment and sustainability in pulse production. Pest management in general and weed management in particular, has tremendous scope for increasing food production. Modern agriculture largely depends on herbicides for chemical weed control, Saxena et al., (1976) reported that initial four to six weeks were the most critical for weed competition.

In India weeds offer serious competition to blackgram, yield losses may be upto 75-100%. This problem is more critical under rain fall when weeds grow luxuriantly and it also hinders the physical weeding operations. This is the reason why chemical control of weeds is of great importance in our country (Gupta and Choudhary, 1986). Like any other crop urd bean is also affected by various diseases, pests and weeds. The most common weeds in urd bean fields are broadleaf weeds and grass weeds. Buckwheat (*Fagopyrum* Munch.), wild oat (*Avena fatua* L.), paradoxa grass (*Phalaris paradoxa* L.), smartweed and dandelion (*Taraxacum officinale* Weber) predominantly affect urd bean fields. Nandan et al., (2012) reported that predominant weed flora in urdbean fields comprised of *Echinochloa colona* (80%), *Cynodon dactylon* (15%) and *Cyperus rotundus* (5%) in monocots whereas among dicot weeds, *Commalina bengalensis* (75%) and *Ageratum conozoides* (15%) were predominant. They also tested efficiency of pre and post emergent herbicides on weed flora of urd bean under rainfed subtropical Shiwalik foothills of Jammu and Kashmir. The weed free treatment produced the highest seed yield and was at par with imazethapyr 250 ml/ha (post-emergence) after 15–20 days sowing. However, among the other treatments, pendimethalin (pre-emergence) 1.0 kg/ha
at 30 DAS was found superior in controlling the weed flora and increasing the seed yield. Unweeded check produced the lowest seed yield.

Das et al., (1990) reported that imazethapyr at 0.150 kg was found to be superior to the other herbicide treatments in reducing weed populations and weed DW. This treatment also gave the highest grain yield in black gram (*Vigna mungo*) cv. T-9. Shekhawat et al., (2002) reported maximum plant height, yield attributes and grain yields of maize and black gram when metolachlor at the rate of 1.0 kg/ha was applied. Rathi et al., (2004) investigated that low dose of pendimethalin (0.5 kg/ha) followed by one hand weeding done at 60 days after sowing demonstrated intended weed control (67.80% WCE), enhanced higher grain yield (379 kg/ha or 119.49%) in Central Uttar Pradesh. Mayfield and Presser, (1988) found that crop topping with paraquat or glyphosate is an effective strategy for controlling ryegrass seed set in some pulse crops without yield loss of the crop.

Singh, (2011) reported that pendimethalin 0.45 kg/ha+hand weeding (HW) 25 days after sowing (DAS) gave maximum yield in summer season in blackgram. Soni and Singh, (1988) found that among the weed control methods two hand weeding and fluchloralin 1 kg/ha per-plant incorporation in soil recorded higher yield in *Vigna mungo*. Ishaya et al., (2008) found that mixture of metolachlor and prometryn at 1.25+0.08 kg a.i./ha was consistent in better growth and seed yield. However, application of mixtures of metolachlor and terbutryn at 0.99+0.50 kg a.i./ha and ametryn and terbutryn at 1.60+1.00 kg a.i./ha gave lower vigour score of the crop, higher crop injury, smaller canopy spread and lower seed yield. It can be concluded that the metolachlor and prometryn mixture gave good selective weed control in cow pea.

Singh and Singh, (1988) evaluated the effects of pre-emergence herbicides (1.0 or 1.5 kg/ha alachlor or 0.75 kg pendimethalin) or manual weeding (at 20 ± 35 d after sowing) for the control of weeds like *Echinochloa* spp., *Ageratum conyzoides*, *Celosia argentea*, *Euphorbia hirta*, *Panicum* spp., *Achyranthes aspera* and *Xanthium strumarium* in *Vigna mungo* cv. T-9 on sandy loam. The herbicides reduced weed DW from an untreated value of 1890 to 890-1210 kg/ha., whereas manual weeding once and twice resulted in 280 and 110 kg DW of weeds, respectively. All treatments increased *V. mungo* seed yields from 235 to 445-695 kg/ha; the highest value resulted from manual weeding twice, but pendimethalin also gave high yields (680 kg).

Shaukat et al., (1980) evaluated tolerance of *Vigna radiata* and *Vigna mungo* to chlorophenoxy acetic acid, chlorophenoxy butyric acid and triazine herbicides. They
suggested that prometryne is the safest herbicide for weed control in *V. mungo* and *V. radiata*.

The efficiency, suitability, selectivity, toxicity and overdose effects of many herbicides on various *Vigna* spp. have been studied. Soltani et al., (2005) studied the effects of some post-emergent herbicides (bentazon, fomesafen, sethoxydim, quizalofop-

*p*-ethyl, imazamox plus fomesafen and imazamox+bentazon) on the growth and yield of adzuki bean (*Vigna angularis*). They recommended that fomesafen, sethoxydim, quizalofop-

*p*-ethyl and imazamox +fomesafen are better suited for weed management in adzuki beans.

O'Makinwa and Akinyemiju, (1990) also demonstrated that a pre-emergence application of 3 kg a.i./ha of a metobromuron+metolachlor mixture was most suitable to control *Euphorbia heterophylla* L. weed in two cowpea cultivars (Ife-Brown and TVx 3236). However, its post-emergence application was lethal to cowpea. Alachlor at 2.5 and 5.0 kg a.i./ha applied pre- and post-emergence was also ineffective for the control of *E. heterophylla*. The herbicide dimethazone severely affected the growth and chloroplast development in cowpea (Duke and Paul, 1986). The affected plastids lacked thylakoids, had irregular envelopes, and contained numerous vesicles. Duke and Kenyon, (1986) demonstrated that dimethazone affected chloroplast development, but it did not have a direct effect on photosynthesis. Almas et al., (2005) studied the effects of glyphosate, metribuzin, fluchloralin and 2,4-dichlorophenoxy-acetic acid (2,4-D) on plant vigor, nodulation, photosynthetic pigments, N content, seed yield and protein content in seeds. The pre-emergence application of the four herbicides, at 2 μg a.i./g of soil, adversely affected these parameters. The maximum increase of 12.5% in seed yield occurred at 0.5 μg a.i./g of glyphosate. However, metribuzin at 0.5 and 1 μg a.i./g decreased the seed yield by 33.3 and 55.5%, respectively. Glyphosate at 0.5 μg a.i./g increased the number of nodules formed per plant by 12.5% and 14.3% at 35 and 60 days after sowing. Metribuzin (0.5 and 1 μg a.i./g) and the higher rates of 2 μg a.i./g of glyphosate, fluchloralin and 2,4-D significantly reduced the nodulation (nodule number and dry mass). The maximum grain protein (24%) was obtained for glyphosate at 0.5 μg a.i./g while minimum grain protein was obtained at 0.5 (10%) and 1 μg a.i./g (8%) of metribuzin application. The study showed that among the herbicides tested, metribuzin showed significant phytotoxicity to the crop. However, the other three herbicides could be used with caution and at appropriate doses.
Mansoor et al., (2004) evaluated the efficacy of various weed management strategies in mung bean. Hand weeding, water extracts of sorghum, *Eucalyptus* and *Acacia*, and a pre-emergence herbicide pendimethalin were compared to assess the utility of each of these three methods for weed control in mung bean. All the treatments significantly affected number of branches per plant, number of pods per plant, 1000 grain weight and grain yield. It was found that the water extract of *Acacia* was most suited, followed by hand weeding and pendimethalin treatment. Dungarwal et al., (2003) conducted a study to determine the most effective herbicidal treatment for controlling weeds in mung bean variety K-851. The herbicides and doses tested were: pre-emergence application of alachlor (1.5 or 2.0 kg/ha), pendimethalin (0.75 or 1.00 kg/ha), metolachlor (0.75 or 1.00 kg/ha), pre-plant incorporation of trifluralin (0.75 or 1.00 kg/ha) and fluchloralin (0.75 or 1.00 kg/ha), and controls. The higher rates of alachlor, pendimethalin, trifluralin, fluchloralin and metolachlor were more effective in reducing weed density and weed dry matter and produced higher mung bean yields than their lower rates. Among the herbicide treatments, 2 kg alachlor/ha recorded the lowest weed density and weed dry matter, followed by trifluralin and pendimethalin (both at 1.0 kg/ha). It was most effective in reducing crop-weed competition, producing a mean yield of 791 kg/ha. Felton, (1979) demonstrated that bentazone herbicide treatment significantly reduced the mung bean yield. Bentazone influenced yield most by reducing the number of pods per plant; there was also a significant reduction in the number of seeds per pod. The grain size was reduced as well with increased and repeated rates of bentazone treatment.

Kaushik and Inderjit, (2006) evaluated the phytotoxicity of three herbicides with different modes of action: atrazine, a photosystem II inhibitor; metsulfuron, an acetolactate synthase inhibitor; and isoxaflutole, a 4-hydroxyphenylpyruvatedioxygenase inhibitor. The parameters studied were shoot height, chlorophyll concentration and cellular damage. It was seen that isoxaflutole inhibited shoot growth and chlorophyll concentration of the mung beans. However, atrazine and metsulfuron did not cause reduction in the shoot growth. Metsulfuron (226, 452, 1356 and 2260 μg/kg soil) and isoxaflutole (452, 1356 and 2260 μg/kg soil) in soil reduced the concentration of leaf chlorophyll.

Watanabe et al., (2001) examined oxyfluorfen-induced lipid hydroperoxide content and changes of fatty acids constituting the neutral lipid in mung bean cotyledons. Oxyfluorfen induces lipid peroxidation, which, in turn, leads to oxidative
stress. The continuous oxidative stress caused by oxyfluorfen exposure led to the destruction of cell membranes. The results of the study demonstrate that oxyfluorfen-induced oxidative stress disrupts the regulation of the biosynthesis of different fatty acid species in mung bean. Parani, (1999) studied the effects of herbicide Machete on growth and metabolism of seedlings of V. radiata and V. mungo. Windley et al., (1999) conducted a screening of some broadleaf herbicides for post-emergent use in mung bean. Nehru et al., (1999) evaluated the effect of some herbicides on post-emergence and seedling vigour. Ameri et al., (2012) surveyed effects of different doses of pursuit herbicides on weed control and yield and yield components of two cultivars of cowpea in city of Ahvaz, Iran. They recommended that for pre-emergence, after complete weeding treatment, Pursuit 1 liter treatments per hectare is best treatment. Singh et al., (2012) assessed the comparative efficacy of salicylic acid in combination with different concentrations of pendimethalin on black gram (Vigna mungo). Seed germination and seedling growth decreased under increasing pendimethalin concentrations. Root length also reduced significantly. Herbicide treatment remarkably declined pigment, protein and sugar contents of the seedlings when compared with control. Total antioxidants and malondialdehyde (MDA) content increased significantly in pendimethalin treated seedlings. Rao et al., (2010) found that pre emergence sand mix application of pendimethalin 1000 g ha$^{-1}$ followed by imazethapyr 50 g/ha at 20 days after sowing (DAS ) significantly reduced weed growth and recorded the highest seed yield (1113 kg /ha) in black gram.

2.3 Glyphosate

The herbicide properties of glyphosate [N-(phosphonomethyl) glycine] were discovered by Monsanto in 1970. Glyphosate was first introduced as a commercial herbicide in 1974 with the trade name Roundup™, and is now it is the most important and the most widely used herbicide in the world (Franz et al., 1997; Baylis, 2000; Powles and Preston, 2006). Glyphosate is a post-emergent, systemic, nonselective, broad-spectrum herbicide that controls annual and perennial weeds and crops in a wide range of conditions. It was initially used as a non-crop and plantation crop herbicide but now widely used for selective weed control in transgenic glyphosate-tolerant crops, such as soybean (Glycine max L.), cotton (Gossypium hirsutum L.), canola (Brassica napus L.), and corn (Zea mays L.) (Baylis, 2000; Shaner, 2000, Woodburn, 2000) and also in non transgenic crops.
Glyphosate is a simple zwitter ionic amino acid which is formulated as many different salts like roundup which is formulated as isopropylamine salt of glyphosate. It is applied as a foliar spray because it tightly binds to soil components and has little or no activity in soil. Glyphosate is easily degraded by several soil microbes (Duke et al., 2003). Glyphosate salts are highly polar, water-soluble molecules with low lipophilic character that probably penetrate the overall lipophilic cuticle via diffusion through a hydrophilic pathway (hydrated cutin and pectin strands) into the apoplast (Caseley and Coupland 1985; Franz et al., 1997). Absorption of glyphosate by plant cells through the plasma membrane into the symplast is a slow process and involves a passive diffusion mechanism, and also an active transport mechanism (phosphate carrier) (Caseley and Coupland, 1985; Franz et al., 1997). Glyphosate is rapidly translocated in most plants. It readily enters the symplast, and its translocation takes place through phloem like source and sink relationship of solute transport (Perez-Jones, 2007).

2.4 Herbicide Resistance and Tolerance

Herbicide resistance is an induced inherent ability of some plant species to survive and reproduce after receiving a lethal dose of herbicide (Prather et al., 2000). In contrast herbicide tolerance can be defined as the inherent ability of plant to survive and reproduce with a herbicide treatment at a normal use rate (Vargas and Wright 2004). In other words tolerance is the ability to compensate the damaging affects of the herbicide with no physiological mechanisms involved (Menalled and Dyer, 2006). The level of tolerance to commonly used herbicides in cereal and pulse varieties has been found to vary between cultivars. The variation in tolerance may be due to any combination of differences in morphological or physiological traits among the varieties (Zerner et al., 2014).

The mechanisms that are involved in herbicide resistance can be categorized into two: (A) target site-based resistance, and (B) nontarget site-based resistance. In target site-based resistance there is a modification of the site of action in such a way that the herbicide has reduced affinity and no longer binds to the altered target enzyme. This results from a single nucleotide change (i.e., mutation) in the gene encoding the enzyme to which the herbicide binds (Devine and Shukla, 2000; Preston and Mallory-Smith, 2001). Target site-based resistance is applied in case of those herbicides that inhibit photosynthetic electron transfer at photosystem II (Gronwald, 1994), acetyl CoA carboxylase (ACCase) (Délye et al., 2005), acetolactate synthase (ALS) (Tranel and Wright, 2002), and tubulin polymerization (Yamamoto et al., 1998). However in
nontarget site-based resistance there is elimination of the herbicide molecule from the target site due to differential uptake and/or translocation, sequestration, or increased metabolic detoxification. Many weed species like blackgrass (De Prado and Franco, 2004), rigid ryegrass (Preston, 2004), and downy brome (Bromus tectorum L.) (Park et al., 2004), have evolved resistance to several herbicides due to increased rates of herbicide detoxification.

Many enzymes are involved in the metabolic detoxification of herbicides, like glutathione transferases, the aryl acylamidases, and the cytochrome P-450 monooxygenases. Decreased translocation and decreased penetration of the herbicide to the active site, as well as increased sequestration of the herbicide in the vacuole, have been suggested as the mechanisms of resistance of barley grass (Hordeum glaucum Steud.) to paraquat (Devine and Preston, 2000; Preston and Mallory-Smith, 2001).

2.5 Mechanism of Glyphosate Action and Tolerance

Glyphosate inhibits the enzyme 5- enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) (Steinrucken and Amrhein, 1980, Amrhein et al., 1980). EPSP synthase is the sixth enzyme of the shikimic acid pathway, which is essential for the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, and also for the production of numerous aromatic secondary metabolites (e.g., auxins, phytoalexins, anthocyanins, and lignin) (Kishore and Shah, 1988); tetrahydrofolate, ubiquinone, and vitamin K (Gruys and Sikorski, 1999). EPSP synthase catalyzes the conversion of shikimate-3- phosphate and phosphoenol-pyruvate (PEP) to yield EPSP and inorganic phosphate (Geiger and Fuchs, 2002). Reduction in aromatic amino acid pools is accompanied with the increase in shikimic acid. This increase in shikimic acid has been related to a decline in carbon fixation intermediates (e.g., ribulose bisphosphate) and a reduction of photosynthesis (Duke et al., 2003). The shikimic acid pathway, present in plants and microorganisms, is completely absent in mammals, fish, birds, reptiles and insects. The inhibition of EPSP synthase leads to killing of plants due to scarcity of the three aromatic amino acids. EPSPS inhibition results in bleaching, chlorosis, and stunted growth of plants. These symptoms are mainly concentrated in metabolically active sink tissues such as immature leaves, shoot tips, buds, and roots (Franz et al., 1997). The yellowing from exposure to glyphosate starts with the older leaves and moves toward the younger leaves.
Rubin et al., (1982) found that activities of enzymes of aromatic amino acid biosynthesis i.e. shikimate dehydrogenase, chorismate mutase, and aromatic aminotransferase, prephenate dehydrogenase and arogenate dehydrogenase were all insensitive to inhibition by glyphosate but 95% inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP synthase-Co) was found in the presence of glyphosate. Shikimate accumulation and in vivo inhibition of both 5-enolpyruvylshikimic acid 3-phosphate synthase and one of the two DAHP synthase isozymes was found in glyphosate-treated seedlings. Amrhein et al., (1983) reported that Aerobacter aerogenes and cultured cells of the higher plant Corydalis sempervirens growing in 10 mM glyphosate showed a 10–30-fold increase in amounts of shikimic acid-3-phosphate and/or shikimic acid. Pinto et al., (1988) studied that glyphosate induces 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase in potato (Solanum tuberosum L.) cells grown in suspension culture. Forlani et al., (1992) proposed that a glyphosate-resistant 5-enol-pyruvyl-shikimate-3-phosphate synthase confers tolerance to a maize cell line to
glyphosate at concentrations as high as 10 mM. Tolerance to the herbicide was correlated with the reduced affinity of the enzyme for the substrate phospho-enol-pyruvic acid.

Milligan et al., (2001) elucidated that the expression of a maize glutathione S-transferase gene in transgenic wheat confers herbicide tolerance, both in plant and in *vitro*. Yuan et al., (2002) suggested triple mechanisms of glyphosate-resistance in a naturally occurring glyphosate-resistant plant *Diciptera chinensis*. They found that glyphosate treatment caused a significant increase in the EPSPS mRNA and protein level but the increase is not due to gene amplification. They also suggested possible posttranscriptional regulation. Ye et al., (2001) reported that plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plastid-expressed EPSPS could provide very high levels of glyphosate resistance, although levels of resistance in vegetative and reproductive tissues differed depending on EPSPS accumulation levels, and correlated to the plastid abundance in these tissues.

### 2.6 In *vitro* Screening for Stress Tolerance

While making selections under the field conditions, plant breeders do not have precise tools, to select the desirable traits. It takes several years and many generations to accomplish the goal. *In vitro* selection can shorten considerably the time for the selection of desirable traits under *in vitro* selection pressure with minimal environmental interaction, and can complement field selection. Table-2.2 lists *in vitro* selection for tolerance to herbicide, disease resistance, and abiotic stress in different crop plants. There are several types of stresses that can be applied in plant tissue culture to obtain different types of objectives like selection of tolerant genotypes for various stresses (Table -2.2), assess the effect of various stresses on cell metabolism and cultures and select tolerant callus/cell lines for different stresses (Table-2.1).

In the present work we investigated the *in vitro* methods of screening for herbicide tolerance in *Vigna mungo* genotypes. Moreover, to the best of our knowledge, this is the first report in which the screening of glyphosate tolerant genotypes has been done. The *in vitro* effects of glyphosate on seed germination, plant morphology, callus induction plant regeneration ability antioxidative responses were also investigated. Six varieties of *V. mungo* were chosen for the study PU-19, PU-31, PU-35, IPU-94-1, Azad-1 and Azad-2. The seeds were procured from the Indian Institute of Pulses Research (IIPR), Kanpur. The isopropylamine salt of glyphosate, RoundUp (Monsanto, USA), was used in all the experiments. The molecular weight of glyphosate mono-
isopropylammonium is 228.2 and it is supplied as a 41% solution. For initial/preliminary screening seeds were soaked for 24 hrs in increasing glyphosate concentrations, the seeds were allowed to germinate in petridishes. After germination the data on various morphological parameters were taken and analyzed. Further selection scheme involved in vitro germination of seeds either in herbicide medium/ selective media or in basal MS media. The conclusions drawn from these experiments have been detailed and discussed in the next chapter.

2.7 In vitro Selection for Herbicide Tolerance

Herbicides are now extensively used to improve crop yields. However, the damage caused by soil residues in crop rotation programs and selective concentrations used for weed control, have narrowed the usefulness of many herbicides to a limited range of crops, and for many crops suitable herbicides are still not available. The high cost of producing, testing and marketing new herbicides has made the development of plants with tolerance to specific herbicides a feasible solution to these crop/herbicide interaction problems. Plant tissue culture has revolutionized the entire scenario of crop improvement. Regeneration of plants from callus and in vitro selection is the most important strategy that has been used to obtain efficient regenerated lines and subsequently genetic manipulation of crops using in vitro techniques. Many attempts to produce herbicide tolerant plants using in vitro selection have been made to date with varying degrees of success.

Tolerance revealed at the cell level is unstable and may vanish when the selection agent is not present. If the tolerance continued in the absence of the selection agent it must still be expressed in the regenerated plant. If herbicide tolerance has a genetic basis the tolerance must be transmitted to the selfed progeny of the regenerated plants. Regenerated plant may be resistant (survive to normally lethal levels of the herbicide) or tolerant (the plant can survive at sub lethal levels as compared to the wild type (Gressel et al., 1978). Sometimes unstable herbicide tolerance develops due to some epigenetic events which develop in response to selection pressure and may be lost when the selection pressure is removed. The main cause of epigenetic changes may be changed levels of gene expression due to in vitro culture conditions rather than from a true mutation of gene regulatory mechanisms (Chaleff, 1983). According to Hughes, (1983) instability may be due to a very low or total absence of expression of the variant trait even though it may be present in the genome. This is because gene(s) for herbicide resistance may be differentially activated, i.e. the mutation is expressed in cultured cells
but not in the regenerated whole plant (Meredith and Carlson, 1982). Tolerance may also be lost if plants are regenerated from chimeric callus composed of both resistant and sensitive cells. The sensitive cells may grow more as compared to the resistant cell on non-selective media, and since these sensitive cells may also have increased organogenic capacity, a sensitive plant may be produced (Pofelis, 1991).

Various methods have been applied for selection of herbicide tolerance in plants. In *in vitro* selection method herbicide is added the culture medium at required concentration. Most of the publications talks about the selection of somaclonal variant cell lines showing resistance to herbicide except that of Yenne et al., (1987) where responses of existing commercial cultivars were compared. In another report *Brassica* microspores were subjected to mutagenic agents, plated, and then early stage embryos subjected to range of herbicides. Survivors completed the regeneration and colchicine doubling phase and plants were regenerated for testing (Beversdorf and Kott, 1987). Resistance to most major herbicide classes has been selected *in vitro*, and success has been met with obtaining partially resistant plants regenerated in many cases. Plants regenerated from resistant cell lines of tobacco showed tolerance to the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl (Chaleff, 1986a; 1986b). Plants regenerated from hybrid poplar leaf explants subjected to selection on media containing sulfometuron methyl were tolerant of herbicide levels lethal to control plants (Michler and Haissig, 1988; Michler, 1988). Regeneration of shoots from poplar leaf explants exposed to glyphosate gave rise to glyphosate tolerant plants (Michler and Haissig, 1988). Yenne et al., (1987) observed that in pea, *in vitro* sensitivity of some commercial varieties showed some correlation with field sensitivity to glyphosate. Picloram tolerant tobacco plants were regenerated from resistant cell lines (Chaleff, 1986b). Instability of the tolerance is a feature of several of these findings like plants of birdsfoot trefoil regenerated from cells surviving in chlorsulfuron media was found to be more sensitive than the controls (MacLean and Grant, 1987).

Roudsari et al., (2009) regenerated glyphosate-tolerant *Nicotiana tabacum* after plastid transformation with a mutated variant of bacterial *aroA* gene. A variant form of EPSPS gene that confers higher resistance to the broad-spectrum herbicide, glyphosate, was substituted against the spectinomycin resistant gene as a sole selectable marker for plastid transformation of *Nicotiana tabacum*. A long preculture incubation period followed by a gradual increase in glyphosate concentration led to sufficient expression of the transgene.
Khalafalla et al., (2005) recovered herbicide-resistant Azuki bean plants via *Agrobacterium*-mediated transformation and obtained plants expressing the hygromycin phosphotransferase (*hpt*), green fluorescent protein (*sgfp*) and phosphinothricin acetyltransferase (*bar*) genes. Zhou et al., (2006) used a directed evolution strategy to identify a glyphosate-resistant mutant of rice 5-enolpyruvylshikimate 3-phosphate synthase. In this method rice (*Oryza sativa*) EPSPS gene, mutagenized by error-prone polymerase chain reaction, was introduced into an EPSPS-deficient *Escherichia coli* strain, and transformants were selected. Three mutants with high glyphosate resistance were identified. Glyphosate resistance assays indicated a 3-fold increase in glyphosate resistance of *E. coli* expressing the P106L mutant. Ling et al., (2006) selected glyphosate- and haloxyfop-resistant embryos through application of these substances to the cultural media with microspore-derived embryos *in vitro* in four oilseed rape (*Brassica napus* L.) F1 hybrids (7039, 7040, 282 and 5102). Genotypes 7039 and 7040 were used to select glyphosate-resistant regenerated plants and genotypes 282 and 5102 to select haloxyfop-resistant plants. The embryos at cotyledonary stage were grown on glyphosate- and haloxyfop-containing MS-2 medium for 2 weeks. The non-resistant embryos collapsed after a short time, while the resistant ones turned green and survived for 2 weeks. The regenerated plants from green embryos showed tolerance to 0.25% sprayed glyphosate, indicating the effectiveness and reliability of this *in vitro* selection method.

*In vitro* culture of rice buds has been developed to induce tolerance to phosphinothricin (PPT) by Liu et al., (2005). At earlier stage of tissue culture with PPT concentrations higher than 0.76 μM inhibited the bud growth of rice cv. Koshihikari. Smaller buds were more sensitive. Through 28-day incubation, microshoot induction was most significantly stimulated by 5.0 μM PPT, as compared with other concentrations; with higher levels resulting in plant death.

Raducanu, (2004) performed *in vitro* screening for roundup resistance in sunflower. A preliminary experiment was performed in order to determine the dose of glyphosate required in the culture medium to inhibit callus growth relative to control by 50% (ID 50). The explants represented by immature embryos (10 days old) were cultivated on Har medium supplemented with NAA and BAP. After the incubation period calli were transferred on the same medium with Roundup (glyphosate) with concentrations: 2.0 ml/l; 3.0 ml/l; 4.0 ml/l; 6.0 ml/l. Genotypes differed regarding their response (callus viability), depending on the herbicide concentration. Callus viability
decreased with increasing herbicide concentration. Venkataiah et al., (2005) selected atrazine-resistant plants by *in vitro* mutagenesis in cotyledon cultures of pepper (*Capsicum annuum*). Low levels of herbicide cause less growth inhibition, with some chlorophyll loss causing albino shoots. Mutagenized cotyledon explants resulted in production of herbicide-resistant plants on medium containing selective levels of sucrose (0.5%) and atrazine (20 mg/l). Complete atrazine-resistant plantlets were obtained after rooting of regenerated green shoots on rooting medium containing 10 mg/l atrazine, 1.0 mg/l IAA and 0.5% sucrose. All regenerants were resistant to the atrazine.

Koch et al., (2009) screened populations of sugarcane from breeding crosses for naturally occurring tolerant genotypes, and produced tolerant genotypes through *in vitro* cell mutagenesis. 11000 seedlings were sprayed with 0.1-1.5 l/ha Arsenal, after which 1.25 l/ha Arsenal was selected to test 12000 seedlings. In the second approach regeneration of herbicide tolerant plants through induced somaclonal variation was used. Somatic embryogenesis calli of N12 were screened for somaclonal variant tolerance to imazapyr, which may have resulted from 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium.

Cha-um et al., (1999) selected Khaw Dawk Mali 105 (KDML 105) calli tolerant to glyphosate on solidified MS selection medium (MS salts and vitamins, 1 mg/l kinetin, and 10^{-4} M glyphosate). Glyphosate caused reduction in calli fresh weight. The selected calli at 4 and 8 weeks (S_{4} and S_{8}) could be regenerated on the glyphosate-free medium. Plantlets were propagated by tissue culture technique and treated with 0, 10^{-5}, 5x10^{-5} and 10^{-4} M glyphosate *in vitro*. The height of S_{4} and S_{8} plantlets was decreased with increasing glyphosate concentrations in culture media while number of tillers and number of roots were increased in culture media with 10^{-5} M glyphosate. The S_{8} plantlets could survive on the culture supplemented with 5x10^{-5} M glyphosate. Increasing the height of plantlets indicated high survival percentage under glyphosate stressed condition. Tolerant plants were transferred to pots and treated with 0, 250, 500, 1000 and 2000 mg/l glyphosate. The growth of plants was inhibited and phytotoxicity was observed when treated with high glyphosate concentrations. Increasing glyphosate concentrations resulted in lesser toxicity in S_{8} plantlets than in S_{4} plantlets.

Chen et al., (2012b) obtained glyphosate-tolerant variants in manilagrass (*Zoysia matrella* L.) by *in vitro* selection and greenhouse screening. Calli were transferred to selection medium containing 2 mM glyphosate. After two rounds of selection calli were transferred to regeneration medium without glyphosate. Regenerated plantlets were then
transferred to regeneration medium containing 0.5 mM glyphosate to select tolerant plantlets. Fully developed plantlets were transferred to a greenhouse and then subjected to greenhouse screening by foliar spraying with 0.05 % glyphosate solution.

Lee, (1981) studied effect of glyphosate on callus cultures or tobacco \textit{(Nicotiana tabacum} and \textit{N. glauca-langsdorffii}, and soybean \textit{(Glycine max} L., cv. Chippewa) Glyphosate inhibited growth both in the dark and light but showed a greater toxicity in the dark. The inhibition of growth was not reversed by simultaneous addition of aromatic amino acids to the medium. The tobacco callus tissue was more sensitive to glyphosate than the soybean callus tissue, showing differential tolerance between plant species. Despite the inhibitory effect of glyphosate, treated tissue revived after being transferred to a glyphosate-free medium.

In vitro culture of barley calluses has been used to produce plants with increased glyphosate tolerance. Calluses from immature embryos were cultured on Murashige and Skoog medium with 10^{-6}, 10^{-5}, 10^{-4}, 5\times10^{-4}, 10^{-3}, or 10^{-2}M glyphosate for one, four or thirty months. Plants were regenerated from calluses maintained in glyphosate medium at 10^{-6}, 10^{-5} or 10^{-4}M for four months, at 10^{-5} or 5\times10^{-4}M for one month and at 10^{-5}M for thirty months (Escorial et al., 1996).

Callus cultures were initiated from hypocotyl segments of oilseed flax \textit{(Linum usitatissimum)} and transferred to selection medium containing chlorsulfuron, the active ingredient in the herbicide Glean\textregistered. After a suitable period, surviving colonies were isolated from the selection medium and placed in regeneration medium, where shoot reorganization in some cases produced fertile plants (Jordan and McHughen, 1987).

Dryanova and Dimitrov, (2000) studied morphogenetic response of callus cultures of 3 triticale genotypes after adding herbicide Stomp 330 (pendimethalin) into the regeneration nutrient medium. The regenerative ability was not affected at the lowest herbicide concentration (0.01%). However, the higher doses (0.05 and 0.1%) reduced regenerative ability at different rates depending on the genotype. Rhizogenesis was completely suppressed. The necrotic calluses increased and the fresh, unorganized growing calluses reduced. Abnormalities such as chromatin pycnosis and micronuclei were observed. The frequency of these abnormalities was proportional to the herbicide concentration. Stomp 330 exerts a strong C-mitotic effect in the callus cells, which is demonstrated by high frequency of polyploid cells.

Toldi et al., (2000) produced rice \textit{(Oryza sativa} L.) tolerant to the herbicide phosphinothricin (PPT) by means of \textit{in vitro} selection. First, sublethal and lethal
concentrations of PPT on 7-day-old seedlings were determined. Differentiation of 6–30 microshoots on 5–40% of the treated plant material was observed on a hormone-free culture medium supplemented with a sublethal concentration of PPT. Fertile plants were grown from those microshoots having PPT tolerance under greenhouse conditions. An elevated GS activity was detected in PPT-tolerant plant material which could result in an elevated PPT tolerance at unchanged concentrations of the herbicide.

A lot of information of biochemical pathways of herbicides, along with an understanding of the different mechanisms involved in plant resistance is now available. Using cell culture techniques, it is possible to screen large populations of somatic cells for resistant variant cells in a limited amount of space and in a short time. The source of genetic variability can already be present in the original tissue, can be generated with the use of a mutagen or be randomly culture induced. Callus, suspension, and protoplast techniques have been used in cell selection programs, each with their own advantages and limitations. Moreover, the application of a selection pressure (i.e. an herbicide in the culture medium) during the selection procedure makes the screening more efficient by giving the variant cells/callus of interest the selective advantage. Selection using cell culture techniques can be a useful tool only when if the character that is being selected for, operates at the cell level, and is also expressed in the whole plant. Metribuzin, for example, a highly effective herbicide on photosynthesizing seedlings, acts on the photosynthetic electron transport system. However, the herbicide was found to have no effect on callus culture growth and development (Ellis, 1978).

2.8 Transgenic Approaches for Development of Herbicide Tolerant Plants

In recent time gene transfer technology has been widely used for screening, development and characterization of herbicide tolerant plants as this technology provide better results in less time and labour. Genetic engineering can complement traditional breeding methods, it is possible to introduce herbicide tolerant genes into the desired germplasm. Nasir et al., (2014) used glyphosate-tolerant gene of 1368 bp cloned directionally under the 35S promoter with the β-glucuronidase (GUS) reporter gene as the transgene. Calli were transformed via the biolistic method with glyphosate-tolerant gene constructs. Acclimatized transgenic sugarcane plants survived the glyphosate spray application of 900 mL/0.404 ha, except for the control nontransformed plants. Van Der Vyver et al., (2013) also obtained herbicide-resistant transgenic sugarcane plants containing mutant forms of a tobacco acetylactate synthase (als) gene were obtained following biolistic transformation. Putative transgenic callus was selectively proliferated
on MS medium containing chlorosulfuron. Glasshouse spraying of putative transgenic plants with 100 mg/l chlorosulfuron dramatically decreased the amount of non-transgenic plants. Herbicide-resistant cowpea were produced by transformation with the bar gene (Aasim et al., 2013). Immature cotyledons were transformed with *Agrobacterium tumefaciens* strain LBA4404 harboring the recombinant binary vector pRGG containing an herbicide tolerance gene (*bar*) along with a uidA (*GUS*) gene under 35S promoter. Phosphinothricin was used as a selectable marker at a concentration of 2.5 mg l\(^{-1}\). Putative transformants were screened by the histochemical GUS assay. The selected transgenic plants showed a resistance to Basta® nonselective herbicide at up to 10 ml l\(^{-1}\) of water. Kwapata et al., (2012) performed genetic transformation of common bean (*Phaseolus vulgaris* L.) via the biolistic bombardment of the apical shoot meristem primordium. Transgenes used were *gus* color marker which visually confirmed transgenic events, the bar herbicide resistance selectable marker used for *in vitro* selection of transgenic cultures and the *HVA1* drought tolerance genes. Torres et al., (1999) produced glyphosate-resistant plants of lettuce (*Lactuca sativa* L.) by using *Agrobacterium tumefaciens* containing a plasmid carrying glyphosate oxidase and EPSPS gene. An *in vitro* assay was performed to determine the sensitivity of ‘South Bay’ leaf discs and seedling explants to varying glyphosate concentrations. The I50 for glyphosate leaf discs was 53.8 mM and for glyphosate seedlings 7.6 mM. There was a high correlation between the response of leaf discs and seedlings to glyphosate based on dry weight. These findings will allow identification of glyphosate-resistant transformants in an early stage of plant development, saving time and reducing the cost in generating an improved cultivar with the glyphosate resistance trait.

**2.9 In vitro Studies in Vigna Group of Pulses**

*In vitro* culture technique helps in the implementation and manifestation of biotechnological investigations and has been extensively used in plants. It is generally accepted that exogenous growth regulators play major role in the formation of callus tissue. In culture both auxin and cytokinin are required for indefinite growth and cell division in callus. After the formation of unorganized mass of cells at the cut ends, gradually the whole tissue gets involved to form callus. The callus gradually increases in mass and cells are added by mitotic division. Estimation of callus growth can be made on the basis of change in fresh or dry weight. Several workers have tried tissue culture techniques in leguminous crops (Kartha and Gamborg, 1978; Mukhopadhyay and Bhojwani, 1978; Bajaj and Dhanju, 1979; Boulter and Crocoma, 1979; Phillips and
Collins, 1979; Hammat and Davey, 1997; Hammat et al., 1986; Gulati and Jaiwal, 1992, 1990; Gill et al., 1988; Polisetty et al., 1997) and have discussed the importance of tissue culture methods in improvement of pulses and their regeneration potential. Success has also been achieved in regenerating the plants, shoot morphogenesis and somatic embryogenesis.

2.9.1 Direct Shoot Organogenesis

2.9.1.1 Cotyledonary Node Explant

Formation of multiple shoot from cotyledonary node explants relies on the number and size of cotyledons attached to the cotyledonary node, type and concentration of cytokinin, presence or absence of shoot tip and genotype. Cotyledonary node explants with or without cotyledon developed multiple shoots on MS (Murashige and Skoog, 1962) or MSB (MS salts and B5 vitamins) medium supplemented with different concentrations of cytokinins (Gill et al., 1987; Ignacimuthu et al., 1997; Sen and Mukherjee, 1998; Franklin et al., 2000; Saini et al., 2003; Mony et al., 2010).

Cotyledonary node explants cultured on medium with 1.0 mg/l BAP for 10 days followed by transfer to hormone-free media gave higher number of shoots (9.33 shoots/explant) compared to those cultured on hormone-free medium for 15 days followed by transfer to medium containing 1.0 mg/l BAP (8.33 shoots/explant). The shortest shoots (1.77 cm) was observed in culture of explants for 15 days on MS medium containing 10 mg/l BAP followed by transfer to hormone free MS medium. The highest concentration of BAP in shoot initiation medium might have a suppressive effect on shoot length (Mony et al., 2010). Presence of BAP is required for multiple shoot production and shoot bud regeneration can be achieved via meristem formation on excised cotyledons on MS basal medium with B5 vitamins supplemented with TDZ (Sen and Mukherjee, 1998). The explant with both the cotyledons without shoot tip was found to be more superior to those with one or no cotyledon. Absence of both the cotyledons delayed the shoot induction response and reduced the regeneration frequency, the number of shoots per explant and the length of shoots (Saini et al., 2003). According to these reports presence of cotyledon on the explant is not necessary for multiple shoot formation, which is contrary with previous findings in which there was either no regeneration (Ignacimuthu et al., 1997) or regeneration of only a single shoot (Sen and Mukherjee, 1998) from such explants without cotyledons. In the presence of cotyledons, the axillary shoots produced from node were healthy and possessed trifoliate leaves whereas in the absence of cotyledons, the shoots were weak and possessed a simple leaf.
(Franklin et al., 2000). The younger explants gave better shoot regeneration response than older ones (Saini et al., 2003). Efficient in vitro plant regeneration was achieved using nodal and cotyledonary node explant of Vigna radiata (L.) Wilczek by Vats et al., (2014). Maximum response in terms of shoot regeneration was observed in MS medium supplemented with BAP (0.5 ppm) and NAA (0.25 ppm). The regenerated shoots were cultured on rooting medium supplemented with different doses of IBA. The best response was observed in 3 ppm IBA.

Direct and efficient multiple shoot regeneration (80–100%) from the cotyledonary nodes was obtained in all epigeal and in hypogeal asiatic Vigna species while two other hypogeal species V. angularis and V. umbellata failed to initiate shoots from the nodes. Adventitious shoots also developed at the basipetal cut (hypocotyl) in 35–67% of V. angularis explants. Cotyledonary node explants were more regenerable than cotyledons. Rooting response was higher in MS basal medium than in MS with 1.0 mg/l IAA (Avenido and Hattori, 1999).

Immature cotyledonary node explants excised from immature pods after 18 d of anthesis, on MS basal medium supplemented with BAP (1.0 mg/l), TDZ (0.1 mg/l) and AdS (15 mg/l) developed a maximum of 28 shoots/ explant at the end of second subculture, i.e. after 45d of culture. Periodic excision of regenerated shoots from explants increased shoot regeneration efficiency during subculture. Elongation and rooting were performed in GA3 (0.6 mg/l) and IBA (1.0 mg/l) containing medium (Muruganantham et al., 2005).

Efficient regeneration of mungbean from cotyledonary node explants was achieved by Gulati and Jaiwal, (1994). The explants were capable of directly developing multiple shoots on basal media devoid of any growth regulators. The shoot multiplication was influenced by media composition, growth regulators, age of donor seedling and explant type. The explants with both the cotyledons attached to the embryonic axis excised from 4-d-old seedlings, produced the highest number of shoots (5 or 6) in 100% of the cultures within 2 weeks on B5 basal medium (BBM) containing BAP or 2-iP, respectively, (at 5x10^{-7}M) and 3% sucrose. Shoots elongated and developed better using BAP.

Double cotyledonary node explants from 3-d-old seedlings germinated on MS and Gamborg’s medium (B5) containing (2.0 mg/l) 6-benzyl aminopurine (BAP) showed multiple shoot bud initials when cultured onto MS B5 medium augmented with different concentrations of BAP and very low concentrations of different auxins (NAA, IAA and
IBA) and cytokinin (Kn). For shoot proliferation shoot bud initials were transferred to MS B₅ media containing reduced concentrations of BAP (Yadav et al., 2010). Axillary and adventitious shoot regeneration was found in Vigna radiata (L.) Wilczek from 3 day old cotyledonary node and hypocotyl explants on MS medium supplemented with TDZ (0.9 μM). An initial exposure to TDZ for 20 d and three successive transfers to fresh medium with reduced TDZ levels (0.09 μM) resulted in the regeneration of 104 shoots per explant from the cotyledon and 30 shoots per explant from the hypocotyl (Amutha et al., 2006).

The shoot multiplication rate was influenced by the presence or the absence of cotyledons in cotyledonary node explants of african cowpea variety (Diallo et al., 2008). Explants with two entire cotyledons produced the greater number of shoots (8.3) supplemented with 1 mg/l BAP. Shoots elongation is optimal on media supplemented with kinetin. Rooting was improved after an induction phase on half strength MS, producing 95.83% of rooted plants.

Shoot regeneration occurred from immature cotyledonary nodes from 18 day-old immature cotyledonary node explants (18 d after anthesis) of black gram (Muruganantham et al., 2005). Multiple shoots produced in MS salts+B5 vitamins containing medium in the presence of BA (1.0 mg/l), TDZ (0.1 mg/l) and AdS (15 mg/l). Maximum shoot proliferation (28 shoot/explant) occurred at the end of second subculture after 45 d. Periodic excision of regenerated shoots from explants increased shoot regeneration efficiency during subculture. The combination of TDZ and AdS with BA significantly increased shoot proliferation. Elongation and rooting were performed in GA₃ (0.6 mg/l) and IBA (1.0 mg/l) containing media, respectively. Muruganantham et al., (2007) performed efficient Agrobacterium -mediated transformation of Vigna mungo using immature cotyledonary-node explants and phosphinothricin as the selection agent. Herbicide (Basta®)-tolerant Vigna mungo L. Hepper plants were produced using cotyledonal-node and shoot-tip explants from seedlings germinated in vitro from immature seeds. Shoot regeneration occurred for 6 week on regeneration medium (MS medium with 4.44 μM BAP, 0.91 μM TDZ, and 81.43 μM AS with 2.4 mg/l PPT, elongation medium (MS medium with 2.89 μM GA and 0.4 mg/l PPT), β-glucuronidase-expressing putative transformants were rooted in MS medium with 7.36 μM IBA and 2.4 mg/l PPT.

Multiple shoots were induced from cotyledonary nodes of grasspea derived from 7-d-old in vitro seedlings on MS medium containing BA, kinetin, or TDZ, BA being the
most effective. Genotype IC-120487 gave the maximum number of shoots (11.3 shoots per explant) on MS medium augmented with 2.0 mg/l BA. Up to 81.8% of the shoots developed roots following transfer to half-strength MS medium containing 0.5mg/l IAA (Barik et al., 2005). Bud proliferation occurred at the cotyledonary nodes of cowpea seedlings three weeks after culture on MS medium containing B5 vitamins supplemented with TDZ. A 10 μmol/l TDZ pre-treatment, shoot tip removal and excision of longitudinal thin cell layers (TCL) at the level of the cotyledonary nodes with subsequent culture on a MSB5 medium supplemented with 1 μmol/l IBA and 1 μmol/l TDZ were the optimal conditions for maximum bud proliferation. Up to 32.5 regenerated shoot buds were produced per TCL. The regenerated plants were true-to-type and successfully transferred to soil (Van Le et al., 2002). Vijayan et al., (2006) developed direct multiple shoots from the cotyledonary node explants of 2-day-old in vitro grown seedlings of mungbean. Maximum number of shoots (an average 12.1 shoots per explant) was obtained on a medium containing MS salts, B5 vitamins and 5.0 mg/l BAP. A medium with lower BAP concentration appeared suitable for rapid shoot elongation. The elongated shoots were rooted on 0.2 mg/l NAA.

2.9.1.2 Shoot Apex Explant

Shoot apices cultured on modified MS basal medium supplemented with PGRs directly elongated into shoots without callus formation and the shoots gradually developed roots at the basal end, resulting into complete plantlets (Agnihotri et al., 2001; Das et al., 1998; Saini and Jaiwal, 2005). Basal medium supplemented with BAP (0.5 – 15 μM) induced callusing at the base of explants followed by multiple shoots differentiation. Maximum 3-4 shoots of 3.5 cm in length were obtained in the 90% of the cultures when BAP concentration was 1.0 μM (Saini and Jaiwal, 2005). Preconditioning of the explant at 10 μM BAP for 3d was most effective in increasing the number of shoots by 3-fold (an average of 13-14 shoots/explant) over those without preconditioning treatment (4-5 shoots/explant). 4 shoots was produced from shoot tip on culture in the presence of two cytokinins, 2.2 μM each of BAP and 2-iP and 0.48 μM NAA. BAP along with 2-iP was more effective than either of the cytokinins alone (Agnihotri et al., 2001).

Complete plants were regenerated directly without an intervening callus phase from shoot tips on basal medium (MS salts+B5 vitamins) in Vigna radiata (L.) Wilczek (Gulati and Jaiwal, 1992). Regeneration frequency varied with genotype, explant size and growth regulator combinations in the medium. Addition of cytokinins induced a variable
amount of callus at the base of the shoot tip, followed by multiple shoot formation. Benzyl adenine (BA), kinetin and zeatin at $5 \times 10^{-6}$ M each, induced multiple shoots in 100% of the explants but the highest number of regenerants per explant (9) was produced with BA. The efficacy of BA for shoot multiplication was not improved when it was supplemented with naphthalene acetic acid (NAA) or indole acetic acid (IAA). NAA or adenine sulphate, when applied alone, induced complete plantlets. The growth regulator requirement of explants for the induction of multiple shoots varied with explant size.

Two endangered aromatic varieties of *Vigna radiata* (L.) Wilczek Sonamung and Tilmung were micropropagated by *in vitro* shoot-tip multiplication (Betal and Raychaudhuri, 1999). The best hormone combination in B5 medium was 0.2 mg of NAA/l and 5 mg of BA/l. Shoot-tip multiplication method was found to be a reproducible and effective method for germplasm preservation of mungbean.

Brar et al., (1997b) studied *in vitro* shoot tip multiplication of cowpea *Vigna unguiculata* (L.) Walp cv.Georgia-21. 5 mM long shoot tips cultured on MS medium containing BA at 1, 2.5, or 5 mg/l or kinetin at 1, 2.5, or 5 mg/l combined with 2,4-D at 0.01, 0.1, or 0.5 mg/l NAA at 0.01, 0.1, or 0.5 mg/l. BA induced greater shoot proliferation as compared to kinetin. The highest number of shoots was produced on BA 5 mg/l in combination with NAA or 2,4-D at 0.01 mg/l (0.05 µM). Roots were formed in the presence of kinetin, but not on BA-containing medium. Shoots were rooted on 0.1 mg (0.5 µM) NAA per liter to form whole plants.

Multiple shoots from shoot meristems of three - five-day-old *in vitro* grown seedlings of turkish cowpea was obtained in MS supplemented with 0.50 mg/l BAP - 0, 0.10, 0.30 and 0.50 mg/l NAA. Increased diameter of calli was recorded on MS medium containing 0.5 mg/l BAP and 0.1, 0.3 and 0.5 mg/l NAA. The highest frequency (%) of shoot regeneration and mean number of shoots per explant was recorded on MS medium containing 0.5 mg/l BAP with out NAA (Aasim et al., 2008). Aasim et al., (2009) compared shoot regeneration on different concentrations of TDZ from shoot tip explant of cowpea on gelrite and agar containing medium. Frequency of shoot regeneration increased with increase in TDZ concentrations in both cultivars on both agar and gelrite gelled medium. Both cultivars showed maximum mean number of shoots per explant in gelrite compared to agar gelled medium. Maximum number of 4.72 and 2.86 shoots per explant were recorded on MS medium containing 0.25 mg/l TDZ in cv. Akkiz and cv. Karagoz respectively. Agar gelled medium had greater shoot length compared to gelrite medium in both cultivars. Regenerated shoots were rooted easily on MS medium.
containing 0.50 mg/l IBA with regeneration of mean number of 4 secondary shoots on cv. Akkiz and 3 on cv. Karagoz. Multiple shoot formation from the plumular apices excised from mature embryos of cowpea cv. Akkiz was obtained after pulse treatment with 10 mg/l BAP for 5 days followed by culture on MS medium containing 0.25, 0.50, 0.75 and 1.00, 1.25 mg/l BAP – with or without 0.10 mg/l NAA. 0.1 mg/l NAA showed positive effect on callus diameter and shoot length. Maximum number of shoots 7.11 per explant was obtained on MS medium containing 1.00 mg/l BAP. Longer shoots were recorded on MS medium containing various concentration of BAP+ 0.1 mg/l NAA compared to those containing various concentrations of BAP singly. All shoots cultured on MS medium containing 1 mg/l BAP were rooted on MS medium containing 0.50 mg/l IBA (Aasim et al., 2010). Shoot organogenesis response was observed from shoot apices of 3–5-day-old seedlings of black eye cowpea. The optimal medium for maximum shoot initiation comprised MS salts, B5 vitamins, 8.88 µM BAP, 1 gl/l casein hydrolysate, 342 µM L-glutamine, 3% sucrose, 0.3% phytagel, adjusted to pH 5.8. A shift in pH from 5.8 to 7.0 had no effect on shoot initiation and on number of shoots per explant (Mao et al., 2006).

Agnihotri et al., (2001) found that among the five explants of V. mungo var. T9 the excised shoot tips gave best response with regard to offshoot formation followed by the embryonal axis explants. Combination of 0.5 mg/l BAP, 0.5 mg/l 2iP and 0.1 mg/l NAA produced 10 offshoots in shoot tip explants, only 3 offshoots were formed in the explants of embryonal axis in a treatment containing 0.5 mg/l BAP and 0.1 mg/l NAA, found optimum for them. Multiple shoots differentiated when growing offshoots were first cultured in a treatment containing 0.1 mg/l BAP, 0.25 mg/l IAA and 5 mg/l CCC (chloroethyltrimethyl ammonium chloride) and then subcultured in the same treatment but having only 1 mg/l CCC. The isolated shoots rooted in 0.5 mg/l IAA, resulting in the formation of complete plantlets.

2.9.1.3 Epicotyl and Hypocotyl Explant

Sen and Mukherjee, (1998) induced multiple shoots and plant regeneration under in vitro condition using cotyledonary nodes, excised cotyledons, and hypocotyl segments of six varieties of Vigna mungo and V. radiata. Multiple shoots developed on cotyledonary node explants in all varieties tested on basal medium containing cytokinin. Presence of both the cotyledons, either full or half, resulted in a maximum number of shoots produced. Shoot bud regeneration was achieved via meristem formation on excised cotyledons on Murashige-Skoog basal medium with B5 vitamins supplemented
with TDZ. Mature plants had normal phenotypes. *V. mungo* var. PS1 and *V. radiata* var. Pusa 105 were found to be the most responsive varieties for shoot regeneration.

Mongomaké et al., (2009) produced *in vitro* regeneration system via direct organogenesis in Bambara groundnut (*Vigna subterranea* L.) using hypocotyl and epicotyl cuttings. Basal MS medium supplemented with BAP, Kn (kinetin) or TDZ with or without NAA were attempted. Multiple shoots were induced from both explants but regeneration efficiency was higher in epicotyl cuttings. BAP (2 mg/l) gave the highest response (73.33 - 97.77%) with the regeneration of 3.7 shoots per explant with hypocotyl and 5.8 shoots per explant with epicotyl. The regenerated shoots were elongated on the same medium as used for induction and rooted on half-strength MS basal medium without any growth regulators. 62% of the plantlets were successfully acclimatized and potted plants were established in soil with 73% survival rate.

The epicotyl explants excised from 3-day-old *in vitro* - raised seedlings planted vertically with basal cut end inserted in the MS basal medium (MSB) with BAP (5 µM) produced multiple shoots at the apical end by direct organogenesis while the basal end formed callus which differentiated into shoots via indirect organogenesis. With increase in the age of explants, the shoot forming response increased at the apical end while at the basal end only callus formation takes place. Substitution of BAP at an equimolar concentration with kinetin, 2-iP, TDZ and AdS or auxins (IAA, IBA, and NAA) did not improve shoot regeneration. The shoot forming response of explants decreased as the distance of the epicotyl segment increased from shoot apex of the mother seedling as well as when the orientation of explants deviates from the normal vertical upright position (Saini and Jaiwal, 2002).

**2.9.2 Shoot Organogenesis via Callus**

Callus induction and shoot regeneration was observed from cotyledonary node, hypocotyl and other explants (epicotyl, axillary bud, immature leaf) on MS basal medium supplemented with B5 vitamins, IAA, NAA, IBA, KIN and BAP either individually or in combination (Geetha et al., 1997). Among these explants, hypocotyls were found to be more efficient in producing callus on MSB medium containing NAA (16.1 µM) and BAP (2.2 µM). Shoots were induced from callus cultures on medium containing different combinations of kinetin (2.3 - 9.3 µM) or BAP (2.2 - 8.8 µM) together with IAA (2.8 µM) or NAA (2.6 µM). The highest frequency of shoot bud regeneration (80.8%) and the highest number of shoots (13.9 shoots/callus) were
observed using cotyledonary node explant with BAP (6.6 µM) and NAA (2.2 µM) (Geetha et al., 1997).

Shoot regeneration via callus formation also occurred on BAP (1 mg/l) alone from cotyledonary node explants (Avenido and Hattori, 1999). Multiple shoot induction from embryo derived callus cultures of cowpea (*Vigna unguiculata* L.) Walp has also been reported (Odutayo et al., 2005). After five weeks, multiple shoots developed from the calli cultures after being subcultured on media with high concentration of cytokinin, BAP. Percentage shoot production from calli grown on media with 1 mM concentration of BAP was 45.5% while calli subcultured on 4 mM BAP produced 87.5% shoot production. Root development was promoted by the action of the auxin, NAA, at low cytokinin concentration.

Cotyledonary tissues of black gram var. Sarala were used first to develop organogenic calli within 4–6 weeks of culture on MS medium supplemented with 3.0 mg/l BAP and 2.0 mg/l NAA. Shoot buds were formed on MS medium supplemented with 2.0 mg/l BAP within 3–4 weeks of subculture (Adlinge et al., 2013). The number of shoots per culture varied from 1.12 to 8.75 in different growth media. The cultures incubated initially on dark photoperiod for 2 weeks and subsequently transferred to 16 h photoperiod showed higher number of shoot bud regeneration. The proliferated shoots were further sub-cultured on similar medium for higher rate of shoot bud regeneration.

The effect of cytokinins BAP, TDZ and Kn on *in vitro* regeneration from cotyledonary explants was studied in black gram var. Vamban-1 (Srilatha, 2014). The regenerated shoots elongated on the same medium. TDZ (4.0 mg/l) was proved to be best for induction of shoots from cotyledon explants. Micro shoots were rooted on MS medium fortified with IBA (3.0mg/l) followed by transfer to greenhouse. TDZ induces significant regeneration as well as shoot elongation from cotyledon in black gram var. Vamban-1.

Callus initiation, shoot regeneration and plantlet formation from cotyledon, hypocotyl, root tip and shoot tip explants were studied in black gram by Mony et al., (2008). Among the explants, hypocotyls showed the best performance in callus formation (92.33%) with 2.5 mg/l BAP and 1.5 mg/l NAA followed by cotyledon, shoot tip and root tip explants, respectively. Shoot regeneration from the calli was also maximum in hypocotyls (56.33%) in medium with 3.0 mg/l BAP and 0.3 mg/l and 0.5 mg/l GA3 (gibberellin) while other explants had no shoot regeneration.
Roy et al., (2003) reported plantlet regeneration in black gram from callus culture and effect of genotype, explant and media composition. Hypocotyl, shoot bud, leaf tip and root tip explants of black gram genotypes B-76, T-9, L-13 and L-7 were cultured on B5 and modified MS media. Hypocotyls and roots of 4- to 7-day old seedlings exhibited earlier and faster callusing. Addition of 2,4-D to the medium was more effective than addition of NAA. Callus induction was faster and more successful in modified MS medium with MS salts and B5 vitamins than in B5 medium. Plantlet regeneration was achieved in modified MS medium. Maintaining an auxin:cytokinin ratio of 1:3 in the culture medium was critical for the success of *in vitro* propagation of black gram.

Primary leaf explants excised from 7-d-old *in vitro* raised seedlings produced calli on MS medium supplemented with 2, 4-D (6.0 µM). 2-week-old leaf derived greenish white friable calli differentiated in to shoot buds on MS liquid medium supplemented with 6.0 µM 2,4-D. The shoot buds were then transferred to MS medium containing 6.0 µM 2,4-D for development of plantlets (Srivastava and Pandey, 2011). NAA failed to induce calli and shoots buds, indicating that leaf segments have different sensitivity to various auxins and their concentrations. Full-strength MS medium was found to be more effective than the other media due to the presence of high levels of nitrogen, particularly the reduced form (NH₄PO₄) in MS medium.

Regeneration of *V. radiata* and *V. mungo* was achieved from callus cultures derived from leaf explants on basal media supplemented with various combinations of growth regulators. Maximum callus was formed with 4 µM each of 2, 4-D and kinetin. Shoot bud formation in *V. radiata* occurred within 10 days after transferring the callus into the MS medium supplemented with 15 µM kinetin + 1 µM NAA, while roots were induced within a week in root induction medium (BM supplemented with NAA). Shoot regeneration from callus decreased with increasing concentration of auxins in the medium, while maximum rooting was observed with 6 µM NAA in the medium. *In vitro* regeneration of *V. radiata* and *V. mungo* plantlets from shoot tip (apical meristem) cultures was observed within 4 weeks on MS medium supplemented with cytokinins. Kinetin (15-17 µM) was found optimum for plant regeneration (Teli and Maheshwari, 2001).

### 2.9.3 Regeneration from Multiple Explants

Rao et al., (2005) reported effect of different growth regulators and their concentration on callus formation and organogenesis in various explants and callus was studied in mung bean [*Vigna radiata* (L.) Wilczek]. 2, 4-D and NAA alone or in
combination with Kn supported callus induction and further growth. 2, 4-D proved better than NAA and addition of Kn at 4.6 (µM/l) further enhanced the growth of callus. Organogenesis was obtained from callus, shoot tip and cotyledonary node explants on BAP medium. In vitro plant regeneration was also achieved via adventitious shoot-bud formation from seedling hypocotyls, cotyledonary node (CN) and root explants from 4-d-old in vitro-germinated seedlings of adzukibeans (Vigna angularis) by Avenido and Hattori, (2000). Explants were grown on medium consisting of MS salts, B5 vitamins, 3.0% (w/v) sucrose and 4.4 µM BA. Shoot buds arose adventitiously at the basipetal cut of the hypocotyl. 8–10 mm CN explants exhibited significantly higher response.

Plantlets were regenerated from different seedling explants of mungbean on medium supplemented with different concentrations and combinations of BAP and NAA by Khatun et al., (2008). Cotyledon explants performed best in callus induction (90.0%) at the combination of 1 mg/l BAP and 2.5 mg/l NAA. The calli derived from cotyledon, hypocotyls and root tip were cultured on medium with different concentrations of Kn, BAP and/or NAA for shoot induction. Regeneration occurred with 62.50% frequency only from cotyledon calli on 5 mg/l BAP and 0.05 mg/l NAA. Shoot tip cultured for direct regeneration in the same media.

Pellegrineschi, (1997) achieved shoot regeneration via organogenesis in cowpea hypocotyls and cotyledons of advanced breeding lines and varieties. Cotyledons and hypocotyls were tested on media with gradients of several hormonal and putrescine combinations. Cowpea cotyledons and hypocotyls exhibited a pattern of shoot formation that occurred in three distinct phases. Wounded region of the primary hypocotyl and cotyledons showed multiple shoots within 45 days in media with high cytokinin.

Brar et al., (1997a) cultured shoot tip and leaf disk explants in two cultivar, Early Scarlet and 91-245 of cowpea (Vigna unguiculata). Multiple shoots were produced from shoot tips cultured on media with 5 mg/l kinetin and 0.01 mg/l NAA. Leaf disks were cultured on MS media supplemented with 0.5 mg/l BAP and 1 mg/l 2,4-D. Callus growth was highest on media of full strength MS with 0.5 mg/l BAP and 1 mg/l 2,4-D. The effects of the media constituents were genotype dependent, with Early Scarlet generally producing larger shoots and greater amounts of calli. Study showed that the plant genotype and growth hormones have the greatest influence on cowpea growth in vitro.

Avenido and Hautea, (1990) reported in vitro organogenesis and flowering in mung bean Vigna radiata (L.) Wilczek. Callus was induced from cotyledon, epicotyl and leaf segments of mung bean on MS medium supplemented with 2 mg/l 2,4-D and 0.1-
0.2 mg/l BAP. Root formation occurred on medium with 2 mg/l BAP. Multiple shoots were directly formed from cotyledonary nodes on medium with 1 mg/l BAP. Plantlets produced from them initiated flowering after two months of subculturing for rooting.

Plant regeneration was achieved using stem, epicotyl and cotyledon explants, while shoots were regenerated from leaf sections of Vigna radiata (Mendoza and Futsuhara, 1990). Explants differed in hormonal requirements included in the MS or B5 basal medium. Stem, leaf and epicotyl explants required the addition of 0.2 mg NAA and 1 or 2 mg kinetin or 6-BA (benzyladenine)/litre to give optimal concentrations. No auxin additions were required for cotyledon cultures wherein regeneration occurred only at the proximal ends; addition of 1 or 2 mg kinetin or 6-BA and 100 mg yeast extract/litre gave efficient plant regeneration. Regeneration ability varied with genotype.

Mathews, (1987) observed morphogenetic response of in vitro cultured seedling explants of mung bean (Vigna radiata L. Wilczek). Direct induction of shoots/plants was possible from shoot tip, cotyledon and cotyledonary node explants. Dedifferentiation of the explants viz; shoot tip, cotyledons, cotyledonary node, primordial leaves, and roots were obtained on basal medium supplemented with auxin and cytokinin. Shoot regeneration was limited to primary calli while rhizogenesis was of common occurrence in established calli. Badere et al., (2002) suggested that medium supplemented with 0.1 mg/l NAA and 0.1 to 0.7 mg/l BAP was most effective in inducing multiple shooting and cotyledonary node with cotyledon was found to be the best explant for multiple shooting.

2.10 Herbicide Induced Oxidative Stress

Several classes of herbicides have been found to result in ROS generation, either by direct involvement in radical production or by inhibition of biosynthetic pathways (Hess, 2000; Garcia-Plazaola et al., 2002; Kim and Lee, 2005; Artetxe et al., 2006). These herbicides exacerbate the production of ROS, causing a rapid photo bleaching when treated plants are exposed to light (Hess, 2000; Artetxe et al., 2006). The herbicide bipyridinium generates ROS directly in light (Kim and Lee, 2005). Compounds such as paraquat (methyl viologen) induce light-dependent oxidative damage in plants. These ROS initiates lipid peroxidation of PUFAs (Hess, 2000). Other compounds, such as acifluorfen methyl, which blocks photosynthetic electron transport, and norflurazon, which inhibits carotenoid (CAR) biosynthesis, initiate photo-oxidative processes most probably via the generation of $^{1}\text{O}_2$ (Artetxe et al., 2006, Karuppanapandian et al., 2011).
2.10.1 Catalase (H$_2$O$_2$: H$_2$O Oxidoreductase; EC 1.11.1.6)

CATs have been implicated in herbicide tolerance in several plant species. Cat1 transcript increased in the norflurazon-treated scutella of maize W64A (standard maize line) and WDN10 (CAT-2/CAT-3 double null mutant) lines (Jung et al., 2006). In another study, Jung, (2003) showed that Cat1 transcript increased greatly in response to norflurazon in leaves and mesocotyls of two maize lines: W64A (an inbred line) and A130-1 (an SOD mutant). In A130-1, Cat2 transcript increased in mesocotyls after upon norflurazon treatment. A study by Ananieva et al., (2004) revealed that treatment of twelve-day-old barley seedlings with 10 µM/l paraquat caused over a 40% increase in the activity of CAT. CATs hold a great significance in plant tolerance towards the herbicide paraquat (trade name for N,N'-Dimethyl-4,4'-bipyridinium dichloride, a viologen).

Lee and An, (2005) showed that three hot pepper (Capsicum annuum L.) CAT genes (CaCat1, CaCat2, and CaCat3) expressed differentially after paraquat treatment. CaCat1 mRNA increased at 4-12 h in leaves in the paraquat-treated plants; however, CaCAT1 mRNA did not show same induction in the paraquat-treated stems. Willekens et al., (1997) used Cat1AS (containing a Cat1 antisense cassette) and co-suppressed CatGH (containing a Cat1 antisense cassette, and a cassette for sense expression of the cotton catalase SU2) transgenic lines of Nicotiana tabacum L. cv. Petit Havana SR1. Both these lines had approximately 10% of wild-type CAT activity. It was found that these CAT-deficient lines showed increased susceptibility towards paraquat as compared to the wild type tobacco plants, thereby confirming the protective role of CAT against paraquat. The study also confirmed that CAT functions as a sink for H$_2$O$_2$.

Batish et al., (2006) reported that 2-benzoxazolinone (BOA), a potential herbicidal candidate and a well-known allelochemical, caused oxidative stress in P. aureus (mung bean) by generation of reactive oxygen species (ROS). A significant increase in the activity of scavenging enzymes like CAT and SOD was found in the root and leaf tissue of the herbicide-treated mung bean plants. The study concluded that BOA induces oxidative stress in mung bean through generation of ROS and upregulation of activities of various scavenging enzymes. Oxyfluorfen and diuron are the two herbicides commonly used in champagne vineyards. These herbicides are known to generate reactive oxygen species (ROS), and thereby causing oxidative stress (lipid peroxidation, DNA damage, and protein degradation). The toxic effects of these herbicides were studied on the microalgae Scenedesmus obliquus (Geoffroy et al., 2002). Oxyfluorfen significantly induced CAT activity. The activities of other antioxidant enzymes were also
increased; the antioxidant enzyme activities were used as biomarkers of toxicity. In three terrestrial plant species, oat (*Avena sativa* L.), Chinese cabbage (*Brassica campestris* L. cv. *chinensis*) and lettuce (*Lactuca sativa* L.), CAT activity and so also the activity of 3 other antioxidant enzymes SOD, peroxidase, and glutathione reductase was found to increase after exposure to herbicide sodium trichloroacetate (Radetski et al., 2000). The activities of all the 4 enzymes showed the increase at the lowest concentration of the herbicide tested. Germination rate and biomass were also decreased significantly. Transgenic plants carrying CAT genes are also found to show increased tolerance to paraquat. Mohamed et al., (2003) showed that tomato transgenic plants carrying the *E. coli* katE gene (*a* CAT) were less damaged than the wild-type plants when exposed to 100 μM paraquat and high light illumination (800 μmol/m² s⁻¹). The transgenic plants showed increased tolerance to the oxidative damage as well. Harper and Harvey, (1978) compared 4 paraquat-tolerant lines and 11 paraquat-susceptible cultivars of perennial ryegrass (*Lolium perenne* L.) grown under controlled conditions. It was found that paraquat-tolerant lines showed significantly higher activities of CAT than susceptible cultivars.

**2.10.2 Peroxidase (EC 1.11.1.7)**

Peroxidases are also known to play significant role in helping plants tolerate higher herbicide doses; and help overcome oxidative stress produced by herbicides. Wang and Zhou, (2006) reported that an increased peroxidase activity was responsible for counteracting the oxidative stress generated after chlorimuron-ethyl herbicide treatment in wheat. Li et al., (2007) studied the response of two rice varieties (that differed in susceptibility to the herbicide) to bound residues of metsulfuron-methyl in paddy soils. The two rice varieties were differentially susceptible to the herbicide: Xiushui 63, a sensitive rice variety, and Zhenong 952, a resistant variety. It was found that peroxidase activity was higher in the roots of the sensitive rice variety. Irrespective of the two varieties tested, the changes in the enzyme activities were greater in the roots as compared to leaves. *Rehmannia glutinosa* L. plants show the unique characteristic of intrinsic tolerance to paraquat herbicide. The enhanced antioxidant enzyme activities are found responsible for conferring intrinsic paraquat tolerance to *R. glutinosa* plants (Choi et al., 2004). The antioxidant activities of *R. glutinosa* were compared with that of soybean. The peroxidase activity was 2.7-fold higher in paraquat-tolerant *R. glutinosa* than in paraquat-susceptible soybeans. Yamato et al., (1994) purified and characterized a protoporphyrinogen-oxidizing enzyme having peroxidase activity. The enzyme was
purified from tobacco cultured cells. The enzyme showed peroxidase activity toward guaiacol and pyrogallol. It was also found that the purified enzyme was not inhibited by herbicides that inhibit protoporphyrinogen oxidase. This showed that this peroxidase enzyme was responsible for giving herbicide resistance in the tobacco cultured cells. Transgenic plants overexpressing peroxidases also show enhanced tolerance towards herbicides. Tateshi et al., (2001) introduced an aspen peroxidase gene prxA3a into tobacco plants. The transgenic plants showed increased herbicide-tolerance against paraquat, DCMU and atrazine; this study further confirms the importance of peroxidases in herbicide tolerance.

### 2.10.3 Superoxide Dismutase (EC 1.15.1.1)

Superoxide dismutases (SOD; EC 1.15.1.1), originally discovered by McCord and Fridovich in 1969 (McCord and Fridovich, 1969), react with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide. The enzyme is central to the defense mechanism against oxidative stress. Plants contain a mitochondrial matrix localized MnSOD and a cytosolic Cu/ZnSOD, with FeSOD and/or Cu/Zn SOD present in the chloroplast stroma. Chloroplastic SOD is generally the most abundant SOD in green leaves, while in germinating seedlings and in etiolated material the cytoplasmic and mitochondrial SODs are prevalent (Foster and Edwards, 1980; Jackson et al., 1978; Kanematsu and Asada, 1989; Kanematsu and Asada, 1990; Tsang et al., 1991). The expression of SODs is to some extent determined by the availability of their metal cofactors (del Rio et al., 1991).

Any perturbation in photosynthetic activity can cause the formation of reactive oxygen species initiating either from photosystem I, ferredoxin, or excited chlorophyll. Therefore, herbicides that directly affect chloroplast activity can stimulate processes that induce damaging oxygen species. Bipyridyl herbicides such as paraquat and diquat increase oxidative stress by generating oxygen radicals. Both these herbicides appear to mediate identical effects. Paraquat, also known as methyl viologen (1, 1’-dimethyl-4,4’-bipyridinium chloride), is a redox-active compound that is photoreduced by photosystem I and subsequently reoxidized by transfer of its electrons to oxygen, forming the superoxide anion (Asada and Takahashi, 1987; Halliwell, 1984; Rabinowitch et al., 1983).

SOD has often been correlated with paraquat survival. In *E. coli*, which contains both MnSOD and FeSOD, the former enzyme is induced by paraquat (Hassan and Fridovich, 1977). Paraquat is found to induce about 40 different proteins in *E. coli*,
including antioxidant and repair enzymes. Some of these are positively regulated at the transcriptional level by a gene product of the *soxR* locus (Greenberg et al., 1990; Tsaneva and Weiss, 1990). The importance of SOD in paraquat survival has been shown by the isolation of an SOD-deficient mutant that is hypersensitive to paraquat (Carlioz and Touati, 1986).

The SOD activity was found to increase in the green alga *Chlorella sorokiniana* grown in sublethal concentrations of paraquat. The increase was found to be due to the synthesis of a new MnSOD isozyme (Rabinowitch et al., 1983). This induced MnSOD activity, together with other protective enzymes, confers resistance to higher doses of the herbicide. Similarly, treatment of duckweed (*Spirodea oligorrhiza* (Kurz) Hegelm.) with benzyl viologen leads to an increase in SOD activity, which is a factor in the plant’s resistance to paraquat (Lewinsohn and Gressel, 1984).

The treatment of *Phaseolus vulgaris* L. (Chia et al., 1982) and *Lemna* (Srivastave and Tel-Or, 1991) leaves caused a general increase in SOD activity. In *Nicotiana plumbaginifolia* L., chloroplastic, cytosolic, and mitochondrial SOD expression was analyzed at the mRNA level, and all three were strongly induced by paraquat (Tsang et al., 1991).

Besides being involved in paraquat survival, SODs are also important in the metabolism of noxious pollutants like ozone and sulfur dioxide (Bowler et al., 1992). However, the importance of SOD in plant protection to sulfur dioxide is well-established, but the evidence for its involvement in ozone resistance is often indirect and inconclusive.

SOD activity is found to increase up to 13-fold in *Iris pseudacorus* L. during the anoxic phase. In the flooding-sensitive *Iris germanica* L., similar increase in the SOD activity is not observed. This increase reflects the importance of SOD in plants during waterlogging and drought. SOD may play a more direct role in the defense of plants against fungi of the genus *Cercospora*, a large group of fungal pathogens that cause damaging leaf spot diseases on a wide range of economically important crops (Bowler et al., 1992). Thus, it is evident that SODs are also pivotal in relieving plants from stresses caused by atmospheric pollutants, waterlogging and drought, and plant pathogens.

**2.11 Effect of Glyphosate on Morphological and Biochemical Parameters**

Glyphosate treatment causes alterations in phyiology and morphology of target crops, nontarget crops and microorganisms. There are several publications that points out effect of glyphosate on morphological and biochemical parameters of plants.
Huang et al., (2012) studied effect of glyphosate on growth of weed cogongrass (*Imperata cylindrical* L.). The photosynthesis, chlorophyll fluorescence, chlorophyll a and b content, proline content and shikimic acid content were assessed at the second, fifth, and ninth day after treatment with different concentration levels of glyphosate (0%, 0.3%, 0.5%, 1.0%, and 2.0%). Chlorophyll a and b content decreased significantly after treatment.

Parween et al., (2012) evaluated the effect of chlorpyrifos on several metabolic and stress related parameters of *Vigna radiata* L. Twenty-day-old plants were exposed to several concentrations of chlorpyrifos, ranging from 0 to 1.5 mM through foliar spray in the field condition. Lipid peroxidation rate, proline, dehydroascorbate, oxidized and total glutathione were all increased. Chlorpyrifos enhanced lipid peroxidation rate and proline content with 1.5 mM at Day 20 whereas dehydroascorbate, oxidized and total glutathione were increased in 1.5 mM at Day 10. Activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase enhanced significantly in all the concentrations at Day 10. Maximum catalase activity was observed at Day 10 in control and it declined thereafter.

Aioub et al., (1993) compared phytotoxic effects of 30 µM mercury (II) chloride and 50 µM acifluorfen on corn (maize) and found it similar. Acifluorfen treatment elevated MDA levels. Ahsan et al., (2008) reported glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Two-week-old rice leaves were subjected to glyphosate or a reactive oxygen species (ROS) inducing herbicide paraquat, and total soluble proteins were extracted and analyzed by two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis. 18 proteins were found up-regulated and 7 proteins were down-regulated. Rubisco large subunit was significantly decreased by the treatment of both herbicides. An increased accumulation of antioxidant enzymes including superoxide dismutase in the glyphosate-treated sample suggests that a glyphosate treatment possibly generates oxidative stress in plants. Increase in thiobarbituric acid reactive substances (TBARS) concentration revealed that the glyphosate application generates ROS, which resulted in the peroxidation and destruction of lipids in the rice leaves.

Chen and Polatnick, (1991) studied comparative effect of artemisinin, 2,4-D, and glyphosate in mung bean. At 5 µM all these compounds inhibited root induction in mung
bean (*Phaseolus aureus*) seedling cuttings. Artemisinin also caused inhibition in growth like glyphosate.

Elevated antioxidant response and induction of tau-class glutathione S-transferase after glyphosate treatment in *Vigna radiata* was reported by Basantani et al., (2011). Elevated expression of the oxidative stress enzymes and induction of tau-class GSTs after glyphosate treatment in the seedling roots of two *Vigna radiata* varieties was found after glyphosate treatment. Nemat Alla and Hasan, (2007) studied that isoproturon induced significant accumulations of H$_2$O$_2$ in the leaves of 10-d-old maize seedlings, the accumulation increased with time and also with herbicide dose. SOD activity was significantly enhanced up to the 12th d whereas ascorbate peroxidase (APX) activity was significantly reduced. Catalase activity were similarly increased during the first 4 d but decreased from the 12th day. Low doses increased SOD and guaiacol peroxidase (GPX) activities but high doses led to diminutions whereas CAT and APX were reduced by all doses.

Chloropyrifos and malathion generate xenobiotic / pollution stress on tomato and brinjal leading to creation of reactive oxygen species (ROS) in them which culminate the plants into death through cellular damages (Nasrabadi et al., 2011). Scavenging of ROS through stimulation of antioxidant enzymes such as SOD, POD and PPO is the most adaptive mechanism for the tolerance of pollution. Both the pesticides have caused highly significant stimulation in the activities of SOD, POD and PPO, and it increased with the increasing dose of chloropyrifos and malathion.

Wang and Zhou, (2006) reported effects of herbicide chlorimuron-ethyl on physiological mechanisms in wheat (*Triticum aestivum*). Chlorimuron-ethyl elevated MDA content in leaves and roots after a 1-day. The 300 mg/kg chlorimuron-ethyl treatment caused significant damage to chlorophyll accumulation. The POD activity in roots was higher than in leaves. The damage to the antioxidative defence systems is affected by the concentration and exposure time, and the defensive effect is completely lost with prolonged exposure. Herbicide caused a significant decrease of soluble protein content and SOD activity in the leaves and roots. Qian et al., (2008) studied effects of glufosinate on antioxidant enzymes, in the unicellular green alga *Chlorella vulgaris*. Exposure to glufosinate increased MDA content by up to 2.73 times compared with the control. Activities of the antioxidant enzymes SOD, peroxidase POD, and CAT also increased markedly in the presence of glufosinate.
Wu and Von Tiedemann, (2002) reported enhancement of plant antioxidative enzymes and the enhanced scavenging of potentially harmful $O_2^-$ by fungicides as a mechanism of protecting plants against noxious oxidative stress from the environment. They found that fungicide treatments of barley plants at growth stage (GS) 32 significantly increased the total leaf soluble protein content and activities of the antioxidative enzymes SOD, catalase (CAT). Treatment of herbicide chlorotoluron in soil in wheat resulted in the peroxidation of plasma membrane lipids in the plant. Proline level increased significantly in chlorotoluron-exposed roots and leaves. The total activity of POD in roots was significantly enhanced. Activities of APX in roots and leaves showed a similar pattern. The CAT activities were generally suppressed under the chlorotoluron exposure (Song et al., 2007). Chao et al., (2007) found that MDA level detected in the leaves of wheat after 1 day exposure of acetochlor which decreased after prolonged exposure, indicated the presence of poisoning AOS. They suggested that chlorophyll and soluble protein could be considered as biomarkers of stress by acetochlor in soil, activity of POD and contents of MDA could not be considered as biomarkers of stress by acetochlor in soil and role of SOD as biomarkers of stress is doubtful.

Nemat Alla and Hasan, (2007) found that after treatment of isoproturon in maize, SOD activity was significantly enhanced up to the 12th day whereas APX activity was significantly reduced after the fourth day onwards. CAT and GPX activities were similarly increased during the first 4 d but decreased from the 12th and the eighth day, respectively. Low doses increased SOD and GPX activities but high doses lead to diminutions whereas CAT and APX were reduced by all doses. Stajner et al., (2003) found that paraquat (1.0 and 2.0 µM) treatment inhibits germination and SOD and CAT activities in seeds. In leaves, lower concentrations of these herbicides increased activities of antioxidant enzymes but at the highest herbicide concentrations (200 M) activities of investigated enzymes declined. The pigment contents the leaves decreased due to alachlor and metolachlor treatment in a concentration dependent manner. Growth of wheat seedlings with isoproturon was inhibited (Yin et al., 2008). Chlorophyll content significantly decreased at the low concentration of isoproturon (2 mg/kg), suggesting that chlorophyll was rather sensitive to isoproturon exposure. Activities of the antioxidant enzymes showed a general increase at low isoproturon concentrations and a decrease at high isoproturon concentrations. Activities of CAT in leaves showed progressive suppression under the isoproturon exposure.
Kitchen et al., (1981) found that glyphosate significantly decreased the chlorophyll content of field-grown soybeans (*Glycine max* L. Merr. ‘Williams’) within 48 h after a 2.24-kg/ha treatment. In studies with 8-day-old etiolated corn shoots, 0.1, 1.0, and 10.0 mM glyphosate decreased chlorophyll content of corn shoots 24, 42, and 50%, respectively, after 12 h of illumination.

**Table 2.1: In vitro selection for abiotic stress tolerance**

<table>
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<tr>
<th>Plants</th>
<th>Agronomic traits</th>
<th>References</th>
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<td>Ametryn</td>
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<td>Wheat</td>
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Table 2.2: *In vitro* selection of stress tolerant genotypes.

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<tr>
<td>Wheat</td>
<td>Frost</td>
<td>Moraru et al., (1997)</td>
</tr>
<tr>
<td><em>Vigna mungo</em></td>
<td>moisture</td>
<td>Yadav et al., (2013)</td>
</tr>
<tr>
<td></td>
<td>deficit stress</td>
<td></td>
</tr>
</tbody>
</table>
3.0 Materials and Methods

3.1 The Plant Material Used in the Present Study: *Vigna mungo* (L.) Hepper

3.1.1 Classification

**Kingdom Plantae** – Plants

**Subkingdom Tracheobionta** – Vascular plants

**Superdivision Spermatophyta** – Seed plants

**Division Magnoliophyta** – Flowering plants

**Class Magnoliopsida** – Dicotyledons

**Subclass**- Rosidae

**Order** - Fabales

**Family**- Fabaceae – Pea family

**Genus** - *Vigna* Savi – cowpea

**Species** - *Vigna mungo* (L.) Hepper – black gram

Black gram is an annual, semi-erect to spreading herb growing to a height of 25-90 cm. Stems are diffuse, branching, sometimes procumbent, and covered with long dense brown or black hairs. It possesses strong tap root system with many laterals. Leaves are pinnately trifoliate, hairy with large ovate to lanceolate and entire leaflets. Flowers are pale yellow, small with a yellow spirally coiled keel. The flowers are borne in clusters of 5-6 on a short hairy peduncle in axillary racemes. Pods are short, erect to sub erect, 4-7 cm long and 0.6 cm wide, brown to black in colour, hairy and with stout hooked beak, containing about 6-10 seeds. Seeds are small, oblong slightly truncated at ends measuring 4-5.2 mm long and 3.5-4.1 mm wide; thousand seeds weight is around 40 g with varying colour from black, dark brown to green. The testa is smooth and hilum white and concave. Pods do not shatter readily. Flowers are self-fertile and self pollinated. Flowering is indeterminate.

3.1.2 Genotypes used in Present Study

1. PU-19
2. PU-35
3. PU-31
4. IPU-94-1
5. Azad-1
6. Azad-2
3.1.3 Germplasm Collection

The six genotypes of leguminous plant *Vigna mungo*, were selected for our investigations. The seeds of these were used as material in the experiment. The seeds were bought from Indian Institute of Pulse Research (IIPR), Kanpur, these were used as starting material for the experiment. As there were seeds of different size and color, so seeds of same average size and of same color were selected to perform experiments. Selection of seeds done carefully so as to select, the healthy and viable seeds, to get most accurate result in the experiment, seed of uniform morphology and color were only used for experiment.

3.2 Methods of Screening

Following *in vitro* methods has been used for screening of herbicide tolerant genotypes

3.2.1. Petridish germination screening

3.2.2 *In vitro* germination screening in MS medium

3.2.3 *In vitro* culture screening

3.2.3.1. *In vitro* assessment of tolerance based on morphogenic response of various explants on herbicide supplemented medium.

3.2.3.2. Gradual stress treatment of callus cultures

3.2.3.3 Shock treatment of callus cultures

3.2.4. *In vivo* evaluation of herbicide effect on selected genotypes in field under wire house conditions.

3.2.1. Petridish Germination Screening (*Petridish Bioassay*)

The seeds were thoroughly washed with double distilled or autoclaved water before conducting any experiment. In order to screen the genotypes for herbicide tolerance the seeds were grown in petridishes, using moist blotting paper method. Glyphosate solutions of increasing concentrations were prepared using isopropylamine salt of glyphosate (41%) in autoclaved water. The glyphosate concentrations employed were, 1 mM, 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM. The control seeds were treated with water only. The seeds of six genotypes of *Vigna mungo* were soaked for 24 hrs in the glyphosate/roundup solutions, or water in case of control. All the treatments were given in triplicate. After soaking the seeds for 24 hrs, they were thoroughly washed with autoclaved water and placed on moist filter paper in petridishes, for germination. The seeds were watered each day. Readings were taken after 2, 7 and 10 days. In order to avoid infection, the blotting paper was changed every 2 days. The growth parameters
studied were germination, survival, shoot length, root length, fresh weight, and dry weight. Fresh weight was taken using electric pan (Eagle digital) balance. Dry weight was taken after drying seedlings for 24 hour at 70°C till the weight stabilized. To assess tolerance various parameters were calculated:

Germination percentage was calculated by

\[
\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100
\]

Survival percentage was calculated by

\[
\text{Survival percentage} = \frac{\text{Number of seedlings survived}}{\text{Total number of seeds}} \times 100
\]

Percentage of phytotoxicity was calculated according to Chou et al., (1978)

\[
\frac{\text{Radicle length of control} - \text{Radicle length of treatment}}{\text{Radicle length of control}} \times 100
\]

Vigour index of seedling was calculated according to Abdul-Baki and Anderson, (1973).

\[
\text{Vigour index} = \text{Germination percentage} \times \text{length of seedlings}
\]

Tolerance index of seedlings was calculated according to Turner and Marshal, (1972).

\[
\text{Tolerance index} = \frac{\text{Mean length of longest root in treatment}}{\text{Mean length of longest root in control}}
\]

3.2.2 In vitro Germination Screening

- Seeds collected from homogeneous population of six varieties of black gram PU-19, PU-35, IPU-94-1, PU-31, Azad-1 and Azad-2 were grown on Murashige and Skoog’ basal medium supplemented with various concentrations of herbicide (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM)
- Readings were taken after 2, 7 and 15 days after inoculation (DAI) and on the basis of observations made on parameters like germination percentage, survival percentage, root length, shoot length, seedling fresh weight they were classified as tolerant, moderately tolerant and susceptible.
- Effect of herbicide was also assessed on biochemical parameters like activity of antioxidative enzymes catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), protein content and proline content in seedlings.

3.2.3. In vitro Culture Screening

3.2.3.1 Screening via Response of Explants in Herbicide Media

- Seeds germination on MS basal medium.
• Explants, like epicotyl, hypocotyl, cotyledonary node, shoot apex and leaf segments obtained from 7-8 days old seedlings transferred on medium with different levels of herbicide (stress) and plant growth regulators. Herbicide free media was treated as control.
• Percentage of morphogenic response, callus fresh weight, number of shoots per explant, shoot length, number of roots per explant and root length were determined.
• On the basis of above said parameterers better performing cultures on high stress media were treated as tolerant.

3.2.3.2 Gradual stress treatment of callus cultures
• After establishing callus, shoot and root cultures in stress free media they were gradually transferred into media with higher concentration of glyphosate with same or altered concentration of plant growth regulators.
• Glyphosate concentrations of 0 mM (control), 0.01 mM, 0.05 mM, 0.1 mM 0.2 mM, and 0.4 mM were added to MS proliferation media. 20 ml media was poured in each test tube. In gradual treatment method, callus and shoot cultures were gradually exposed to herbicide stress by first culturing them on non stress media/non selective media without herbicide (control). Callus grown on control media were incubated for three weeks. After three weeks a portion of callus was subcultured on control media and another portion was subcultured on media with 0.01 mM glyphosate. After three weeks one portion of callus was subcultured on media with 0.01 mM and another portion on media with 0.05 mM. Similarly calluses were subcultured up to 0.4 mM media. After four weeks, cultures surviving on 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM were used for data collection i.e. fresh weight, tolerance index and percent of surviving cultures.

Tolerance index (callus) was calculated according to LaRosa et al., (1989)

\[ \text{Tolerance index} = \left( \frac{\text{Fresh weight of callus in stress media}}{\text{Fresh weight of callus in control media}} \right) \times 100 \]

3.2.3.3 Shock treatment of callus cultures
• Initially cultures were grown in herbicide free media. Then these cultures were transferred to stress media
• Callus and shoot cultures cultures grown in stress free media were directly transferred to high stress media 0.1, 0.2 and 0.4 mM. Among 60 cultures grown
in stress free media 15 cultures were transferred to media of each herbicide concentration.

3.2.4. **In Vivo Evaluation of Tolerant Genotypes**

Three better performing genotypes of black gram screened on the basis of petridish screening, *in vitro* germination screening and *in vitro* culture screening were sown in field under wire house conditions. At the three leaf stage post emergence spray treatment of herbicide glyphosate was given to plants. Toxicity symptoms and change in biochemical parameters like chlorophyll content, protein content, proline content, MDA content and status of antioxidative enzymes like catalase, peroxidase and superoxide dismutase were studied after 3-4 days of treatment. Effect of herbicide on growth and yield parameters were also assessed in three better performing genotypes.

3.3 **Surface Sterilization of Plant Materials**

For the *in vitro* screening of tolerance seeds of six genotypes were first washed thoroughly under filtered running tap water for 10 minutes and then treated with 2-3 drops of tween -20 per 50 ml, for 10 min and then washed with distilled water 2-3 times. Addition of few drops of detergent helps in removing the waxy coating and also reduces surface tension (acts as surfactant). Subsequently they were further sterilized under aseptic conditions, in a laminar flow cabinet with 0.1% HgCl₂ solution for 10 minutes and washed 4-5 times with distilled water. Lastly material was treated with quick dip of 90% alcohol and washed with autoclaved distilled water.

3.4 **Sterilization of Nutrient Media and other Items**

Sterilization of nutrient media, instruments, beakers, petridishes, distilled water etc was done by autoclaving at 1.08 kg cm⁻² for 15 minutes.

3.5 **Inoculum and Inoculation**

3.5.1 **Inoculum**

Sterilized seeds were inoculated in MS basal media. 1-2 seeds were inoculated in each test tube containing 20 ml of 0.8% agarified MS medium (either basal or supplemented with plant growth regulators) with flamed/sterilized forceps. Various explants like hypocotyl, epicotyl, cotyledonary node leaf segments and shoot apices were excised from 7-8 day old *in vitro* grown seedlings. These explants were inoculated in various selective (herbicide supplemented) and non selective media (herbicide free) to assess effect of herbicide on callus induction, organogenesis, callus weight, number of shoots and roots.
3.5.2 Inoculation

Explants to be inoculated were cut from seedlings under aseptic conditions in a laminar air flow cabinet and inoculation was carried out by using distilled water and 95% alcohol. Besides, a spirit lamp was also used for periodic resterilization of used instruments during inoculation. During inoculation neck of test tubes and test tube caps were warmed over flame to avoid contamination. In most of the experiments one or two explant were inoculated per culture tube with cut basal end inserted in agarified medium supplemented with hormones and herbicides.

3.6 Culture Media

Different growth media were used for the initial experiment but since the best response was obtained in Murashige and Skoog medium (1962) it was used for all the subsequent experiments. Four different stock solutions of major elements, minor elements and vitamins were prepared in distilled water. All the stock solutions were stored in a refrigerator at 4-5°C. To prepare one liter of solution, desired quantities of sucrose (30.0g) and agar (8.0g) were weighed. The required quantities of stock solutions (A, B, C and D) containing major elements, minor and the organic nutrients were measured in conical flask. Sucrose was used as carbon source at a concentration of 30g/l. The pH of the medium was adjusted to 5.7 by using 1N HCl and 1N NaOH. The final volume was made up to 1 liter with distilled water. In the end weighed agar was added and the solution was boiled with constant stirring to homogenize the contents and the growth regulators were added as required. The medium was again boiled so as to ensure complete blending of growth regulators. About 15-20ml of medium was dispensed into each test tube and plugged with nonabsorbent surgical cotton wrapped in muslin/or autoclavable test tube caps. The medium was autoclaved at 15-kg/cm³ pressure for 15-20 min at 121°C and the solidification was done at room temperature. Apart from this, flasks containing distilled water, petridishes, instruments like a pair of forceps, blades etc. were also autoclaved at same pressure, wrapped with aluminum foil. The equipment was taken out when the pressure of the autoclave came down to zero, and was placed in an aseptic room. The composition of Murashige and Skoog (1962) medium is as follows in (Table -1 and 2).

3.6.1 Variations of Media used in Study

1. MS basal media without growth hormone and herbicide.
2. MS basal media with herbicide but without growth hormone.
3. MS basal media with growth hormone but without herbicide (non selective media).
4. MS basal media with growth hormone and various concentrations of herbicide.

Table -3.1: Inorganic Constituents

<table>
<thead>
<tr>
<th>Major element</th>
<th>Amount (mg/l)</th>
<th>Minor elements</th>
<th>Amount (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>1650.00</td>
<td>ZnSO₄.7H₂O</td>
<td>8.60</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900.00</td>
<td>MnSO₄.4H₂O</td>
<td>22.30</td>
</tr>
<tr>
<td>CaCl₂H₂O</td>
<td>440.00</td>
<td>CuSO₄.5 H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370.00</td>
<td>CoCl.6H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170.00</td>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.00</td>
<td>H₂BO₃</td>
<td>0.62</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>27.00</td>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table -3.2: Organic Constituents

<table>
<thead>
<tr>
<th>Amino acid and Vitamins</th>
<th>Amount (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100.00</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxin HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The stock solutions were prepared in distilled autoclaved water and then stored in the refrigerator.

3.7 Growth Regulators

Depending upon the nature of the experiment the basal medium was supplemented with various concentrations and combinations of the following growth regulators, which were prepared as follows in (Table -3.3)
Table-3.3: Growth Regulator Preparation

<table>
<thead>
<tr>
<th>Growth Regularos</th>
<th>Abbreviations</th>
<th>Amount (mg)</th>
<th>Dissolved in (ml)</th>
<th>Final volume with H₂O (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α- Naphthalene acetic acid</td>
<td>NAA</td>
<td>50</td>
<td>5 ml 80% ethanol</td>
<td>50</td>
</tr>
<tr>
<td>Indole acetic acid</td>
<td>IAA</td>
<td>50</td>
<td>5 ml 80% ethanol</td>
<td>50</td>
</tr>
<tr>
<td>2-4Dichlorophenoxy acetic acid</td>
<td>2-4-D</td>
<td>50</td>
<td>5 ml 80% ethanol</td>
<td>50</td>
</tr>
<tr>
<td>6-Benzylamino Purine</td>
<td>BAP</td>
<td>50</td>
<td>5 ml of warm 0.1 N NaOH</td>
<td>50</td>
</tr>
<tr>
<td>6- Furfurylamino Purine</td>
<td>Kn</td>
<td>50</td>
<td>5 ml of warm 0.1 N NaOH</td>
<td>50</td>
</tr>
</tbody>
</table>

The composition of culture medium is the most important factor in the successful establishment of a tissue culture protocol. MS medium consists of a balanced mixture of micronutrients, macronutrients, vitamins, carbon source, organic growth factors and a source of reduced N₂ supply.

3.8 Explants

Hypocotyls, epicotyls, cotyledonary node, node (explants from field grown plants) shoot apices and leaf segments of seedlings were used for tissue culture studies.

3.9 Maintenance of Culture

All cultures were grown in an air-conditioned culture room, illuminated by 40-watt fluorescent tubes. Culture room temperature was maintained at 25±2°C with 16 hours photoperiod at a photo flux density of 1000 Lux approximately. Sub-culturing was done approximately every 30 -40 days of growth according to the need.

Morphogenetic response has been represented in terms of:
- Percentage of explants that produced callus, roots or shoots
- Number of roots per culture
- Number of shoots per culture
- Length of roots per culture
- Length of shoots per culture
- Callus fresh weight and dry weight.
The percent callus response or percent shoot response was calculated using the following formula:

\[
\text{Percent callus/shoot response} = \frac{\text{Number of explants showing callusing/shooting}}{\text{Total Number of explants inoculated}} \times 100
\]

### 3.10 Screening of Glyphosate Tolerant Genotypes at Multiple Shoot and Callus Levels

Mainly three types of selection schemes were employed to screen tolerant genotypes for herbicide tolerance. The purpose of making such selections is to assess natural tolerance of black gram genotypes and to improve tolerance by *in vitro* methods. In tissue culture method, percent callus response, callus fresh weight, shoot buds and multiple shoot induction, number and length of shoots were taken as criteria for screening of tolerance among genotypes. Gradual and shock stress treatment method were also employed to assess tolerance among genotypes.

For *in vitro* studies, first culture conditions were standardized and various explants of seedlings have been exposed to various levels of glyphosate induced herbicide stress. Stock solution of 30 mM was prepared and to make MS medium with different glyphosate concentration calculated amount of stock were added into medium. For example to prepare 25 ml stock of 30 mM glyphosate solution, 419.99 µl glyphosate was added. To prepare 500 ml medium of 0.01 mM 0.166 µl of stock was added. For *in vitro* germination of seeds in herbicide media no growth regulator was added. While to observe/assess effect of herbicide on morphogenic response on black gram genotypes glyphosate were added in MS media supplemented various combinations of growth regulator.

### 3.11 Chemicals

All the chemicals used in the present investigation were analytical grade of high quality, and purchased from authentic sources. Tris buffer, glycine, EDTA, PMSF, CDNB, reduced glutathione (GSH), NaOH, potassium phosphate were purchased from HiMedia Ltd, Mumbai., methanol, acetic acid were obtained from Qualigens Fine Chemicals, Mumbai. Bradford Reagent was purchased from BioRad, USA (Bio-Rad, Inc. Hercules, California USA). Isopropylamine salt of glyphosate (41%) was obtained from Monsanto, USA.

The stock solutions were prepared in double distilled water or autoclaved water. The tris-buffer, phosphate buffer were autoclaved before use. PMSF stock solution was prepared in iso-propyl alcohol and CDNB stock was prepared in ethanol.
3.12 Glyphosate

Glyphosate is a post-emergent, systemic and non-selective (or broad-spectrum) herbicide used in both agricultural and non-agricultural areas. Recommended application rates do not exceed 5.8 kg active ingredient per hectare (a.i./ha). It is used to kill all plant types including grasses, perennials, and woody plants. It is mainly absorbed into the plant through the leaves and then transported throughout the plant where it acts on the plant’s enzyme systemic a post-emergent, systemic and non-selective (or broad-spectrum) herbicide used in both agricultural and non-agricultural areas. In present investigation round up formulation is used in all experiment which is 41% salt of isopropylamine +59% inert materials. Rate of application of roundup herbicide in modern agricultural practices is 2-3 litres of roundup per hectare in 450 litres of water.

3.12.1 Various Glyphosate Concentrations used in Present Study

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Glyphosate Concentrations Used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Vivo Studies</td>
</tr>
<tr>
<td>PU-19</td>
<td>Control</td>
</tr>
<tr>
<td>PU-35</td>
<td>1 mM</td>
</tr>
<tr>
<td>PU-31</td>
<td>2 mM</td>
</tr>
<tr>
<td>IPU-94-1</td>
<td>4 mM</td>
</tr>
<tr>
<td>Azad-1</td>
<td>6 mM</td>
</tr>
<tr>
<td>Azad-2</td>
<td>8 mM</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
</tr>
</tbody>
</table>

In developing a screening/selection scheme to select tolerant genotypes, it is important to know how the crop reacts to herbicide, identify mechanism of herbicide tolerance. Once the mechanism is identified different genotypes could be studied in order to determine genotypic differences for tolerance.

3.13 Protein Extraction

Protein content were measured in in vitro grown seedlings and leaves of treated plants sunder selective media and in plants after 3-5 days of post-emergence spray treatment of herbicide grown in wire house. In case of in vitro grown seedlings whole
seedling and in treated field plants upper leaves were taken for protein extraction. The
seedlings were harvested after 15 days while leaves were taken after 5 days of treatment
for protein extraction. The extracted protein was used for total soluble protein estimation.
The seedlings (0.5 g) were crushed in liquid nitrogen in a mortar and pestle and
homogenized in 2 volumes (w/v) of extraction buffer (0.2 M Tris-Cl pH 7.8, 1 mM
EDTA, 20% glycerol and 2 mM PMSF). The homogenate was centrifuged at 14,000 rpm
for 20 mins. The supernatant was used for protein estimation and enzyme activity assays.
The protein extracts were stored at -20ºC until further use.

3.13.1 Bradford Protein Estimation

Protein was estimated according to the Bradford dye-binding assay (Bradford,
1976). The Bradford dye consists of Coomassie Brilliant Blue G-250, which binds to
arginine, aromatic amino acid and histidine residues of the protein. The assay is based on
the observation that the absorbance maximum for an acidic solution of Coomassie
Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both
hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible
color change (Bradford, 1976).

The 5X Bradford dye (Bio-Rad, USA) was diluted to 1X with water and filtered
through Whatman filter paper no 1. The estimation was done according to
manufacturer’s instructions (Bio-Rad Protein Assay Instruction Manual, #LIT33).
Bovine serum albumin (BSA) was used as the standard. To perform the assay, 5-10 µL
plant protein extract was diluted to 50 µL with water and mixed with 2.5 ml of 1X
Bradford dye. The mixture was allowed to stay at room temperature for 5 to 10 minutes.
Then optical density was read at A595. First, the standard graph was plotted between A595
and the known amounts of BSA. Thereafter, the A595 of unknown plant protein samples
was observed. The A595 of plant protein samples was compared with A595 of BSA of
known quantities. The protein concentration was expressed in mg protein per g of fresh
weight (FW) tissue.

3.14 Enzyme Assays
3.14.1 Catalase Enzyme Assay

Catalase activity was measured according to Euler and Josephson, (1927). A
2.5% protein extract was prepared. 2 ml citrate phosphate buffer (pH 7.0), 1 ml water
and the protein extract were taken in two sets of test tubes. One of the sets was labeled
blank and the other as sample. In the sample, 1ml H2O2 was added and exactly after 10
min the reaction was stopped by adding 2ml 4N H2SO4. In the blank, 2ml 4N H2SO4 was
added first and then 1ml H₂O₂ was added. The reaction mixtures were titrated against 0.01N KMnO₄ till the end point was (light pink) reached. The catalase activity was expressed as µmole H₂O₂ decomposed per 100mg fresh weight (FW) of tissue.

3.14.2 Peroxidase Enzyme Assay

Peroxidase activity was estimated by a modified method of Luck, (1963). 2.5% protein extract was prepared. 2ml citrate phosphate buffer (pH 6.0), 1ml H₂O₂ and 1ml p-phenylenediamine were taken in two sets of test tubes; one was blank and the other was labeled sample. In the sample, the protein extract was added and allowed to stand for 10 min. After 10 min 2ml 4N H₂SO₄ was added to stop the reaction. In the blank, 2ml 4N H₂SO₄ was added and then 1ml enzyme extract was added. All the reaction mixtures were allowed to stand for 1 hr at room temperature in the dark. After 1 hr A₄₈₅ was measured with the spectrophotometer. The enzyme activity was expressed as the difference in OD between blank and sample per 100mg fresh weight (FW).

3.14.3 Superoxide Dismutase Enzyme (SOD) Assay

The activity of SOD was assayed by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT). The assay was conducted according to the method of Beauchamp and Fridovich, (1971). The reaction mixture contained 50 mM potassium buffer (pH 7.8), 10 mM methionine, 1.17 mM riboflavin and 56 mM NBT and the enzyme extract. The final volume of the reaction mix was made up to 3.0 ml. Riboflavin was added in the last. The reaction mixture was incubated in light. The blanks (without enzyme) were not illuminated, and incubated in dark. The reaction was allowed to take place for 30 min. After the reaction was complete the absorbance of the reaction mixtures was measured at 560 nm. One unit of SOD represents the amount of enzyme that inhibits the NBT reduction by 50%.

3.15 Proline Estimation

Extraction and determination of proline was performed according to the method of Bates et al., (1973). 500 mg plant tissue was homogenized in 5 ml of 3% sulphosalicylic acid. Extracts (2 ml) were held for 1h in boiling water by adding 2 ml ninhydrin regent(1.25 g ninhydrin + 30 ml glacial acetic acid + 20 ml 6 M phosphoric acid) and 2 ml glacial acetic acid, after which cold toluene (4ml) was added. Absorbance of supernatant was read at 520 nm using double beam UV-VIS spectrophotometer UV5704SS and calculated as µmol/g fresh weight (FW) against standard proline.

3.16 Malondialdehyde (MDA) Content Estimation/Lipid Peroxidation
The level of lipid peroxidation in plant tissue was measured by the method of Heath and Packer, (1968) in terms of malondialdehyde content, a product of lipid peroxidation determined by the thiobarbituric acid reaction. Fresh control and treated leaf tissue (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3,500 rpm for 20 min. To 1 ml of aliquot of the supernatant, 1 ml of 20% TCA containing 0.5 % (w/v) TBA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in ice bath. The contents were centrifuged at 10,000 X g for 15 min and then absorbance was measured at 532 nm using double beam UV-VIS spectrophotometer UV5704SS. Value for non–specific absorbance at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$ and expressed as nmol/g fresh weight (FW) tissue.

3.17 Pigment Estimation

Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were estimated. The pigment estimation was performed as described by Arnon, (1949). The seedling leaves were homogenized in chilled acetone. 100 mg leaf tissue was ground in 10 ml of 80% acetone (1:10 w/v). The homogenate was centrifuged at 5000 rpm for 10 mins. After centrifugation the supernatant was collected and the absorbance was read at 645, 652, 663 nm for chlorophyll; and 480, 510 nm for carotenoid estimations using double beam UV-VIS spectrophotometer UV5704SS. The amount of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid, and was calculated according to the following formulae. The amount of each of these was expressed in mg/g fresh weight (FW).

$$\text{Chl a} = [12.7(A_{663}) - 2.69(A_{645})] \times \frac{V \times 1}{1000 \ \text{Wt}}$$

$$\text{Chl b} = [22.9 \ (A_{645}) - 4.68(A_{663})] \times \frac{V \times 1}{1000 \ \text{Wt}}$$

$$\text{Chl total} = A_{652} \times \frac{V}{34.5 \ \text{Wt}}$$

$$\text{Carotenoids} = 7.6(A_{480}) - 1.49(A_{510}) \times \frac{V \times 1}{1000 \ \text{Wt}}$$

$V$ = volume, $Wt$ = weight in g
3.18 Statistical Analysis

The data were maintained in minimum three replicates and data recorded from all the screening methods was subjected to statistical analysis. Groups were compared by either one-way analysis of variance (ANOVA) or two-way ANOVA and the significance of mean difference of control group with other treatment groups was done by LSD test. One-way analysis of variance (ANOVA) was performed by statistical software SPSS ver.19.0 whereas two-way ANOVA was calculated by Graph Pad Prism (version 5.0).

Mean:

It was calculated by following formula:

\[ X = \frac{\Sigma X}{N} \]

Standard Deviation (SD) (\( \sigma \)):

It was calculated by squaring the deviation of each observation from the mean, adding the squares, dividing by a number of observations and extracting the square root by using the following formula:

\[ \text{S.D.}(\sigma) = \sqrt{\frac{\Sigma fd^2 - (\Sigma fd)^2}{N-1}} \]

Where:
- \( f \) = frequency
- \( d \) = deviation
- \( \Sigma \) = sign of summation
- \( N \) = number of observations.

Standard Error:

It is a measure of the mean differences between the sample estimate of mean and the population parameter i.e. it is the measure of uncontrolled variation present in a sample. It is estimated by dividing the estimate of standard deviation by the square root of the number of observation in the sample. Thus,

\[ SE = \frac{SD}{\sqrt{N}} \]

Percent of Control:

To calculate the percent control value following formula was applied.

\[ \text{Percent of control} = \frac{\text{Value in treatment}}{\text{Value in control}} \times 100 \]
**Assessment of Variability:**

The structure for analysis of variance (ANOVA), which was used for the experiments laid out in random block design is given below.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Means sum of squares</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>r-1</td>
<td>S</td>
<td>S/(r-1)=v</td>
<td>V/w</td>
</tr>
<tr>
<td>Treatment</td>
<td>t-1</td>
<td>P</td>
<td>P/(t-1)=u</td>
<td>U/w</td>
</tr>
<tr>
<td>Error</td>
<td>(t-1)(r-1)</td>
<td>Q</td>
<td>q/(t-1)(r-1) = w</td>
<td></td>
</tr>
</tbody>
</table>

Where:

- \( t \) = Number of treatments
- \( S \) = Sum of square due to replications
- \( P \) = Sum of square due to treatments
- \( q \) = Sum of square due to error
- \( F \) = Variance ratio
- \( v \) = Mean sum of squares due to replication
- \( u \) = Mean sum of square due to treatments
- \( w \) = Error mean square
Weed control is the primary yield limiting factor in pulse production, which impacts essentially every acre in commercial production worldwide. Cost effective, efficient, environmentally friendly weed control methods are essential to sustainable pulse production. During present study black gram genotypes have been screened for tolerance to glyphosate herbicide at practical application levels. These genotypes were screened via in vitro selection and did not involve transgenic approaches. This study offers the possibility of creating non-transgenic, glyphosate tolerant black gram cultivars and eliminating market resistance and perception issues against transgenic crops. This selective method will lead to enhanced profitability and likewise promoting economic and environmental sustainability in black gram farms.

The present study was performed to investigate the in vitro methods that can be used for screening of herbicide tolerant genotypes in Vigna mungo. There is lot of literature on screening of stress tolerant plants in various crops using in vitro methods (in vitro selection) but unfortunately there is no any report to the best of our knowledge on screening of herbicide tolerant plants/genotypes in black gram which is one of the most valuable pulse crop in India. The study was performed, keeping in view that post emergent herbicides are generally used only to eradicate weeds in the crop fields including pulses but broad spectrum herbicides like glyphosate can not distinguish between weeds and desired crops so they cause harm to the latter. If we can screen the tolerant cultivars which may occur naturally in environment we can use post emergent herbicides for weed eradication during standing crop season.

In the present study we investigated the response of Vigna mungo genotypes using petri dish bioassay or petri dish germination test at varying level of glyphosate concentration and studied effect of different concentration of glyphosate herbicide on morphological parameters like germination percentage, survival percentage, root length, shoot length, seedling fresh weight and dry weight. Under in vitro studies we first screened genotypes by in vitro germination on MS basal media supplemented with different concentration of glyphosate herbicide and also by inoculating various seedling explants (grown in MS basal free from herbicide) into selective herbicide media. For further confirmation of tolerance we chose indirect (gradual treatment) and direct (shock treatment) methods. In in vitro germination method screening was done by observing effect of glyphosate herbicide on the above mentioned morphological parameters.
Activity of antioxidants like catalase, peroxidase superoxide dismutase, proline content, lipid peroxidation and total protein content was also investigated. Impact of herbicide on morphogenic response, percent callus response, number of shoots and roots and length of shoots and roots were also studied in all genotypes used in study. The isopropylamine salt of glyphosate, RoundUp (Monsanto, USA), was used in all the experiments. The molecular weight of glyphosate mono-isopropylammonium is 228.2 and it is supplied as a 41% solution. For petridish screening procedure seeds were soaked for 24 hrs in increasing glyphosate concentrations, after thorough washing the seeds were allowed to germinate in petridishes. For *in vitro* germination screening procedure sterilized seeds were inoculated in test tubes. After germination the data on various morphological and biochemical parameters were taken and analysed. For indirect (gradual treatment) and direct (shock treatment) various explants obtained from *in vitro* grown seedlings are inoculated in MS media supplemented with various growth hormone and varying glyphosate concentrations. The conclusions drawn from these experiments have been detailed and discussed in the present chapter.

4.1 Petridish Germination Test

The morphological parameters like seed germination and survival percentages, seedling root length and seedling fresh weight and dry weight of germinated seedlings were measured after the treatments.

4.1.1 Germination Percentage

Mean values for germination percentage (GP) of six black gram genotypes differed significantly after herbicide stress induced by glyphosate treatment. Treatment with glyphosate led to a decrease in germination percentage in all the varieties (*Plate-1*). In control maximum germination was in PU-19 (100.00±0.00%) and minimum in PU-31 and Azad-2 (91.11±2.22%). Glyphosate caused maximum percent decrease over control in germination percentage at 10 mM of PU-35 and Azad-2 where germination was reduced up to 92.68% while minimum percent inhibition over control was found in Azad-1(86.36%). Among treatment there was no significant difference on lower doses 1 mM and 2 mM but showed significant (P<0.05) reduction in GP at higher doses (4, 6, 8 and 10 mM). The glyphosate concentration which led to about 50% inhibition over control in germination was different for all the six genotypes. It was 8 mM for PU-19 (46.88%) and PU-35(51.56%), and 6 mM for IPU-94-1, PU-31, Azad-1 and Azad-2 (43.18, 43.90, 47.73 and 56.10% respectively) (*Table-4.1, Figure-4.1A*). Two way
anova showed a highly significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between two factors. Factor 1- F_{5,84}=8.713, p<0.0001; Factor 2- F_{6,84}=461.3, p<0.0001; factor 1x2- F_{30,84}=3.187, p<0.0001 (Appendix Table -1).

4.1.2 Survival Percentage

The seedling survival showed a marked difference in all the six genotypes. The percentage survival of seedlings at the highest glyphosate concentration (10 mM) was 8.89±2.22 in PU-19, 4.44±2.22 in PU-35, 6.67±3.85 in IPU-94-1 and 6.67±0.00 Azad-1 whereas it was only 2.22±2.22 in Azad-2 and PU-31. The percentage survival showed a gradual decrease with increasing glyphosate concentrations in all the six genotypes (Table-4.2, Figure-4.1B). In PU-19 maximum survival was in control (88.89±4.44) and minimum at 10 mM (8.89±2.22) with percent reduction of 90.00 %. In PU-35, maximum survival was in control (80.00±0.00) and minimum at 10 mM (4.44±2.22) with percent reduction of 94.44 %. In IPU-94-1 percent reduction of 91.89 % was obtained between maximum survival in control and minimum at 10 mM. % In PU-31, Azad-1 and Azad-2 percent reduction of 97.06 %, 91.43% and 96.88% was obtained between maximum survival in control and minimum at 10 mM. The glyphosate concentration which led to 50% or reduced survival was different for all the six genotypes. It was 6 mM (52.50%) for PU-19, 4 mM (41.67%, 48.65%) for PU-35 and IPU-94-1, 6 mM (58.82%, 45.71%) for PU-31 and Azad-1 and 4 mM (53.13%) for Azad-2 but percent reduction with respect to control in survival percentage at 10 mM was PU-19 (90.00), PU-35 (94.44), IPU-94-1(91.89), PU-31(97.06), Azad-1(91.43) and Azad-2(96.88). Two way anova revealed a significant effect of genotype (factor -1) and concentration (factor -2) but not of interaction between two factors (genotype and concentration).Factor 1- F_{5,84}=9.741, p<0.0001; Factor 2- F_{6,84}=215.1, p<0.0001; factor 1x2- F_{30,84}=1.114, p<0.34 (Appendix Table-2).

4.1.3 Seedling Root Length

This parameter is most sensitive for herbicide among all the parameters studied showing significant (P<0.05) reduction in root length at 2 mM in all the genotypes except Azad-2 (Table-4.3). With increasing concentration there was progressive decline in root length in all the genotypes (Plate-1C). Highest root length was observed in control and seedling root length in various genotypes was: PU-19(6.06±0.27cm), PU-35 (5.77±0.22cm), IPU-94-1(5.87±0.18cm), PU-31(5.90±0.21cm), Azad-1(5.83±0.09cm) and Azad-2(4.80±0.12cm) and at 10 mM PU-19 and Azad-2 had highest and lowest root
length (0.49±0.03 and 0.23±0.12 cm) respectively. 1 mM concentration did not show significant difference on root length but concentration greater than it caused significant reduction in root length in all genotypes. In concentrations higher than 2 mM roots were stunted and there was decrease in root hair. Percent reduction with respect to control of root length increased with increase in glyphosate concentration in all the genotypes. Percent reduction (%) with respect to control in highest dose 10 mM was PU-19 (91.96), PU-35 (93.87), IPU-94-1 (90.91), PU-31 (94.58), Azad-1 (91.77) and Azad-2 (95.21) (Figure- 4.2A). This clearly indicates the relative tolerance among genotypes and we can conclude that the genotypes with less inhibition at highest glyphosate concentration (10 mM) can better tolerate the herbicide stress. Two way anova for root length showed significant difference of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F $F_{5,84}=2.583, p<0.318$; Factor 2- $F_{6,84}=789.3, p<0.0001$; factor 1x2- $F_{30,84}=3.325, p<0.0001$ (Appendix Table -3).

4.1.4 Seedling Fresh Weight

Glyphosate treatment reduces fresh weight of plants. A reduction in fresh weight of seedlings was also observed during present study. Significant (P<0.05) differences was observed in seedling fresh weight between herbicide concentration and genotypes (Table-4.4). The fresh weight of germinated seedlings of V. mungo genotypes was taken and it was found to decrease after treatment in all the six genotypes. The fresh weight (g) of control seedlings was PU-19(1.15±0.18), PU-35 (0.91±0.03), IPU-94-1(1.23±0.09), PU-31(0.85±0.03), Azad-1(0.95±0.03) and Azad-2(0.83±0.02). At the highest glyphosate concentration fresh weight was 0.28±0.04 in PU-19, 0.30±0.03 in PU-35, 0.33±0.01 in IPU-94-1, 0.23±0.02 in PU-31, 0.30±0.03 in Azad-1 and 0.24±0.02 in Azad-2 (Figure - 4.3A). The statistical comparison of the six varieties by LSD test showed that decrease in fresh weight was significant for the treatments but not genotypes. Maximum and minimum reduction of fresh weight at 10 mM was observed in PU-31(73.44%) and PU-35 (66.91%). Two way anova for fresh weight of seedlings showed extremely significant difference of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F $F_{5,84}=35.55, p<0.0001$; Factor 2- $F_{6,84}=418.2, p<0.0001$; factor 1x2- $F_{30,84}=5.125, p<0.0001$(Appendix Table -4).

4.1.5 Seedling Dry Weight

Glyphosate also caused reduction in dry weight of seedlings in all genotypes. Significant (P<0.05) differences were observed in seedling dry weight between herbicide concentration and genotypes (Table-4.5). The dry weight of germinated seedlings of V.
mungo genotypes was taken and it was found to decrease after the treatment in all the six genotypes. The dry weight (g) of control seedlings was PU-19 (0.33±0.02), PU-35 (0.27±0.02), IPU-94-1 (0.28±0.01), PU-31 (0.27±0), Azad-1 (0.25±0.02) and Azad-2 (0.20±0.01). At the highest glyphosate concentration dry weight was 0.06±0.02 in PU-19, 0.06±0.01 in PU-35, 0.05±0 in IPU-94-1, 0.02±0.03 in PU-31, 0.04±0 in Azad-1 and 0.03±0.02 in Azad-2. Significant differences in dry weight were observed for the treatments in all the genotypes. Maximum and minimum reduction of dry weight at 10 mM was observed in PU-31 (90.24%) and PU-35 (75.61%). Dose dependent decrease was observed in dry weight on increasing glyphosate concentration in all varieties (Figure-4.3B). Two way anova for dry weight of seedlings showed extremely significant difference of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F s=11.13, p<0.0001; Factor 2-Fs=257.3, p<0.0001; factor 1x2-Fs=2.823, p<0.0001 (Appendix Table -5).

4.1.6 Vigour Index
Vigour index of seedlings decreased with increasing concentration of herbicide in all the treatments and genotypes. The maximum vigour index (PU-19, 1217), (PU-35, 906), (IPU-94-1, 1284), (PU-31, 964), (Azad-1, 1232) and (Azad-2, 1184) was observed at control at 10th day of treatment (DAT). The minimum vigour index (PU-19, 12), (PU-35, 4), (IPU-94-1, 9), (PU-31, 3), (Azad-1, 10) and (Azad-2, 5) was observed in 10 mM concentration (Table 4.6).

4.1.7 Tolerance Index
The maximum tolerance index was observed in PU-19 (0.88), PU-35 (0.63), IPU-94-1 (0.74), PU-31, (0.72), Azad-1 (0.80) and Azad-2, (0.77) while minimum tolerance index was observed in 10 mM (PU-19, 0.88), (PU-35, 0.63), (IPU-94-1, 0.74), (PU-31, 0.72), (Azad-1, 0.80) and (Azad-2, 0.77). Tolerance index decreased with increasing concentration of glyphosate (Table 4.6, Figure 4.2B).

4.1.8 Percentage of Phytotoxicity
The maximum percentage of phytotoxicity was observed in 10 mM (PU-19, 91.85), (PU-35, 93.99), (IPU-94-1, 90.91), (PU-31, 94.35), (Azad-1, 91.83) and (Azad-2, 95.54) while minimum percentage of phytotoxicity was observed in 1 mM (PU-19, 12.48), (PU-35, 37.57), (IPU-94-1, 25.57), (PU-31, 28.25), (Azad-1, 19.43) and (Azad-2, 23.57). Percentage of phytotoxicity increased with increasing concentration of glyphosate (Table 4.6).
4.2 *In Vitro* Germination Test

In the second step of screening of herbicide tolerant genotypes seeds were grown in basal MS media supplemented with various concentrations of glyphosate in test tubes. During *in vitro* germination studies chosen concentrations were much lower than petridish screening depending upon the response of *in vitro* grown seeds and seedlings. Since screening of herbicide tolerant genotypes under field conditions requires large investment and involves interactions of other environmental factors making the study difficult, *in vitro* screening is an alternative approach which is more convenient, efficient and takes less time and space for screening large number of genotypes for herbicide tolerance. During *in vitro* germination screening, effect of glyphosate on growth parameters like germination percentage, survival percentage, root length, shoot length and fresh weight were studied. Effect of glyphosate on activity of antioxidative enzymes like catalase, peroxidase, superoxide dismutase and other biochemical parameters like total proline and total protein were also analysed.

4.2.1 Germination Percentage

Herbicide had profound effect on seed germination and seedling growth. Genotypes showed significant variation for seed germination. Effect of herbicide also showed significant change in seed germination (*Table-4.7*). Highest percentage of seed germination at control was observed in PU-19 (96.67±1.67) and minimum was in PU-31 and PU-35 (86.67±3.33). Percentage of decrease in seed germination with respect to control at highest glyphosate concentration was highest in PU-31 (75.00%) and lowest in PU-19 (51.72%). Concentrations higher than 0.4 mM cause significant reduction in germination percentage except Azad-1 and IPU-94-1. EC$_{50}$ (glyphosate concentration which led to 50% or reduced germination) for germination percentage was different in different genotypes. It was 0.8 mM for PU-19, IPU-94-1 and Azad-1, 0.4 mM for PU-35 and PU-31 and 6 mM for Azad-2 (*Plate-2, 3; Figure-4.4A*).

Two way anova for germination percentage of seedlings showed extremely significant difference of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F$_{5,72}$=23.60, $p<0.0001$; Factor 2- F$_{5,72}$=251.8, $p<0.0001$; factor 1x2-F$_{25,72}$=4.141, $p<0.0001$ (*Appendix Table-6*).

4.2.2 Survival Percentage

Glyphosate also reduced survival percentage of *in vitro* grown seedlings in all the genotypes studied. It was decreased gradually in a dose dependent manner. Significant variation was observed in survival percentage among genotypes and significant reduction
was shown in all concentrations. Highest percentage of seedling survival at control was observed in PU-19 (85.00±2.89) and minimum was in PU-31 and PU-35 (80.00±2.89). Percentage of decrease in survival percentage with respect to control at highest glyphosate concentration 0.8 mM was maximum in PU-31 (79.17%) and lowest in PU-19 (64.71%). Significant reduction in survival percentage was observed in 0.4 mM and onwards (Table-4.8, Figure-4.4B).

Two way anova for survival percentage of in vitro grown seedlings showed significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F$_{5,72}$=10.88, $p<0.0001$; Factor 2-F$_{5,72}$=243.6, $p<0.0001$; factor 1x2-F$_{25,72}$=1.750, $p<0.0346$ (Appendix Table-7).

4.2.3 Root Length

Root length was drastically reduced by all concentrations of herbicide in all the genotypes studied. As compared to petridish germination method, in vitro germination in MS media supplemented with herbicide showed clear relative tolerance among genotypes for the herbicide glyphosate and it can be used as a better method of screening for herbicide tolerance among various cultivars growing naturally. With increasing concentration there was gradual reduction in root length in all the genotypes (Plate-2, 3). Highest root length was observed in control and seedling root length in various genotypes was: PU-19 (9.93±0.28cm), PU-35 (9.00±0.29cm), IPU-94-1(8.50±0.58cm), PU-31(8.07±0.30cm), Azad-1(6.80±0.78cm) and Azad-2(6.63±0.32cm) and at 0.8 mM PU-19 and IPU-94-1 had highest (1.20±0.21, 1.13±0.32cm) and lowest root length was in PU-31 (0.53±0.03cm) respectively (Table-4.9, Figure-4.5B). All the concentration caused significant difference/reduction in root length in all genotypes. In concentrations higher than 0.4 mM roots were stunted, proximal ends of roots were swollen and there was reduction in number of root hair. Percent reduction with respect to control of root length increased with increase in glyphosate concentration in all the genotypes. Percent reduction with respect to control in highest dose 0.8 mM was PU-19 (87.92), PU-35 (89.26), IPU-94-1(86.67), PU-31(93.39), Azad-1(88.73) and Azad-2 (89.45). This clearly shows that the genotypes with less inhibition at highest glyphosate concentration (0.8 mM) can better tolerate the herbicide stress. The F-ratio (5, 12 DF) was found to be statistically significant at 1% (i.e. $p < 0.01$) for all treatments of glyphosate in all the genotypes.

Two way anova for root length of in vitro grown seedlings showed significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between
genotype and concentration. Factor 1-\( F_{5,72}=32.28, p<0.0001 \); Factor 2-\( F_{5,72}=544.6, p<0.0001 \); factor 1x2-\( F_{25,72}=5.544, p<0.0001 \)(Appendix Table-8).

### 4.2.4 Shoot Length

Effect of glyphosate herbicide on shoot length of six genotypes of *Vigna mungo* was also studied in *in vitro* grown seedlings after 15 days of inoculation and it was observed that glyphosate caused inhibition in shoot length but the effect was less prominent as compared to that on root length. According to Table 4.10 maximum shoot length was observed in control and minimum at 0.8 mM. In control, among genotypes shoot length was highest in PU-35 (18.83±0.73cm) and lowest in Azad-1 (11.00±0.58cm) and at highest herbicide dose it was maximum in PU-19 (3.37±0.59cm) while minimum in Azad-2 (1.77±0.26cm) (Figure-4.5A). Maximum percent decrease over control was found in Azad-2, PU-31 (87.07, 84.81) and minimum in PU-19 and Azad-1 (76.24, 76.67). The F-ratio (5, 12 DF) was found to be statistically significant at 1% (i.e. \( p < 0.01 \)) for all treatments of glyphosate in all the genotypes.

Two way anova for shoot length of *in vitro* grown seedlings showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-\( F_{5,72}=18.74, p<0.0001 \); Factor 2-\( F_{5,72}=485.5, p<0.0001 \); factor 1x2-\( F_{25,72}=5.864, p<0.0001 \)(Appendix Table-9).

### 4.2.5 Fresh Weight of Seedlings

A reduction in fresh weight was also observed in *in vitro* germination test like petridish germination test in our investigations. The fresh weight of germinated *V. mungo* seedlings was taken after 15 days of incubation and it was found to decrease after the treatment. The fresh weight (g) of control seedlings was 2.85±0.15 in PU-19, 2.80±0.10 in PU-35, 3.42±0.22 in IPU-94-1, 2.70±0.13 in PU-31, 2.91±0.08 in Azad-1 and 2.76±0.20 in Azad-2. It showed a gradual decrease with increasing glyphosate concentration in all genotypes. At the highest glyphosate concentration 0.8 mM maximum value was obtained in Azad-2 while minimum value was obtained in PU-31 but maximum percent decrease over control at 0.8 mM was found in Azad-1 (Table-4.11 Figure-4.6). The F-ratio (5, 12 DF) was found to be statistically significant at 1% (i.e. \( p < 0.01 \)) for all treatments of glyphosate in all the genotypes.

Two way anova for fresh weight of *in vitro* grown seedlings also showed significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-\( F_{5,72}=4.09, p<0.0025 \); Factor 2-\( F_{5,72}=328.6, p<0.0001 \); factor 1x2-\( F_{25,72}=2.376, p<0.0023 \)(Appendix Table-10).
4.2.6 Catalase Activity

The catalase activity in *V. mungo* seedlings was measured as the amount of H$_2$O$_2$ decomposed per gram fresh weight (µmole H$_2$O$_2$ decomposed/100mg FW). Amongst the controls, catalase activity was lowest, 91.67±4.41 µmole H$_2$O$_2$ decomposed/100 mg FW, in the seedlings of PU-35 and highest, 200.0±30.55 in Azad-1. The catalase activity was clearly found to increase after glyphosate treatment in all the six genotypes (Figure-4.7A). At the highest dose 0.8 mM glyphosate dose, the maximum activity was 773.33±40 µmole H$_2$O$_2$ decomposed/100mg FW in PU-19 while minimum activity was in 446.67±35.13 µmole H$_2$O$_2$ decomposed/100mg FW (Table-4.12). Percent increase over control at 0.8 mM was highest in PU-19 (728.57%) while lowest in PU-31 (179.17%). According to LSD test, the increase in activity across the treatments was significant in the six genotypes. It was assumed that to overcome the oxidative stress caused by glyphosate, catalases might be playing a pivotal role in *Vigna mungo* genotypes. The protective role of catalase in PU-31 and Azad-1 was not sufficient enough to protect the plants from herbicide injury as shown by survival percentage and seedling root length in PU-19. The induced catalase activity in PU-19 and IPU-94-1 might be playing a supportive role causing less damaging symptoms at higher doses. The F-ratio (5, 12 DF) was found to be statistically significant at 1% (i.e. p < 0.01) for all treatments of glyphosate in all the genotypes.

Two way anova for catalase activity of *in vitro* grown seedlings showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F$_{5,72}$=33.68, p<0.0001; Factor 2- F$_{5,72}$=183.4, p<0.0001; factor 1x2-F$_{25,72}$=3.115, p<0.0001 (Appendix Table-11).

4.2.7 Peroxidase Activity

The peroxidase activity in *in vitro grown* seedlings of six genotypes of *V. mungo* was measured as the change in optical density (ΔOD/100mg FW). Peroxidase activity was lowest in control seedlings. Among genotypes it was maximum in PU-19, 4.51±0.46 ΔOD/100mg FW, and minimum in PU-35, 3.51±0.55 ΔOD/100mg FW. Like catalase, peroxidase activity was also found to increase after glyphosate treatment in all the six genotypes (Figure-4.7B). The peroxidase activity was highest at 0.8 mM, it was highest, 10.44±0.28 ΔOD/100mg FW in IPU-94-1, and lowest, ΔOD/100mg FW in Azad-1. The increase in peroxidase activities was found significant from 0.4 mM in all genotypes. The percent increase over control in peroxidase activity was highest at 0.8 mM in PU-19(76.48%), PU-35 (130.93%) and IPU-94-1(154.30%) but it was highest at 0.6 mM in
PU-31 (10.99%), Azad-1 (70.53%) and Azad-2 (82.33%) (Table 4.13). The F-ratio (5, 12 DF) was found to be statistically significant at 5% (i.e. p < 0.05) and 1% (i.e. p < 0.01) for all treatments of glyphosate in all the genotypes.

Two way anova for peroxidase activity of \textit{in vitro} grown seedlings also showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-\textit{F}_{5,72}=7.458, p<0.0001; Factor 2- \textit{F}_{5,72}=29.25, p<0.0001; factor 1x2-\textit{F}_{25,72}=4.318, p<0.0001 (Appendix Table -12).

4.2.8 Superoxide Dismutase Activity

SODs are also known to play important role in herbicide tolerance in several plant species. We also measured SOD activity in \textit{in vitro} grown \textit{V. mungo} seedlings after glyphosate treatment. It was expressed as unit activity/100 mg FW. As compared to other oxidative enzymes SOD activity was not significantly increased after glyphosate exposure. From control to 0.6 mM it was increased but after that it decreased except in PU-19, Azad-1 and Azad-2 where it was increased. SOD activity in the controls was maximum, 13.86±0.84 in PU-19 while minimum, 11.15±1.01 in Azad-1 (Table 4.14, Figure 4.8A). The SOD activity at 0.8 mM was highest 30.48±2.32 in PU-19 and lowest (11.07±1.03) in PU-35. The data indicates that the SOD activity is genotype-dependent. The increase in SOD activity also suggests that glyphosate is inducing oxidative stress in \textit{V. mungo}.

Two way anova for SOD activity of \textit{in vitro} grown seedlings also showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-\textit{F}_{5,72}=38.54, p<0.0001; Factor 2- \textit{F}_{5,72}=21.83, p<0.0001; factor 1x2-\textit{F}_{25,72}=5.268, p<0.0001 (Appendix Table -13).

4.2.9 Protein Content

Biotic or abiotic stress at the germination phase causes a decrease in normal metabolic activities and consequent, reduction of growth. In this phase protein synthesis, anabolic process like photosynthesis and transport of metabolites is badly affected. As glyphosate inhibits amino acid metabolism by inhibiting shikimic acid pathway it also showed profound effect on soluble protein content. Glyphosate caused decrease in protein content of \textit{in vitro} grown seedlings in all the treatment and in all the genotypes. Maximum protein content was in control and value was highest for Azad-1 (10.17±0.54 mg/g fw), followed by IPU-94-1 (10.08±0.05 mg/g fw) and PU-35 (9.07±0.22 mg/g fw) while lowest for Azad-2 (8.17±0.41 mg/g fw) (Table 4.15, Figure 4.8B). Significant
reduction was observed in all treatments. Maximum percent reduction over control at 0.8 mM was in Azad-2 (50.61%) while minimum in IPU-94-1 (31.52%).

Two way anova calculated for protein content of in vitro grown seedlings also showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F<sub>5,72</sub>=6.960, p<0.0001; Factor 2- F<sub>5,72</sub>=48.98, p<0.0001; factor 1x2-F<sub>25,72</sub>=2.571, p<0.0001 (Appendix Table -14).

4.2.10 Proline Content

We analyzed impact of abiotic herbicide stress on proline content and found that there was highly significant difference for proline content for the treatment and genotype. In control seedlings proline content was lowest and among genotypes maximum in PU-31 (0.17±0.02 μmol/g FW) and minimum in Azad-1 (0.14±0.02 μmol/g FW). It was found to increase at all the doses and all the genotypes except Azad-2 at 0.1 mM where it was slightly decreased. 0.4 mM glyphosate in media showed significant increase in all varieties (Table-4.16, Figure-4.9). At the highest concentration maximum percent increase over control was found in Azad-1 (552.38%) following PU-19 (453.33%) and IPU-94-1 (369.05%) while minimum percent increase over control was in PU-35 (206.38%) and Azad-2 (271.43%).

Two way anova calculated for protein content of in vitro grown seedlings also showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F<sub>5,72</sub>=7.741, p<0.0001; Factor 2- F<sub>5,72</sub>=121.5, p<0.0001; factor 1x2-F<sub>25,72</sub>=2.362, p<0.0001 (Appendix Table -15).
4.3 *In vitro* Culture Screening

Tissue culture study was performed in order to understand the response of seedling explants of genotypes in different concentrations and combination of auxin and cytokinin for nonselective (control) and selective media and to screen herbicide tolerant black gram genotypes. The explants viz; hypocotyl, epicotyl, cotyledonary node, shoot apex and leaf segments obtained from one week old seedlings of control seeds on basal-MS medium were used for the experiments. These explants were grown in MS medium with different concentration of glyphosate supplemented with different concentrations of NAA, BAP, and Kn. The explants showed different type of response, some without intermediate phase (direct organogenesis from explants leading to plantlet, shoot and root etc.) and other with intermediate phase (indirect organogenesis by callus and bud). Among various concentrations and combinations of phytohormones best media was selected for callus formation (callusing), shoot formation (shooting), root formation (rooting). After that morphogenic response of various explants of six genotypes were evaluated on the basis of percentage of explants forming callus, shoots and root, root length, shoot length and callus fresh weight under stress (selective) media and the genotypes were categorized in tolerant, moderately tolerant and susceptible.

4.3.1 Media Tested for Callus Formation

Various combinations of auxins were tested for callus formation in *V. mungo*. Usually all explants showed callusing in all combinations formation varies according to explant and genotype (Table-4.17). Among them best two media (A) and (C) were selected to study response of six genotypes to herbicide stress in terms of percent callus response (percent of response that responds for callusing) and callus fresh weight.

4.3.1.1 NAA-0.5 +BAP-1.0 mg/l- (A)

The combination containing NAA and BAP in the proportion 0.5:1.0 mg/l was responsible for positive callusing responses in all the seedling explants for control as well as for herbicide treated cultures (Table-4.18). Cotyledonary node was most superior for callus induction followed by hypocotyl, epicotyl and shoot apex. The overall % of response after 30 days ranged from 100% to 5% (Table- 4.19).

4.3.1.1.1 Epicotyl as Source of Explants

The epicotyl initially produced creamish to light green callus. The callus formed for 0.05, 0.1 and 0.2 mM, while root and callus was observed for control and 0.01 mM. Callus induction also depended upon method of inoculation. When explants were inoculated in horizontal manner then at initial stage callusing observed only towards the
ends of explants later throughout the surface of explants (Plate-4 E) whereas in vertical inoculation basal callusing was observed in which compact greenish brown colored callus was formed (Plate-4 D).

Percent callus response and callus biomass showed dose dependent decrease in herbicide treated media. Callusing was observed up to 0.05 mM in all genotypes but at 0.1 mM only Azad-1 showed very few callusing. At 0.2 mM explants did not show any kind of response in this combination in any genotype.

In control maximum % callus response was observed in IPU-94-1 (100%) and minimum in PU-35 (75%). At 0.05% all genotypes showed callusing but maximum % callus response with 56.67% were observed in PU-19 followed by PU-35 (46.67%) while minimum in PU-31 (25.00%) (Table-4.19).

Callus fresh weight taken after 30 days also decreased in dose dependent manner. Maximum fresh weight in control was recorded in in Azad-2 (1.91±0.12g), minimum in PU-35 (1.62±0.12g) whereas in 0.05 mM greatest fresh weight was in IPU-94-1 (0.89±0.14g) and minimum in PU-31 and PU-35 (0.54±0.09, 0.56±0.09 g).

4.3.1.1.2 Cotyledonary Explants as Source of Explants

These explants gave best response for callusing in this combination. Shooting response was also seen for control and 0.01 mM in PU-19 and Azad-1 (Plate-5 E, F). Merely, callus was produced for 0.05 mM, 0.1 mM from cotyledonary node explants (Plate-5 G). In 0.2 mM callusing response was not observed in PU-31 and Azad-2. In control maximum callus response was found in IPU-94-1 and minimum in Azad-2 (Figure-4.10 A). In 0.1 mM on the basis of percent callus response from highest to lowest values genotypes could be arranged as IPU-94-1(18.33)>Azad-1(16.67)>PU-19(15.00)> Azad-2(10.00)> PU-35(8.33)>PU-31(6.67).

Effect of herbicide on callus fresh weight (CFW) was less harsh on cotyledonary node explants as compared to other explants. There was not great variation in CFW among genotypes in control and lower doses but genotypic differences were observed in higher doses (Figure-4.10 B). In control highest CFW was recorded in PU-19 (2.19±0.13g) and lowest in PU-31 (1.77±0.10g) whereas at 0.2 mM lowest CFW was found in PU-35 (0.18±0.02g) and highest in IPU-94-1 (0.31±0.06g) (Table-4.20).

Reduction in callus fresh weight (RCFW) was also proved a good screening parameter for assessment of tolerant genotypes in black gram. Percent reduction in callus fresh weight in comparison of control increased in a dose dependent manner. At 0.4 mM complete reduction observed as no callus formed even after 30 days in any genotypes.
Genotype PU-31 and Azad-2 showed 100% reduction in 0.2 mM whereas IPU-94-1 scored least reduction in CFW (85.51%) (Figure-4.10 C). Thus if we compare tolerance of genotypes on the basis of percent callusing response of cotyledonal explant in callusing media-C supplemented with PGR NAA-0.5mg/l + BAP 1.0mg/l IPU-94-1, Azad-1 and PU-19 were more tolerant than PU-35, PU-31, Azad-2.

Two way anova for percent callus response and callus fresh weight of cotyledonal explants of six genotypes in this combination showed highly significant effect of herbicide treatment, genotype and their interaction (Table-4.21 and 4.22).

4.3.1.1.3 Hypocotyl as Source of Explants

Hypocotyl explants showed superior callusing than epicotyl and shoot apex in this combination. Callus was initially soft creamish green or whitish green in control while in treated media it became brownish green or black (Plate -4 A, B, C). Callusing was observed up to 0.05 mM in all genotype but in 0.1 mM only Azad-1 and Azad-2 showed callusing while at 0.2 mM PU-19 and Azad-2 showed callusing at very less extent. Percent callus response and callus growth were decreased with increase in glyphosate concentration. In control genotype PU-35, IPU-94-1, PU-31 and Azad-1 showed higher callus response (90%) than PU-19 (80%) and Azad-2 (75%) where as at 0.05 mM Azad-2 showed maximum callus response (33.33%) followed by PU-19 (25.00%), IPU-94-1 (21.67%) but minimum callus response was found in PU-31 (10%). At 0.1 mM more callusing was found in PU-19 (20%) than Azad-2 (15%) and Azad-1(10%).

Callus fresh weight in hypocotyl explants were also found decreased in all the concentrations of glyphosate. There was not great variation in CFW among genotypes at 0.05 mM but at 0.1 mM genotype PU-19 showed more CFW (0.33±0.06g) than Azad-1(0.21±0.05g) and Azad-2 (0.10±0.02g) (Table-4.20). Thus it is evident effect of herbicide on cultures of hypocotyl explants was not as prominent as on cotyledonal cultures.

4.3.1.1.4 Shoot Apex as Source of Explants

As described earlier among the all explants shoot apex was most susceptible to herbicide toxicity and did not responded well for callusing in control and herbicide media. Moderate compact greenish brown or green callus formed at base and apex dried and became dead (Plate-5A, C; Plate 7 A, B). PU-31 and Azad-1 showed highest (63.33%) and lowest (40.00%) callus response respectively in control. In 0.05 mM PU-19 showed maximum callus response (30.00%) and PU-31 and IPU-94-1 minimum
callus response (15%) (Table-4.19). As compared to other explants CFW was very less in shoot apex. In control genotype with maximum CFW was IPU-94-1 (1.08±0.11g) and Minimum was in PU-19 (0.75±0.11g). In 0.05 mM highest CFW was again in IPU-94-1 (0.78±0.08g) and lowest in Azad-2 (0.33±0.11g) (Table-4.20).

4.3.1.2 NAA-1.5+BAP-1.0 mg/l- (C)

Callus media-C with PGR combination of NAA and BAP in the proportion 1.5:1.0 mg/l also showed good callusing responses in all the seedling explants like leaf segments, cotyledonary node and hypocotyl (Table-4.23). Nature, color and site of callus initiation varied according to explants. In leaf segments callus initiated at cut ends of leaf and it was green compact and nodular (Plate-6 G). In cotyledonary node callus started at nodal portion as compact light brown callus while hypocotyl explants showed initiation at either basal end (when inoculated vertically) or at both ends (when inoculated horizontally) as greenish white or creamish white callus (Plate-6-C).

4.3.1.2.1 Leaf Segments as Source of Explants

Leaf explants showed moderate callusing in all genotypes but callus growth and percent callusing response decreased with the increasing glyphosate concentration. In few genotypes like Azad-2 and PU-19 compact greenish white callus was formed while in IPU -94-1 and PU-35 greenish friable callus was observed which was initiated at cut ends and later extended to midrib regions (Plate-6 G, H). Response was observed up to 0.05 mM in all genotypes but in 0.1 mM explants of PU-31 and PU-19 did not form callus. In 0.2 mM only Azad-1 showed very less callusing (Plate-6 B) while in 0.4 mM callus was not formed at all in genotype.

4.3.1.2.2 Cotyledonary Node as Source of Explants

In callus media–(C) cotyledonary node explants was found to be superior for callusing than other explants (Table-4.23). Callus was formed up to 0.2 mM in all the genotypes but in 0.4 mM very less callusing was observed only in IPU-94-1 (Plate-6F). Percentage of explants that responded for callusing decreased with the increasing concentration. In control maximum percent callus response was found in IPU-94-1 and Azad-1 (84.44) and minimum in PU-35 (75.55). At highest concentration on the basis of percent callus response from highest to lowest values genotypes could be arranged as PU-19(15.55)>IPU-94-1, Azad-1 (13.33)> PU-35 (11.11)> PU-31(8.89) >Azad-2(10.00) PU-35(4.45) (Table-4.24, Figure-4.11 A).

Significant effect of herbicide on callus fresh weight (CFW) of cotyledonary node cultures was also observed. Generally cotyledonary node cultures showed higher
CFW followed by hypocotyl and leaf cultures. There was variation in CFW among genotypes in control and treated media. In control highest CFW was recorded in IPU-94-1 (2.86±0.15g) and lowest in Azad-2 (2.06±0.14g) whereas at 0.2 mM lowest CFW was found in IPU-94-1 (0.30±0.02g) and highest in Azad-1 (0.62±0.04g) (Table-4.25, Figure-4.11 B). Percent reduction in callus fresh weight in comparison of control increased in a dose dependent manner in all the genotypes. In 0.2 mM maximum reduction was found in Azad-2 (94.28%) and minimum in PU-19 (80.56%). At 0.4 mM only IPU-94-1 showed little callus with minimum 88.23% reduction while other genotypes showed 100% reduction (Figure-4.11 C).

Two way anova for percent callus response and callus fresh weight of cotyledonary explants of six genotypes in this combination showed highly significant effect of herbicide treatment, genotype and their interaction (Table 4.26 and 4.27).

4.3.1.2.3 Hypocotyl as Source of Explants

Hypocotyl explants produced only callus and roots and shoots were not observed in this combination (Table-4.23). Callus induction and callus growth was better in hypocotyl cultures than leaf segments and shoot apex. Soft, friable, creamish green or whitish green callus was formed in control while in treated media it became brownish green (Plate-7 F, H). Callusing was observed up to 0.2 mM in all genotypes but in 0.4 mM no response was observed in any genotype. Percent callus response and callus growth were decreased with increase in glyphosate concentration. In control genotype PU-31 and IPU-94-1 showed highest percent callus response (70.00%) and minimum response found in PU-35 (60.00) where as at 0.2 mM IPU-94-1 showed maximum callus response (23.33%) followed by PU-19 and Azad-1 (8.33%) but minimum callus response was found in Azad-2 (3.33%) (Table-4.24).

Callus fresh weight in hypocotyl explants were also reduced in all the concentrations of glyphosate except in IPU-94-1 and Azad-2 where it was slightly increased in 0.01 mM than control. At 0.2 mM genotype IPU-19 showed greatest CFW (0.75±0.28g) and lowest in Azad-2 (0.21±0.07g) (Table-4.25).
4.3.2 Media Tested for Shoot Formation

Various combinations of auxin and cytokinin were tested for adventitious and non adventitious shoot formation in black gram (Table-4.28). Among them best three media-(D), media-(H) and media-(I) were selected to study response of six genotypes to herbicide stress in terms of percentage of shoot response, shoot number and shoot length.

4.3.2.1 NAA-1.0+BAP-1.5+Kn-0.5 mg/l – (D)

Among the four seedling explants cotyledonary node and shoot apex were showed better positive response for shoot formation than epicotyl and hypocotyl in control as well as herbicide media in all the six genotypes (Table-4.29). Overall percentage of shoot regeneration response after 30 days ranged from 83.33% to 6.67% (Table-4.30).

4.3.2.1.1 Epicotyl as Source of Explants

In this combination, the epicotyl explant in control and stress media produced only callus to varying degree depending on genotype. Shoot was not observed in any treatment as well as genotype (Table-4.29). Creamish green callus started at basal end of explant that later become brownish (Plate-9 G, H). Callus size and percent callus response decreased with increasing herbicide dose.

4.3.2.1.2 Cotyledonary Node as Source of Explants

The cotyledonary node showed good shooting and callusing in control as well as stress media. Cotyledonary node showed first callusing at basal end of explant while multiple shoots as well as shoot buds proliferated from axillary buds. Initially compact greenish brown callus formed but as shoot length increased callus become brown (Plate-9B). There was progressive decline in percent shoot response, shoot number per explant and shoot length as glyphosate concentration increased in all genotypes but response varied with the genotype. At control and lower concentration of glyphosate i.e. 0.01 mM and 0.05 mM usually all genotypes showed shooting. At higher glyphosate concentration 0.1 mM shooting was observed in PU-35, PU-31 and IPU -94-1 while only IPU-94-1 showed shooting at 0.2 mM (Table-4.29).

Maximum percent of response (percent regeneration of shoots) was found in control and among genotypes it was highest in IPU-94-1(83.33) and lowest in Azad-2(61.67) but at 0.05 mM highest response was recorded in Azad-1 (65.00) while lowest at PU-35 (35.00) (Figure-4.12 A). Shoot number and length of shoots also decreased with the increasing dose of herbicide. Highest number of shoots per explant was found in control. Among genotypes it was highest in genotype IPU-94-1 (9.25±0.75) (Plate-8A)
while lowest in PU-19 (5.33±0.88) (Plate-9A) (Figure-4.12 B). At 0.05 mM concentration of herbicide maximum number of shoots per explant was recorded in Azad-2 while minimum in PU-31(2.75±0.48) and at the same herbicide concentration longest shoots were recorded in Azad-1(2.13±0.20cm) and shortest in PU-35(1.17±0.12cm) (Figure-4.12 C).

4.3.2.1.3 Hypocotyl as Source of Explants

Hypocotyl showed varied response in treatments and genotypes. It showed usually callusing but shooting and shoot bud formation recorded only in control in genotypes PU-35, PU-31 and Azad-2 (Plate-9F). Usually callus was formed at apical portion of explant and it was soft friable, light yellowish green in color. Extent of callus proliferation and callus growth was reduced with increasing concentration of herbicide. At high concentrations 0.2 and 0.4 mM no any response was observed even after 45 days of inoculation and explant become brown after some swelling (Table-4.29).

4.3.2.1.4 Shoot Apex as Source of Explants

The shoot apex explants in control and stress media in this combination produced callusing as well as shoots. Shoots with callusing was observed usually in all genotypes. Callusing occurred at basal cut end of explants while shoot and multiple shoots were observed at apex having shoot apical meristem. Callus were light green to dark green and compact, nodular in control while brownish green and compact in 0.2 mM. Shoots without callus phase obtained for 0.05 mM and 0.1 mM in genotypes PU-19 and Azad-1, while multiple shoots were noticed in control media by genotype IPU-94-1 and azad-2 as well as by PU-19 in 0.05 mM. Shoot number per explant as well as shoot length was decreased in all genotypes with increasing concentration of herbicide. Maximum number of shoots per explant was obtained in control, and order of maximum to minimum was PU-19(4.33±0.67), PU-35(3.00±0.41), IPU-94-1 (4.5±0.65), PU-31(3.75±0.48), Azad-1(3.75±0.48) and Azad-2 (2.25±0.95). At 0.05 mM maximum number of shoots was found in PU-19 (2.93±0.33) and minimum in Azad-2. Maximum average shoot length was observed in control for PU-31 (3.15±0.12cm) and minimum in PU-19 (1.40±0.17cm). In stress media of 0.05 mM greatest shoot length was found in azad-1(1.54±0.23cm) lowest in PU-19 (0.87±0.20cm) (Table-4.30).

Among all the explants, cotyledonary explant were best and for this explant two way ANOVA showed extremely significant effect of herbicide treatment, genotype and their interaction for the percent shooting response, shoot number per explant and length of shoots (Table-4.31, Appendix Table 16, 17, 18).
On the basis of percent shooting response of cotyledonary node explant in media-D at 0.05 mM (in which all genotypes showed shooting response) the genotypes can be grouped according to their relative tolerance as tolerant (Azad-1-65.00%, PU-19-60.00%), moderately tolerant (IPU-94-1-48.33%, Azad-2-46.67%) and susceptible (PU-31-40.00, PU-35-35.00%).

4.3.2.2 NAA-0.5+BAP-2.0+Kn-1.0 mg/l- (H)

In this combination hypocotyl, cotyledonary node and shoot apex showed good response in control and stress media. Best shooting response was observed in cotyledonary explant followed by shoot apex and hypocotyl. In hypocotyl first callus were formed then shoot buds and shoots were initiated. In cotyledonary explant directly shoots and shoot buds arised with callusing (Plate-10A). In shoot apex explants either callusing starts at cut basal end and shoots at apical portion or only shoots formed at apex (Table-4.30). Percentage of explants that showed shooting response ranged from 61.11% to 6.67% (Table -4.32).

4.3.2.2.1 Cotyledonary Node as Source of Explants

In media–(H), cotyledonary node showed good shoot formation, shoot bud formation and callus formation in control and herbicide stress media (Plate-10). As glyphosate concentration increases there was progressive reduction in % shoot response, shoot number and shoot length in all genotypes but response varied with the genotype. Shooting was observed in control as well as herbicide media of 0.01, 0.05 mM while at 0.1 mM response was not observed in PU-35 and PU-31. At 0.2 mM response was observed only in PU-19 and Azad-1. No any response was observed at 0.4 mM in any explant and genotype (Table-4.32).

As compared to media–(D) percent shooting response was comparatively less in media–(H). Highest percent of response (percent shoot formation) was found in control and it was highest in Azad-1 (61.11) and lowest in PU-31 (46.59). At lowest herbicide dose 0.01 mM maximum response was recorded in PU-19 (51.66) and minimum at PU-31 (33.33). At 0.05 mM highest percent response was again in PU-19 and minimum in PU-35 and PU-31 (Table-4.33). At higher herbicide concentration 0.1 mM where all genotypes showed positive response maximum value were found in Azad-1(27.77) while minimum in PU-35 and PU-31(11.11) (Figure-4.13 A). Number of shoots per explant and length of shoots are also decreased with the increasing dose of herbicide. In control highest number of shoots per explant was recorded in Azad-1(6.00±0.58) followed by PU-31, Azad-2(5.67±0.33), PU-19(5.33±0.33), PU-35(4.67±0.33), IPU-94-1(4.33±0.58)
At highest stress level 0.4 mM highest number of shoots per explant were recorded in Azad-1 (2.33±0.33) followed by IPU-94-1, PU-19, Azad-2 (2.00±0.58), PU-35 (1.00±0.58) (Figure-4.13 B). At 0.2 mM only PU-19 showed shoots with 14.44% response, shoot number of 1.33±0.33 and shoot length of 0.93±0.12. Effect of herbicide on shoot length was more severe than on shoot number. In control media longest shoots (7.07±0.30 cm) were observed in PU-19 while shortest in PU-35 (3.77±0.52 cm). At 0.1 mM on the basis of shoot length from highest to lowest values genotypes could be ranked as IPU-94-1 (1.60±0.25 cm) > Azad-1 (1.30±0.06 cm) > PU-19 (1.24±0.17 cm) > PU-35 (1.19±0.15 cm) > Azad-2 (1.17±0.12 cm) > PU-31 (1.03±0.26 cm) (Table-4.33, Figure-4.13 C).

ANOVA showed that in case of cotyledonary explant mean values in percent shoot response, number of shoots per explant and average shoot length for both factors (factor -1-genotype and factor-2- herbicide treatment) and their interaction were significant (except interaction effect for percent response) (Table-4.34 and Appendix Table-19, 20, 21).

4.3.2.2.2 Hypocotyl as Source of Explants

Unlike media–(D), media–(H) with combination of PGRs NAA-0.5+ BAP-2.0+ Kn-1.0 mg/l in which BAP and Kn amount was increased and NAA amount was decreased hypocotyl explants showed good positive response for shooting in control as well as herbicide treated media. Initially callus was induced and then shoots buds and shoots were formed (Plate-11 E, Plate-12F, Plate-13G). However percent response was less as compared to media –D but in this combination 0.01 mM also showed shooting response. At 0.05 mM shoots were not formed except in Azad-1 with 15% response. At 0.01 mM maximum percent response was recorded in Azad-1 (41.67%) while minimum was found in PU-35 (31.11%) and PU-31 (31.67%). Number of shoots per explant was found decreased in this combination also. In control genotype genotype Azad-1 showed best response with maximum number of shoots (3.33±0.33) while minimum numbers of shoots were showed by PU-31 (1.98±0.23). Similarly longest shoots at 0.01 mM were showed by genotype Azad-1 (2.28±0.45 cm) and shortest were found in PU-19 (1.07±0.09 cm) (Table-4.33).

4.3.2.2.3 Shoot apex as Source of Explants

Response of shoot apex explants was some what similar to media –D as shooting response was observed at 0.05 mM in all genotypes. In 0.1 mM genotype PU-35 and IPU-94-1 showed shoot response while in 0.2 mM only genotype IPU-94-1 showed
response. Explants formed shoots at apex and callus was initiated at cut basal end (Plate-10 C; Plate-12 H, E; Plate-13 H, I). Percent response, number of shoots and shoot length were decreased in dose dependent manner. In control maximum percent shoot response was found in PU-35(48.89%) and minimum in IPU-94-1 and PU-31 (38.33%) while at 0.1 mM PU-35(15.00) showed more response than in IPU-94-1(6.66). As compared to cotyledonary node explant shoot number and shoot length were less in shoot apex. Maximum number of shoots in control was showed by genotype PU-31(3.33±0.33) and minimum were recorded in Azad-1(2.00±0.58). At 0.01 shoot length from maximum to minimum in genotypes were PU-19(2.22±0.21cm)>IPU-94-1(2.15±0.23cm)>Azad-1(2.06±0.34cm)>Azad-2(1.56±0.12cm)>PU-31 (1.28±0.19cm)>PU-35 (1.21±0.08cm) (Table- 4.33).

4.3.2.3 NAA-2.0+BAP-2.0+Kn-1.0 mg/l -(I)

In this combination of PGR only two explants cotyledonary and shoot apex responded and genotypic variation in herbicide tolerance among six black gram genotypes was observed. Both the explants showed multiple shoots, shoot buds and shoots with callus in control and herbicide treated media but cotyledonary node were found superior for callusing as well as shooting (Plate-14 A, G, H). Only in media–(I) response was observed in 0.4 mM treated media in which all genotypes except Azad-1 and Azad-2. Percentage of explants that showed shooting response ranged from 76.67% to 3.33% (Table-4.35 and 4.36).

4.3.2.3.1 Cotyledonary Node Explant as Source of Explants

Morphogenic response in cotyledonary node was similar to media–(D) and media–(H). Shooting were observed in control and herbicide treated media but herbicide showed adverse effect on shoot regeneration and callogenesis (Plate-14,15). Percent shooting response decreased in concentration dependent manner. In control genotype PU-35 showed maximum percent shooting response (76.67%) and genotype IPU-94-1 showed minimum response (60.00%) (Figure-4.14 A). At 0.1 mM where all genotypes showed organogenesis highest and lowest response were observed in PU-19 and Azad-2 respectively.

Like other combination of media (D and H) number of shoots per explant and length of shoots were decreased with increasing herbicide dose. In control maximum number of shoots was found in PU-19 (9.00±0.58) and minimum in PU-35 and Azad-2 while in 0.1 mM maximum shoot number were again noticed in PU-19 (3.00±0.58) and minimum in Azad-2 (Figure-4.14 A). Length of shoots was also reduced in this
combination of PGR with the increasing herbicide dose. At 0.2 mM longest shoots were recorded in PU-19 (1.27±0.05cm) followed by IPU-94-1(1.10±0.26cm) and Azad-1 (0.97±0.03cm) while shortest shoots were formed in genotype PU-31 and Azad-2 (0.60±0.06cm) (Figure-4.14 C). At 0.2 mM on the basis of percent shooting response from highest to lowest genotypes can be placed in order of PU-19 (26.67)>Azad-1,IPU-94-1 (15.00)>PU-31, PU-35(10.00)>Azad-2(8.33). At highest dose of 0.4 mM PU-19, IPU-94-1 and PU-35 showed shooting but to a very less extent (Table-4.35) Two way ANOVA showed that in case of cotyledonary explant mean values in percent shoot response, number of shoots per explant and average shoot length in media-(I) for both factors (factor -1-genotype and factor-2- herbicide treatment) and their interaction were significant (Table-4.37; Appendix Table- 22, 23, 24).

4.3.2.3.2 Shoot apex as Source of Explants

Percentage of explants that responded for shooting was low as compared to cotyledonary node in control and herbicide treated media. Morphogenic response was almost similar to other combinations of PGR but callus formed at base of explant was more compact and brownish green in color. At apex green young shoots were proliferated that not increased their length after 45 days and became chlorotic and dead. In some genotypes only leaf like structure of moderate length formed at apex (Plate-14 I). In control media greatest percent response was recorded in Azad-2(46.67) followed by PU19 (45.00), Azad-1, IPU-94-1(40.00) and lowest in PU-31(33.33). At 0.1 mM genotype PU-19 showed highest regeneration response (35.00) followed by PU-35 (33.33), Azad-1(25.00) while genotype Azad-2 (10.00) (Table-4.36).

Shoot number per explant and shoot length consistently decreased with increasing concentration of herbicide in all genotypes. Maximum number of shoots in control was found in genotype IPU-94-1(4.00±0.58) while minimum in Azad-1 and PU-19(3.33±0.88). At 0.1 mM genotype PU-19 and IPU-94-1 showed maximum shoot number (2.00±0.58) and Azad-1 and Azad-2 showed least shoot number (1.00±0.58). There was not great variation in shoot length among genotypes. Longest shoots in control were found in PU-31(2.70±0.15cm) and shortest in PU-35(2.20±0.18cm) while in case of 0.1 mM media maximum shoot length was in Azad-1 (1.37±0.22cm) and minimum in Azad-2(1.07±0.03cm) (Table-4.36).
4.3.3 Media Tested for Root Formation

4.3.3.1 NAA-3.0+BAP-1.0mg/l

In this combination four types of seedling explants: leaf segments, cotyledonary node, hypocotyl and shoot apex were used to assess effect of glyphosate on root formation, root number and root length as there are many reports in which glyphosate caused reduction in root number and root length. All the explants showed positive response in this combination but hypocotyl and cotyledonary node explants showed better response than leaf segments and shoot apex (Table-4.38). In all seedling explants first callusing was observed and then roots initiated at different places depending upon the explant. In control rooting was observed in all explants and in all genotypes but in herbicide treated media rooting was observed usually in leaf segments and hypocotyl explants. Herbicide showed also showed negative effect on percent root response, number of roots per explant and root length and they were found decreased on increasing herbicide concentration.

4.3.3.1.1 Leaf Segments as Source of Explants

Leaf segments initially formed callus at cut ends and midrib region then root were proliferated from callus that appeared originate from midrib tissue (Plate-16 A, B). Roots were observed up to 0.05 mM except in Azad-2 at 0.01 mM. Percent root response were maximum in IPU-94-1 (85.00) followed by Azad-1 and PU-35 (80.00) and minimum in PU-19 (68.33). At 0.01 mM percent root response was decreased with respect to control in all genotypes except in PU-19 where it was increased up to 80.00%. At 0.05 mM highest percent root response was recorded in PU-19 (65.00) while lowest in PU-31 and PU-35 (35.00). Maximum number of roots per explant was found in control and among genotypes highest in PU-19 (5.89±0.40) and minimum in Azad-2 (2.78±0.06). In 0.05 mM maximum number of roots was observed in PU-19 (3.78±0.29cm) and minimum in PU-35 (1.89±0.07). Root length in control was also maximum in PU-19 (3.67±0.22cm) and minimum in PU-31 (2.15±0.14cm). At 0.05 mM maximum root length was in PU-19 (2.57±0.23cm) and minimum in Azad-1 (1.57±0.21cm) (Table-4.39, Plate-16).

4.3.3.1.2 Cotyledonary Node as Source of Explants

Cotyledonary node explants also gave good response for rooting with sufficient callusing (Plate-16 J; Plate-17 F, H, I). In some genotypes like PU-35 few shoots were also observed with roots and callus (Plate-17 H). Percentage of explants that showed rooting (% rooting response) was decreased as glyphosate concentration in media was
increased. Highest percent root response was recorded in control and among genotypes highest value reached in genotype PU-19 (75.00) and minimum in PU-31 where no response was observed in any treatment. At 0.01 mM only PU-19, PU-35 and Azad-1 showed rooting in cotyledonary node explants while at 0.05 mM PU-19, IPU-94-1 and Azad-2 showed response and maximum value was found in IPU-94-1 (40.00). Number of roots per explant and root length did not showed definite pattern of decrease on increasing herbicide concentrations like other parameters in other responses like callus fresh weight, shoot number and shoot length. Root number was highest in control media and among responding genotypes Azad-1 showed maximum value (16.18±0.34) and Azad-2 showed minimum value (5.98±0.08). At 0.05 mM maximum roots were found in IPU-94-1 (6.78±0.98) and minimum in Azad-2 (1.56±0.45). Usually longest roots were in control but in some genotypes PU-35 and Azad-1 average root length was slightly increased in 0.01 mM. At 0.05 mM longest roots were in IPU-94-1 (3.09±0.24cm) and shortest in PU-19 (2.43±0.22cm) (Table-4.39, Plate-16, 17).

4.3.3.1.3 Hypocotyl Explant as Source of Explants

Hypocotyl explants were found best for rooting and callusing in PGR combination of NAA-3.0+BAP-1.0 mg/l. Rooting were observed in all genotypes in control, 0.01 mM (except Azad-2), 0.05 mM (except IPU-94-1), 0.1 mM (except Azad-2) and at 0.2 mM in PU-19, PU-35 and Azad-1 (Plate-17 B, C, E). Percent root response decreased in a dose dependent manner. In control maximum response was found in Azad-1 (71.67) and minimum in PU-19 and Azad-2 (60.00) (Figure-4.15 A). At 0.1 mM genotype IPU-94-1 (30.00) showed maximum root response while Azad-2 did not show response. Root number per explant and root length was usually higher than other explants. In control maximum roots were recorded in IPU-94-1 (14.89±0.99) and minimum in Azad-2 (10.22±0.62) where as at 0.1 mM maximum roots were recorded in Azad-1 (5.00±0.19) and minimum in Azad-2 (Figure-4.15 B). Longest roots were found in control and among genotypes IPU-94-1 showed maximum value (5.83±0.19cm) and minimum in Azad-2 (3.70±0.46cm) while shortest one were observed in 0.1 mM. At 0.1 mM maximum root length was in PU-19 (1.87±0.52cm) and minimum in PU-31 (1.20±0.26 cm) (Plate-17 L) (Figure-4.15 C). ANOVA showed that in case of hypocotyl explant mean values in percent root response, number of roots per explant and average root length for both factors (factor -1-genotype and factor-2- herbicide treatment) and their interaction were significant (Table-4.40, Appendix Table-25, 26, 27).
4.3.3.1.4 Shoot Apex as Source of Explants

Shoot apex explants was least responding and most sensitive for herbicide glyphosate among all explants and produced roots only in control and lower concentrations. In shoot apex explants callusing was observed at base and at apex very few shoots produced. From callus roots were proliferated later (Plate-16 G, H, I). Maximum root response in control was noticed in genotype PU-19 (65.00%) and minimum in IPU-94-1 (0.00%). At 0.01 mM roots were not found in IPU-94-1, PU-31 and Azad-2 whereas at further concentrations response was not found in any genotypes. At 0.01 mM maximum roots were obtained in PU-19 (2.33±0.19) and minimum in PU-35 (1.97±0.31) while longest roots were recorded in Azad-1(1.87±0.32cm) and shortest in PU-35 (1.54±0.07cm) (Table-4.39).
4.4 Gradual Stress Treatment of Callus Cultures of Selected Tolerant Genotypes

The effect of herbicide treated media on callus growth is summarized in Table 4.39. Callus fresh weight data were analyzed using the ANOVA procedure for a completely randomized design. Glyphosate treatment significantly inhibited callus growth as observed by consistent decrease in fresh weight with the increasing concentration of herbicide. At 0.01 mM significant (p < 0.05) decrease in fresh weight was observed only in Azad-1, however at next herbicide level 0.05 mM, all genotypes showed significant reduction in fresh weight and maximum fresh weight was observed in PU-19 (1.61±0.09g) whereas IPU-94-1 showed minimum fresh weight (1.48±0.06g) (Table 4.41, 4.42). The selection procedure was stopped at 0.4 mM where a marked decrease in callus growth was seen and the herbicide treatment was observed to be lethal to all genotypes. The callus at this stage was blackish brown with some small greenish areas (Plate 19 G).

When cultures were gradually subcultured on media with higher concentration of glyphosate, inhibition was observed in all growth parameters but it was less than shock treatment. Range of percent of surviving cultures was 93 to 10%. Maximum culture survival was in control and usually decreased on increasing glyphosate concentration except in PU-19 at 0.05mM where it was increased (Figure 4.17). At highest stress level (0.4 mM) maximum percentage of surviving cultures were found in PU-19 (30%) and minimum in Azad-1 (10%).

Response of genotypes for callus fresh weight was similar to shock treatment and it further confirmed the higher tolerance of IPU-94-1 and PU-19 than Azad-1. Tolerance index was also decreased with increasing herbicide concentration. Tolerance index was maximum in 0.01mM and lowest in 0.4mM. At 0.01 highest tolerance index was in PU-19 (61.47) and lowest in Azad-1 (54.01). Accordingly tolerance index was also highest for IPU-94-1 at 0.4mM. (Figure 4.18).

4.5 Shock Treatment of Callus Cultures of Selected Tolerant Genotypes

In direct shock treatment at highest stress level (0.4 mM) maximum percentage of surviving cultures was observed in IPU-94-1(10%) while minimum value obtained in PU-19 (6.67%) and in Azad-1 there was no cultures survived at this concentration. Callus fresh weight (CFW) after four weeks of subculture (directly on high stress media) was also reduced significantly at all stress levels (Plate 18). CFW was found highest in control media and lowest on stress media of 0.4 mM. Among genotypes maximum callus fresh weight at highest stress level was found in IPU-94-1 with minimum 86.74%
reduction over control while Azad-1 showed 100% reduction in fresh weight because of complete death of inoculated callus in 0.4 mM. Similarly tolerance index at 0.4 mM was found maximum in IPU-94-1 and lowest for Azad-1 (Figure-4.18) (Table-4.41, 4.42).

On comparing both types of stress treatment it was concluded that shock treatment of herbicide stress was inhibitorier than gradual treatment on callus growth in all three genotypes.
4.6 In vivo Assessment of Effect of Herbicide and Tolerance in Vigna mungo Genotypes

Healthy seeds (soaked in water for 24 hr) of three better performing genotypes PU-19, IPU-94-1 and Azad-1 screened via in vitro methods were first soaked in water for 24 hours and then sown in field under wire house conditions. After 21 days of sowing plants were subjected to post emergent treatment of different concentration of glyphosate herbicide i.e., control, 1 mM, 2 mM, 4 mM, 6 mM and 8 mM. Effect of herbicide on biochemical parameters were assessed 4-5 days after treatment, survival percentage was taken after 15 days of treatment while growth and yield parameters were taken after harvesting. The parameters studied were survival percentage, plant height, number of nodes, internodal length, number of pods, pod length, number of seeds per pod, number of seeds per plant. Changes in the plant morphology and yield were genotype-dependent, responding differently to glyphosate.

4.6.1 Morphological and Yield Parameters

4.6.1.1 Survival Percentage

The F-ratio was found to be statistically significant at 1% (i.e. p < 0.01) for all treatments of glyphosate. Survival percentage taken 15 DAT showed dose dependent decrease in all the genotypes and treatments. The data was taken only up to 6 mM dose as survival at 8 mM was nil. Glyphosate treatment of even lowest dose showed adverse impact on survival percentage in all the three black gram genotypes. Survival percentage was highest in control and lowest in 6 mM. At highest concentration, 6 mM genotype PU-19 showed maximum survival percentage (31.11±2.22%) whereas Azad-1 showed minimum (22.22±2.22%) (Table-4.43, Figure-4.18 A). Significant difference with respect to control was observed in all treatments and genotypes except PU-19 in 1 mM. Thus on the basis of response of three genotypes at 6 mM PU-19 can better tolerate the herbicide toxicity as compared to IPU-94-1 and Azad-1.

Two way ANOVA calculated for survival percentages of three genotypes grown in field showed significant effect of only concentration (factor 2) while effect of genotype (factor 1) and interaction between genotype and concentration was not significant. Factor 1-F_{2,30}=3.019, p=0.0639; Factor 2- F_{4,30}=143.1, p<0.0001; factor 1x2-F_{8,30}=1.398, p=0.2378 (Appendix Table-28)

4.6.1.2 Plant Height (cm)

The F-ratio was found to be statistically significant at 1% (i.e. p < 0.01) in all genotypes of Vigna mungo. The over all maximum and minimum height was observed to
be at control and 6 mM treatment of glyphosate respectively. The plant height of *Vigna mungo* genotypes in all herbicide treatments ranged from 43.33±2.41 cm (control) to 9.67±0.33 cm (6 mM). Plant height decreased at all concentration of herbicide in all genotypes (Plate-20). Among controls maximum height was observed in genotype IPU-94-1 (43.33±2.41 cm) and minimum in Azad-1 (37.67±1.45 cm) whereas as highest concentration tallest plants were observed in PU-19 (13.00±1.00 cm) and shortest in Azad-1 (9.67±0.33 cm) (Table -4.43, Figure-4.18 B).

Two way ANOVA calculated for plant height of three genotypes grown in field showed significant effect of concentration (factor 2) and genotype (factor 1) while effect of interaction between genotype and concentration was not significant. Factor 1- \( F_{2,30}=10.55, p=0.0003 \); Factor 2- \( F_{4,30}=247.1, p<0.0001 \); factor 1x2- \( F_{8,30}=0.922, p=0.5125 \) (Appendix Table-29).

### 4.6.1.3 Number of Nodes per Plant

The F-ratio was found to be statistically significant at 1% (i.e. \( p < 0.01 \)) in all genotypes of *Vigna mungo*. The over all maximum and minimum number of nodes was observed to be at control and 6 mM treatment of glyphosate respectively. Like height of plants number of nodes of all genotypes of *Vigna mungo* in all herbicide treatments decreased with increasing herbicide dose. On comparing the means, number of nodes at 2 mM, 4 mM and 6 mM were found to be significantly (\( p<0.05 \) or \( p<0.01 \)) lower as compared to control in all genotypes (Table-4.44). In control maximum and minimum number of nodes was observed in IPU-94-1 (22.46±0.92) and Azad-1 (18.47±0.60) respectively whereas at 6 mM maximum and minimum number of nodes was found in PU-19 (9.88±0.76) and IPU-94-1 (8.93±0.75) respectively (Figure-4.18 C).

Two way anova for number of nodes showed extremely significant effect of genotype (factor 1) and concentration (factor 2) and significant effect of interaction between genotype and concentration. Factor 1- \( F_{2,30}=12.27, p<0.0001 \); Factor 2- \( F_{4,30}=88.15, p<0.0001 \); factor 1x2- \( F_{8,30}=3.518, p=0.0055 \) (Appendix Table-30).

### 4.6.1.4 Internodal Length

The F-ratio was found to be statistically significant at 1% (i.e. \( p < 0.01 \)) in all genotypes of *Vigna mungo*. Negative effect of glyphosate was also observed on internodal length of treated plants and it was significantly (\( p<0.01 \)) reduced in all the treatments except at 1 mM in PU-19 and Azad-1 (Table-4.44). The over all maximum and minimum internodal length was observed to be at control and 6 mM treatment of glyphosate respectively and it ranged from 2.59±0.18 cm (control-IPU-94-1) to
1.10±0.07cm (6 mM -IPU-94-1) among all genotypes. Among 6 mM maximum internodal length was observed in Azad-1(1.19±0.03 cm) and minimum in IPU-94-1(1.10±0.07cm) (Figure-4.19 A).

Two way ANOVA calculated for internodal length showed extremely significant effect of only concentration (factor 2) but effect of genotype (factor 1) and of interaction between genotype and concentration was not significant. Factor 1- F$_{2,30}$=2.984, p<0.0658; Factor 2- F$_{4,30}$=36.35, p<0.0001; factor 1x2-F$_{8,30}$=3.518, p=0.1902 (Appendix Table-31).

4.6.1.5 Number of Pods per Plant

The F-ratio was found to be statistically significant at 1% (i.e. p < 0.01) in all the genotypes of Vigna mungo. The effect of glyphosate was also seen on the yield as reflected by a gradual decrease in the number of pods per plant, pod length and seeds per pod. The number of pods per plant was 16.10±0.88 in PU-19, 16.93±0.90 in IPU-94-1 and 15.03±1.09 in Azad-1 in the control (Table-4.45). It was decreased significantly (p<0.05 or p<0.01) after glyphosate treatment in all treatments and all genotypes. At 6 mM, the number of pods per plant was 8.94±0.61 in PU-19, 7.75±0.43 in IPU-94-1 and 7.29±0.47 in Azad-1 (Figure-4.19 B).

Two way ANOVA calculated for number of pods per plant showed extremely significant effect of only concentration (factor 2) but effect of genotype (factor 1) and of interaction between genotype and concentration was not significant. Factor 1- F$_{2,30}$=0.429, p<0.6546; Factor 2- F$_{4,30}$=65.36, p<0.0001; factor 1x2-F$_{8,30}$=1.254, p=0.3034 (Appendix Table-32).

4.6.1.6 Length of Pods (cm)

The F-ratio was found to be statistically significant at 1% (i.e. p < 0.01) in all the genotypes of Vigna mungo. Glyphosate treatment affected pod length as well. Significant difference in pod length was observed in 2 mM, 4 mM and 6 mM. It was 4.26±0.14cm in PU-19, 4.95±0.05cm in IPU-94-1 and 4.58±0.28 in Azad-1 in the control (Table-4.45). A gradual decrease in the pod length was observed after glyphosate treatment. At 6 mM, the pod length was 3.51±0.22cm in PU-19, 3.32±0.13cm in IPU-94-1 and 3.50±0.06cm in Azad-1 (Figure-4.19 C).

Two way ANOVA calculated for pod length plant showed extremely significant effect of only concentration (factor 2) but effect of genotype (factor 1) and of interaction between genotype and concentration was not significant. Factor 1- F$_{2,30}$=1.442, p<0.2523; Factor 2- F$_{4,30}$=23.91, p<0.0001; factor 1x2-F$_{8,30}$=1.535, p=0.1870 (Appendix Table-33).
4.6.1.7 Number of Seed per Pod

The number of seeds per pod was also decreased with increasing glyphosate concentration. It was 5.15±0.20 in PU-19, 5.50±0.26 in IPU-94-1 and 5.65±0.34 in Azad-1 in the control (Table-4.45). At 6 mM, the number of seeds per pod was 3.35±0.17 in PU-19, 3.40±0.20 in IPU-94-1 and 3.66±0.04 in Azad-1.

Two way ANOVA calculated for number of seed per pod plant showed extremely significant effect of only concentration (factor 2) but effect of genotype (factor 1) and of interaction between genotype and concentration was not significant (Appendix Table-34).

4.6.2 Biochemical Parameters

4.6.2.1 Pigment Estimation

The effect of glyphosate was also seen on leaf pigments. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were measured in the leaves of control and treated plants. All of these registered a decrease after glyphosate treatment.

4.6.2.1.1 Chlorophyll a, b and Total Chlorophyll

In the controls, Chl a was 1.39±0.01 mg/g FW in PU-19, 1.65±0.06 mg/g FW in IPU-94-1, and 1.47±0.05 mg/g FW in Azad-1 (Table-4.46). At 6 mM glyphosate dose chl a content decreased to 0.14±0.02 mg/g FW in PU-19, 0.31±0.02 mg/g FW in IPU-94-1, and 0.32±0.01 mg/g FW in Azad-1. Thus, chl a content was highly reduced in PU-19 as compared to IPU-94-1 and Azad-1 (Figure-4.21 A). In control plants, Chl b was 0.66±0.02 mg/g FW in PU-19, 0.78±0.02 mg/g FW in IPU-94-1, and 0.62±0.02 mg/g FW in Azad-1 (Figure-4.21 B). It also showed a significant decrease after glyphosate treatment. At 6 mM chl b content decreased to 0.34±0.04 mg/g FW in PU-19, 0.35±0.05 mg/g FW in IPU-94-1, and 0.22±0.03 mg/g FW in Azad-1. In case of Chl b Azad-1 showed the more decrease as compared to IPU-94-1 and Azad-1.

Total Chl was 1.39±0.01 mg/g FW in PU-19, 2.46±0.07 mg/g FW in IPU-94-1, and 2.03±0.05/g FW in Azad-1. Herbicide treatment caused significant decrease in all the treatments and genotypes. At 6 mM total Chl decreased to 0.74±0.03 mg/g FW in PU-19, 0.69±0.04 mg/g FW in IPU-94-1, and 0.66±0.05/g FW in Azad-1 (Figure-4.20 C).

4.6.2.1.2 Carotenoids

Carotenoids were also estimated, and like chlorophyll they too showed reduction after glyphosate exposure. In the control plants carotenoids were 0.70±0.02 mg/g FW in PU-19 and Azad-1, 0.72±0.04 mg/g FW in IPU-94-1 (Table-4.47, Figure-4.21 C).
Azad-1 showed significant decrease in carotenoids in all concentrations while other two genotypes showed significant reduction only in 4 mM and 6 mM. At 6 mM glyphosate concentration carotenoids decreased to 0.30±0.02 mg/g FW in PU-19, 0.32±0.03 mg/g FW in IPU-94-1, and 0.29±0.01 mg/g FW in Azad-1.

Thus the above results show that glyphosate severely affects pigment content. The reduction in pigment content suggested that glyphosate disturbed pigment metabolism in plants.

4.6.2.1.3 Catalase Activity

In field experiment catalase activity was measured after 4 days of treatment in leaves of three genotypes of Vigna mungo. Amongst the controls, catalase activity was lowest, 166.67±13.35 µmole H₂O₂ decomposed/100mg FW in leaves of IPU-94-1. In PU-19, it was 200.00±11.56 µmole H₂O₂ decomposed/100mg FW and 186.67±17.66 µmole H₂O₂ decomposed/100mg FW in Azad-1 (Table-4.48, Figure-4.20 A). The catalase activity was increased in a dose dependent manner after glyphosate treatment in all the three genotypes except in Azad-1 where activity slightly declines in 6 mM. At 6 mM glyphosate dose, the activities were 960.00±23.12 µmole H₂O₂ decomposed/100mg FW in PU-19, 773.33±29.09 µmole H₂O₂ decomposed/100mg FW in IPU-94-1, and 486.67±17.66 µmole H₂O₂ decomposed/100mg FW in Azad-1. According to LSD test, the increase in activity in all the treatments as compared to control was significant in all the three genotypes.

Two way ANOVA for catalase activity in herbicide treated leaves showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1-F₂,30=150.4, p<0.0001; Factor 2- F₄,30=361.6, p<0.0001; factor 1x2-F₈,30=26.79, p=0.0001 (Appendix Table- 39).

4.6.2.1.4 Peroxidase Activity

In field experiment peroxidase activity was measured after 4 days of treatment in leaves of three genotypes of Vigna mungo. In control plants genotype IPU-94-1 showed maximum activity (3.73±0.32 ΔOD/100mg FW) and genotype PU-19 showed minimum peroxidase activity (2.37±0.10 ΔOD/100mg FW). Peroxidase activity also significantly increased like catalase with the increasing herbicide concentration in 1 mM and 2 mM and then it was decreased but in case of PU-19 it was consistently increased up to 4 mM and the decreased. At 6 mM highest activity was obtained in Azad-1 (6.88±0.40 ΔOD/100mg FW) where as lowest activity was shown by IPU-94-1 (5.06±0.32 ΔOD/100mg FW) (Table-4.48).
Two way ANOVA for peroxidase activity in herbicide treated leaves showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1- $F_{2,30}=30.83, p<0.0001$; Factor 2- $F_{4,30}=90.11, p<0.0001$; factor 1x2- $F_{8,30}=12.67, p=0.0001$ (Appendix Table- 40).

### 4.6.2.1.5 Superoxide Dismutase Activity

Activity of antioxidative enzyme SOD was also measured in herbicide treated leaves of three *Vigna mungo* genotypes. In the controls it was 10.70±0.63 in PU-19, 11.99±1.05 in IPU-94-1, and 12.38±0.78 in Azad-1. After the glyphosate treatment, the activity was found to increase significantly ($p<0.05$ or $p<0.01$) in all the three varieties from 2 mM except in IPU-94-1 where it was decreased sharply at 6 mM (Table-4.49, Figure-4.20 B). The SOD activity at 6 mM was 18.32±0.53 in PU-19, 11.99±1.05 in IPU-94-1, and 18.96±0.63 in Azad-1. The increase in SOD activity also suggests that glyphosate is inducing oxidative stress in *V. mungo*.

Two way ANOVA for SOD activity in herbicide treated leaves also showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as interaction between genotype and concentration. Factor 1- $F_{2,30}=44.57, p<0.0001$; Factor 2- $F_{4,30}=26.24, p<0.0001$; factor 1x2- $F_{8,30}=4.570, p=0.0010$ (Appendix Table- 41).

### 4.6.2.1.6 Total Protein Content

Post emergent treatment of glyphosate caused a general increase in total protein content as compared to control in all treatments and genotypes. In IPU-94-1 there was not significant difference in protein content at any concentration (treatment) and here slight increase was occurred at 6 mM while other two genotypes showed significant differences in all concentrations but there was slight decline in protein content at 6 mM as compared to 4 mM. In controls minimum protein content was in Azad-1 (5.69±0.25 mg/g fw) and maximum in IPU-94-1 (6.78±0.30 mg/g fw). At 6 mM minimum protein content was found inPU-19 (6.20±0.09 mg/g fw) and maximum in IPU-94-1 (7.75±0.99 mg/g fw) (Table-4.49, Figure-4.22 C).

Two way ANOVA for protein content in herbicide treated leaves revealed that there was not significant effect of genotype (factor 1) and interaction between genotype and concentration on protein content but there was significant effect of concentration (factor-2. Factor 1- $F_{2,30}=1.811, p<0.0001$; Factor 2- $F_{4,30}=3.602, p<0.0001$; factor 1x2- $F_{8,30}=0.6117, p=0.0010$ (Appendix Table- 42).
4.6.2.1.7 MDA Content

To assess effect of herbicide glyphosate on lipid membranes of leaves malondialdehyde content was analysed in leaves of three *Vigna mungo* genotypes. In the controls it was 68.67±0.52 nmole/g FW tissue in PU-19, 60.60±1.30 nmole/g fw tissue in IPU-94-1, and 58.99±1.50 nmole/g FW tissue in Azad-1. After the glyphosate treatment, MDA was found to increase significantly (p<0.05 or p<0.01) in all the three varieties in 4 mM and 6 mM in all the three genotypes. (Table-4.50, Figure-4.22 A). MDA content at 6 mM was 99.00±0.82 nmole/g FW tissue in PU-19, 68.69±0.90 nmole/g FW tissue in IPU-94-1, and 71.01±0.54 nmole/g FW tissue in Azad-1. The increase in MDA content indicates that glyphosate caused lipid membrane damage in *V. mungo*.

Two way ANOVA for MDA content in herbicide treated leaves showed extremely significant effect of genotype (factor 1) and concentration (factor 2) as well as for interaction between genotype and concentration. Factor 1-F$_{2,30}$=172.1, $p<0.0001$; Factor 2- F$_{4,30}$=82.20, $p<0.0001$; factor 1x2-F$_{8,30}$=20.59, $p=0.0001$ (Appendix Table- 43)

4.6.2.1.8 Proline Content

Under many abiotic stresses proline levels are found to be increased. Thus in our studies effect of herbicide glyphosate on proline content was also estimated in leaves of three *Vigna mungo* genotypes. In the controls it was 0.65±0.02 µmole/g FW tissue in PU-19, 0.61±0.01 µmole/g FW tissue in IPU-94-1, and 0.67±0.05µmole/g FW tissue in Azad-1. After the glyphosate treatment, proline was usually found to increase in all treatments and genotypes but significant (p<0.05 or p<0.01) increase was found only at 4 mM and 6 mM. (Table-4.50, Figure-4.22 B). Proline content at 6 mM was 1.01±0.04 µmole/g FW tissue in PU-19, 1.12±0.08 µmole/g FW tissue in IPU-94-1, and 1.35±0.14 µmole/g FW tissue in Azad-1.

Two way ANOVA for proline content in herbicide treated leaves showed extremely significant effect of genotype (factor 1) and concentration (factor 2) but not for interaction between genotype and concentration. Factor 1-F$_{2,30}$=6.181,$p=0.0057$; Factor 2- F$_{4,30}$=44.21, $p<0.0001$; factor 1x2-F$_{8,30}$=1.662, $p=0.1493$(Appendix Table-44).
Crop improvement relies on the ability to generate genetic variation and selection of individuals with improved characteristics. Improvement in legumes by conventional breeding is limited due to available narrow genetic base as well as natural barriers of crossing between species. Tissue culture is a technique based on the phenomenon of totipotency of plant cell which underlines its genetic potential to produce the complete plant and its ability to perform all the functions of development which are characteristics of zygote (Haberlandt, 1902). With respect of crop improvement plant cells and tissue culture is also potent approach which provides diverse type of input to plant breeding efforts.

In recent past several novel techniques developed from plant tissue culture approach viz. micropropagation, anther culture, in vitro selection, embryo rescue, somaclonal variation, somatic hybridization and transformation which can be used to increase the efficiency of the breeding process, to improve the accessibility of existing germplasm and to create new variation and screen germplasm for desired traits.

Present study was aimed to investigate methods for screening of herbicide tolerant genotypes in urd bean. Herbicide tolerant genotypes screened in this study will be useful resources for development of herbicide tolerant cultivars as well as for undertaking genetic and physiological studies on herbicide tolerance in Vigna mungo.

Genetic variability for herbicide tolerance among genotypes of Vigna spp and other crops was studied earlier by many workers. Si et al. (2012) identified a number of highly tolerant yellow lupin genotypes to metribuzin and carfentrazone-ethyl herbicides. LD50 of the two herbicides were used to screen diverse yellow lupin landraces and wild types to quantify the diversity of the germplasm. Considerable variation in response to the two herbicides was observed among the genotypes screened, ranging from complete plant death to no symptoms. Genotypes with no damage were considered as tolerant while genotypes showing complete death were susceptible. Farnham and Harrison, (1993) screened broccoli cultivars for tolerance to oxyfluorfen herbicide after post emergent and pre emergent application of herbicide. Tolerant cultivar (Pinnacle) showed least foliar damage and reduction in fresh weight while Green Goliath was very susceptible to oxyfluorfen. Gaur et al., (2013) found that large genetic variability
occurred in chickpea for tolerance to herbicides imazethapyr and metribuzin and the sensitivity of the genotypes to metribuzin was higher compared to that for imazethapyr.

In our studies screening of herbicide tolerant genotypes has been carried out by *in vitro* as well as *in vivo* approaches. Tolerant genotypes for other abiotic stresses have also been selected in many crops by both *in vivo* and *in vitro* methods viz. in *Beta vulgaris* for drought tolerance (Ober and Luterbacher, 2002), in *Vicia faba* for drought tolerance (Link et al., 1999), in *Pisum sativum* for cadmium toxicity (Metwally et al., 2005). Genotypic variation was also observed in response to increased carbon dioxide concentration for growth and yield of *Vigna mungo* genotypes (Jyothi Lakshmi et al., 2013).

During present investigations, first *in vitro* screening for herbicide tolerance in *Vigna mungo* genotypes was done by petri dish germination bioassay method in presence of herbicide. In spite of development of many analytical methods, bioassay is still a main tool for qualitative and quantitative determination of herbicides phytotoxicity. Bioassay methodologies are usually more economical, less difficult to perform and do not require as much expensive equipments as in chemical analytical methods. Among many bioassays petri-dish bioassay is more commonly used as the basis of germination test that allows simple and inexpensive analysis of biological activity of environmental pollutants, including herbicides and other hazardous agrochemicals. O’Donovan et al., (1996) used seedling bioassay to study response of wild oat to triallate herbicide on filter paper in petri dishes. Petri dish bioassays have been effectively used in green foxtail for study of herbicide resistance to trifluralin (Beckie et al., 1990), in wild oat for aryloxyphenoxypropionate and cyclohexanediione herbicides (Murray et al., 1996).

### 5.1 Seedling Parameters

*Germination percentage, survival percentage, root length, shoot length, fresh weight, dry weight*  

Roundup is widely used in modern agriculture as a weedicide and being nonselective it also causes damage to nontarget crops. There are several reports on detrimental effect of glyphosate on seed germination and seedling growth. In our study too an adverse affect of herbicide on germination was observed in both petri dish germination and *in vitro* germination on MS media. Germination, seedling survival and all other seedling parameters showed an inverse relationship with herbicide dose. Maximum germination percentage was in control and minimum in 10 mM dose. Maximum percent decrease over control in germination percentage at 10 mM in PU-35 and Azad-2 while minimum percent inhibition over control was found in Azad-1.
Reduction in germination caused by herbicides has been explained due to delay or inhibition of physiological and biological processes necessary for seed germination which include enzyme activity. Jain and Kumari, (2012) suggested that inhibition of germination by glyphosate was due to effect of herbicide on amylase activity in *Cajanus cajan*. Blackburn and Boutin, (2003) found that roundup (1%, 10% or 100% of a 890 g a.i./ha label rate solution) reduce germination and growth of the F₁ generation of seeds produced by plants sprayed with the herbicide. Shaukat et al., (1980) reported that 2,4-D and 2,4,5-T were highly inhibitory for seed germinaton and early seedling growth of *Vigna radiata* and *Vigna mungo* while MCPB (Methyl-chlorophenoxybutyric acid) suppressed germination, root and shoot growth.

Pfeilstetter et al., (2000) performed rapid and efficient screening of phosphinothricin tolerant oilseed rape (*Brassica napus*) from non transgenic rape seedlings with a novel germination test. The method was based on the germination of rape seeds on filter paper soaked with a 0.005% phosphinothricin solution. Under these conditions inhibition of seedling development by the herbicide can be observed after 10 days. The germination test gains an advantage over the routinely used herbicide spraying, because it is rapid, needs little space and allows efficient screening of huge numbers of seeds.

Salazar and Appleby, (1982) also found that glyphosate reduced germination of two legumes and three grasses and reduced seedling growth of all species when applied at rate of 1.0 and 3.0 kg/ha and paraquat (1,1′-dimethyl-4,4-bipyridinium ion) at 0.6 kg/ha to exposed seeds of six grasses and two legume species. Paraquat was toxic only to the grasses. Glyphosate mainly affected germination and growth of legumes when applied directly to their seeds. Preharvest glyphosate applications lead to a general decrease in germination percentage. Baig et al., (2003) observed that preharvest application of glyphosate in pea reduced seed germination and seedling emergence. In another experiment two *Triticum aestivum* varieties were assessed for effects of preharvest glyphosate treatment. Glyphosate was applied at 0.62 or 0.84 kg ae/ha at the milk, soft dough, or hard dough stage of wheat development; or 7 d following the hard dough treatment, and 1 d prior to wheat harvest. Glyphosate application at milk stage affected seed germination (Yenish and Young, 2000). McLaren and Don, (2002) showed that preharvest treatment of Derkado barley crops with glyphosate resulted in decreased levels of germination while Grey and Prostko, (2010) found that glyphosate application reduce peanut pod yield and peanut seed mass but glyphosate applied at any rate or
timing did not affect seed viability. Rajashekar et al., (2012) reported that herbicide pendimethalin reduced seed germination, radicle and plumule length in maize.

After glyphosate treatment root length was significantly decreased with increasing concentration of herbicide glyphosate in all genotypes of Vigna mungo and in all treatments. Percent reduction in seedling root length can be used as an important parameter for assessment of herbicide tolerance among cultivars. In our experiments at higher dose of herbicide genotypes showing lower percent reduction viz. PU-19, IPU-94-1 and Azad-1 could better tolerate the herbicide stress as compared to PU-35, PU-31 and Azad-2. Many reports have clearly demonstrated that glyphosate decrease root length. The reason for reduced root length may be genotoxic effect of herbicide on root meristem. Glyphosate induced inhibition of root length by herbicide in various crops has been reported by many workers (Agnieszka et al., 2011; Jain and Kumari, 2012). Pre emergent treatment of glyphosate in Vigna radiata seeds caused a significant decrease in seedling root length (Basantani et al., 2011). Hampton and Hebblethwaite, (2006) found that after preharvest application of 1 and 2 litres ha⁻¹ IPA glyphosate (isopropylamine salt of glyphosate) to a seed crop of perennial ryegrass, germination was significantly reduced and abnormal seedlings were produced. The germination of seed harvested in the previous year from glyphosate treated plots decreased with storage. Seed vigour, germination rate and field emergence were also significantly decreased as a result of glyphosate application.

In black gram seedlings glyphosate treatment at 8 and 10 mM in petri dish experiment caused stunting and thickening of primary roots that lacked root hairs whereas in in vitro seed germination experiment there was complete absence of roots at concentrations higher than 0.8 mM. It may be due to inhibited growth and death of radicle, hypocotyl and cotyledons caused by glyphosate as proposed by Kamble, (2008). He also found that glyphosate causes inhibition of linear growth of seedlings and causes swelling in Hibiscus cannabinus Linn. Glyphosate caused more damage to radicle as compared to hypocotyl. Rubin et al., (1982) also found that glyphosate treated seedlings were short and stubby, and the roots had few or no secondary roots and percent relative growth of roots were decreased with the increasing glyphosate concentration. Stunted roots with somewhat swelling was observed in treated seedlings of higher doses. These altered morphogenic responses under stress conditions might had involved inhibition of cell elongation, localized stimulation of cell division and alterations in cell differentiation status. The common molecular processes such as increased ROS-
production and altered phytohormone transport and/or metabolism occurs under abiotic stresses. The stress-induced morphogenic response is postulated to be part of a general acclimation strategy, whereby plant growth is redirected to diminish stress exposure (Potters et al., 2007).

Hassan, (1988) reported that glyphosate treatment at different concentrations did not affect the germination percentage in wheat cultivars but in comparison to the control, the herbicide treatment caused significant reduction in seedling root and shoot lengths. The reduction increased proportional to increasing in herbicide concentration. Treatment with high concentrations of the glyphosate caused significant reduction in total root number per seedling. Wheat cultivar showed clear variation in susceptibility to glyphosate treatment. In our studies also differential genotypic response has been observed for glyphosate toxicity in black gram. Reduction in shoot growth after treatment of sulfonylureas herbicide amidosulfuron was reported in target plant Chenopodium album and non target plant Hordeum vulgare by Žaltauskaitė and Brazaitytė, (2011). De Felipe et al., (1986) found significant effect of herbicide isoproturon on root growth of wheat. Nacheva et al., (2010) found that herbicide (Terbacil, Pendimethalin and Napropamide) caused suppression of the root growth of rooted plants under in vitro conditions in Prunus cerasifera embryos and some micropropagated rootstocks of fruit trees and also found inhibition of root meristem growth and browning of cotyledons after the treatment with pendimethalin. Pendimethalin and napropamide showed a depressing effect on the root formation.

Shimina and Neelamegam, (2009) found that the herbicide Excel Mera-71 inhibited the growth of black gram seedlings, except root/shoot ratio and the inhibitory effect was increased with increasing concentration of herbicide. The adverse effect of herbicide foliar spray treatment was higher in 7 day old seedlings than in 15 day old seedlings. The tolerance index of black gram seedlings against herbicide foliar spray treatment was more in 15 day old seedlings while, it was reduced in 7 day old seedlings.

Deleterious effect of glyphosate was observed on fresh weight and dry weight of seedlings of all genotypes of black gram in our experiments both petri dish and in vitro grown seedlings. At the highest glyphosate concentration fresh weight was maximum in IPU-94-1 and minimum in PU-31. Maximum and minimum reduction of dry weight at 10 mM was observed in PU-31 and PU-35. It was concluded that fresh weight measurement is more convincing parameter to assess the relative tolerance of cultivars for agrochemicals as compared to dry weight. Other studies also reported that the
application of glyphosate led to a continuous decrease in the fresh weight of plants like maize (Sergiev et al., 2006), pea (Baig et al., 2003). Glyphosate has also been known to reduce leaf dry matter accumulation in Phaseolus vulgaris L. (Brecke and Duke, 1980). Deleterious effect of herbicide on seedling dry weight was observed at all concentration of herbicide glyphosate in Lolium multiflorum (Perez and Kogan, 2003). There was significant decrease in shoot fresh and dry weight after atrazine treatment (Nemat Alla and Hassan, 2006) and after isoproturon treatment in maize (Nemat Alla et al., 2008b). El Tayeb and Zaki, (2009) also found that seedling fresh weight and dry weight reduced on increasing concentration and duration of roundup. Ali and Fletcher, (1978) also found that glyphosate application caused reduction in plant height, leaf length, and shoot fresh weight up to 62, 60, and 84% respectively in corn seedlings. The lowest concentration of glyphosate (0.28kg/ha) inhibited plant growth more than the inhibition caused by highest concentration of amitrole (3.36 kg/ha). The root growth of plants treated with 1.12 kg/ha glyphosate was inhibited more than 80%, whereas with amitrole at 1.68 kg/ha was 40%. Primary site of action of glyphosate in corn seedlings is in the roots whereas the effect of amitrole is in the shoot.

Similarly Reddy et al. (2001) reported that in glyphosate-resistant soybean glyphosate at the rate of 2.24 kg/ha, reduced shoot and root dry weight by 25 to 30%. Nemat Alla et al., (2008a) reported that recommended field dose of metribuzin, butachlor and chlorimuron-ethyl to 10-days-old wheat and maize seedlings differentially reduced shoot fresh and dry weights. Acuna et al., (2009) studied the effect of herbicide, dose and soil organic matter content using the bioassay technique where radicle length and fresh weight of pre germinated seeds of Avena sativa were used as indicators. Increasing herbicide dose Pendimethalin, Bensulide, Prodiamine and Dithiopyr caused significant decrease in the radicle length but had no effect on fresh weight.

Patil et al., (2012) reported decrease in shoot length of plants, dry weight of plant shoot after treatment of herbicides 2, 4-D amine salt, Roundup and Atrazine. Michałowicz and Duda, (2009) found decrease in fresh weight, dry weight and shoot length of 12 day old plants of reed canary grass leaves (Phalaris arundinacea) after treatment of 2,4,5-Trichlorophenol (herbicide and fungicide).

Seedling vigour index, tolerance index and percentage of phytotoxicity were determined in petri dish grown seedlings. In all Vigna mungo genotypes herbicide treatment caused decrease in seedling vigour index and tolerance index while percentage of phytotoxicity increased with increasing herbicide dose. At 10 mM glyphosate
concentration, on the basis of tolerance index PU-19 and Azad-1 were more tolerant than the other genotypes. Narain et al., (2012) found that lower concentration of tannery effluent increased vigour index while decreased at higher concentrations in *Cicer arietinum*. Increasing level of tannery effluent stress in *Vigna mungo* genotypes caused increase in percent phytotoxicity (Indra and Mycin, 2009).

**5.2 Morphological/Growth and Yield Parameters**

In wire house experiment tolerance of three better performing genotypes of *Vigna mungo* were studied *in vivo*. Detrimental effects of glyphosate were observed in form of reduced survival percentage, plant height, number of nodes per plant, internodal length, number of pods per plant and number of seeds per pod. At highest dose of glyphosate (8 mM), survival was not observed in any genotype while at 6 mM genotype PU-19 and Azad-1 showed maximum and minimum survival percentage respectively. Plant height was significantly reduced in all glyphosate treatment and in all genotypes. Maximum plant height at 6 mM was found in PU-19 and minimum in Azad-1. Leaf necrosis and chlorosis with curling of leaves was observed in 4 and 6 mM in all the three genotypes and it was increased with the increase in days after treatment. The results of the present study visibly revealed that glyphosate affects the overall growth and morphology of plants, the effects persist even during the flowering and fruiting of plants and plants could not endure high herbicide doses. In previous studies also it has been shown that glyphosate affects plant morphology. Mahakavi et al., (2014) found that black gram plants treated with quizalafo p-ethyl (1 %, 1.5 and 2 %) showed significant decrease in plant height. Yield attributing characters like number of pods/plant, seeds/plant and weight of 100 seeds were also decreased at 1 %, 1.5 and 2 %. Quizalafo p-ethyl treatment also decreased the photosynthetic pigments at 0.5 %, 1 %, 1.5 % and 2 % at 5 DAT, 10 DAT and 20 DAT over their respective controls. They observed that chl a, chl b and carotenoid contents reached maximum in 15 days after flowering and rapidly decreased after 33 days of flowering. According to Cole, (1985) chlorophyll level is highly sensitive to glyphosate, and plants exposed to sublethal doses of the herbicide have been found to show an achlorophyllous subsequent foliar growth. Glyphosate concentrations as low as 10 µM cause more than 50% inhibition of seed germination. On the contrary, a treatment with 2.4 mM to plantlets at the five-leaf stage does not inhibit growth. There are several reports on deleterious effect of glyphosate on plant morphology. Forlani and Racchi, (1995) found that herbicide glyphosate at a concentration of 1.2 mM to plantlets at the three-leaf stage resulted in the onset of
damage symptoms like mild bleaching and slow development after one week of treatment but in the next two weeks damage of seedlings was overcome and growth was like control. Out of 11 inbred lines, treatment of 2.4 mM concentration caused complete leaf necrosis in few lines after three weeks, while plantlets of other inbreds were much less affected. Ozlem and Dane, (2007) used four different doses of Fusilade (0.25%, 0.5%, 1% and 1.5%) in (Fluazifop-p-butyl) Lens culinaris seeds and leaves. He reported that shoot growth and lateral root growth was reduced in Fusilade treated groups and leaf deformations like chlorosis, curling, expansion and asymmetry were observed. Santos et al., (2005) demonstrated that glyphosate affects leaf growth and morphoanatomy of Eucalyptus. The treatments were 0, 43.2, 86.4, 172.8 and 345.6 g a.e. glyphosate/ha, applied 40 days after transplanting. Phytotoxicity in relation to the control was evaluated 7, 15, and 30 days after application (DAA). Wilting, chlorosis and curling were observed in plant apices sprayed with 17.8 and 345.6 g a.e. glyphosate/ha. The treated plants also showed abnormal branching and necrosis of the tissue.

During field testing of relative tolerance of genotypes, PU-19 and IPU-94-1 showed higher tolerance than Azad-1. Cowpea cultivars for bentazone tolerance in the field were evaluated by Harrison and Fery, (1993) and they found that the most susceptible selections were killed or severely injured by bentazon at 2 kg a.e. ha$^{-1}$; the most tolerant selections were not severely injured by bentazon at 16 kg ha$^{-1}$. Reduction in seed number per pod in higher concentration of 0.375 kg a.i./ha in the pre-emergence application of pendimethalin, pursuit plus and the early post emergence application of pursuit plus in Vigna unguiculata was reported by Olorunmaya, (2010).

Gilreath et al., (2000) reported that plant vigor declined with increased glyphosate rates and younger plants were more sensitive than older plants. Plant height decreased as glyphosate rate increased. Deeds et al., (2006) determined winter wheat response to simulated drift rates of glyphosate and imazamox at early jointing or the early flower stages of growth and found that wheat injury and yield loss increased as herbicide rate was increased. This reduction was greater in case of glyphosate than imazamox. Cerdeira et al., (1985) reported that treatment of cowpea plants with glyphosate when pods were 7 and 10 days after flowering (DAF) prevented accumulation of the major storage protein polypeptides. The accumulation of these polypeptides was not inhibited as much when pods were 11, 12, or 13 DAF at the time of treatment with glyphosate. Pod length and seed fresh weight were inhibited by glyphosate treatment of plants bearing pods 7, 10, 11, and 12 DAF. Pod width, seed dry
weight, and seed length were inhibited by glyphosate when plants bearing pods 7, 10, and 11 DAF were treated. Growth reduction of several plant species was reported after the application of sulfonyle group herbicides, a group of Metosulam (Scarponi et al., 1998; Yan et al., 2000). Norsworthy et al., (2001) studied the cause of differential susceptibility of barnyardgrass, hemp sesbania, pitted morningglory, and prickly sida to glyphosate was examined. It was found that the biomass of barnyardgrass and prickly sida was reduced by 95% by Roundup Ultra®. Hemp sesbania and pitted morning glory showed more tolerance, with 66 and 51% average biomass reduction, respectively.

The present and other investigations show that glyphosate affects growth and morphology of the plant, and remains in the plant till flowering and fruit set. However, these studies do not tell if the phytotoxic effect is due to glyphosate or its degradation metabolite, aminomethylphosphonic acid (AMPA). Reddy et al., (2004) demonstrated that glyphosate-resistant soybean (developed by integration of a foreign gene that coded insensitive EPSP synthase) exposed to AMPA had reduced chlorophyll content and shoot fresh weight, similar to glyphosate-isopropylammonium treatment.

5.3 Biochemical Parameters

Effect of glyphosate on biochemical parameters was studied in in vitro grown seedlings of six genotypes and glyphosate treated plants of three genotypes grown in wire house. Among studied biochemical parameters chlorophyll content, protein content were decreased whereas proline content, MDA content and activity of antioxidative enzymes CAT, POX and SOD were increased with the increasing herbicide concentration.

5.3.1 Antioxidative Enzymes

On the basis of several reports (Fan et al., 2013, Romero et al., 2011, Sergiev et al., 2006, Moldes et al., 2008,) where it was suggested that round up and its main constituent glyphosate causes oxidative stress in plants and animals, we measured the activity of three antioxidant enzymes, catalase and peroxidase and SOD after glyphosate treatment. In both types of experiment, in vitro germination of seeds in herbicide supplemented MS media and wire house experiment in which post emergence treatment was given to young plants, activity of antioxidative enzymes was found increased in dose dependent manner. Catalase activity was consistently increased in all treatments and genotypes in in vitro grown seedlings while in treated plants Azad-1 showed a slight decrease at 6 mM with respect to 4 mM. Peroxidase activity was increased consistently in PU-19, PU-35, IPU-94-1 and PU-31 but slightly decreased at 0.8 mM in Azad-1 and
Azad-1 with respect to 0.6 mM. In treated plants also peroxidase activity was slightly decreased at highest concentration 6 mM with respect to 4 mM. SOD activity also increased in a similar manner in vitro grown seedlings except in PU-35 at 0.8 mM where it was decreased with respect to control whereas in treated plants some what different activity was observed. Among three genotypes PU-19 showed continuous increase up to 6 mM while in IPU-94-1 and Azad-1 activity was slightly decreased at 6 mM. Thus we can conclude that herbicide exposure both in vivo and in vitro cause production of ROS in treated seedlings and plants of *Vigna mungo* and hence elevation of antioxidative defense system.

Oxidative damage induced by ROS, is prevented by elevation of the antioxidant defense systems that operate with synchronized actions of a number of enzymes including SOD, POD, and CAT. Increased production of 'OH might generate O$_2^-$ and H$_2$O$_2$, and the SOD activity in black gram was increased as an antioxidant response to protect the plants. Activities of these antioxidative enzymes system are related to the plant resistance/tolerance, and eventually embodied in adjustment to adversity or resistance induction of plants. SOD is the first stage enzyme that removes O$_2^-$ radicals generated from normal physiological activities and exposure to oxidative stress (Bowler et al., 1992). It can catalyze the disproportionation of two O$_2^-$ radicals to H$_2$O$_2$ and O$_2$ and probably plays a key role in reducing toxic ROS accumulation (Salin, 1988). As an effective reaction of antioxidant enzyme systems, induction of SOD usually coincided with the increased activities of POD and CAT which are important H$_2$O$_2$ scavengers by catalyzing H$_2$O$_2$ to water and oxygen.

Catalases are involved in the metabolism of oxidative stress causing herbicides and protect plants from the stress generated by herbicide overdose. Catalases involved in herbicide tolerance, or an increase in catalase activity during herbicide exposure, have been reported from several plant species (Jung et al., 2006; Jung, 2003; Radetski et al., 2000). Moldes et al., (2008) found that glyphosate caused slight increase in CAT activity in susceptible cultivars of soyabeans. Okuda et al., (1992) reported increase in catalase activity by paraquat and cold treatment in wheat leaves. A few earlier reports have also shown an increase in catalase activity in *Vigna* spp. after herbicide exposure. A herbicide 2-benzoxazolinone (BOA) was found to cause oxidative stress in mung bean plants, which responded by an increase in the activity of ROS scavenging enzymes like catalase and SOD in the root and leaf tissues (Batish et al., 2006). Sergiev et al., (2006) demonstrated that catalase activity was increased after 6 and 10 days of glyphosate
application in maize plants. Lukatkin et al., (2013) reported that aryloxyphenoxypropionate class herbicide TOPIK induced changes, predominantly increases in lipid peroxidation (LPO) intensity, superoxide anion $O_2^-$ generation, total antioxidant activity (AOA), catalase and ascorbate peroxidase activity, although the response by plants was nonlinear and depended on the herbicide concentration and duration of treatment. As TOPIK concentration increased, so too did LPO and AOA in leaves, confirming the presence of oxidative stress in the cells of all three cereals: winter wheat, winter rye and maize.

Besides increasing catalase activity in plant tissues, glyphosate has been known to induce catalase activity in animal tissues as well. Langiano and Martinez, (2008), determined the toxicity of RoundUp for the Neotropical fish *Prochilodus lineatus*. Short-term (6, 24 and 96 h) toxicity tests were performed to evaluate the effects of sub-lethal concentrations of the herbicide (7.5 and 10 mg L$^{-1}$) to *P. lineatus*. It was observed that catalase activity in the fish liver was significantly increased in the fish exposed to 10 mg L$^{-1}$ of the herbicide. This increase in activity was suggestive of the activation of antioxidant defense after RoundUp exposure. Pieniazek et al., (2004) studied the effects of exposure of human erythrocytes to different concentrations of Roundup Ultra 360 SL and its active compound glyphosate. The human erythrocytes were incubated with 100–1500 ppm of Roundup Ultra 360 SL and glyphosate. Both Roundup Ultra 360 SL and glyphosate caused statistically significant increase in catalase activity in the erythrocytes.

Peroxidase is a general indicator of stress in plants and changes in peroxidase activity could be used to analyse stress in plants. (Sprecher et al., 1993) An increase in activity of peroxidase in response to glyphosate suggests the active participation of the enzyme in scavenging reactive oxygen species under stressed conditions. Increase in peroxidase activity was also found in *Vigna radiata* plants treated by glyphosate (Basantani et al., 2011). The pattern of peroxidase activity induction in the six varieties could again be explained in light of catalase activity elevation. The highly significant induction of both the antioxidant enzymes in PU-19, PU-35 and IPU-94-1 suggests that they are more capable to combat oxidative stress and thus under herbicide stress these two enzymes are upregulated.

The protective role of both the antioxidant enzymes in other three genotypes, however, does not seem to be sufficient to protect the plants from glyphosate injury. Peroxidase, an antioxidant enzyme, has been clearly implicated in herbicide metabolism in plants. Peroxidase upregulation after herbicide exposure has been demonstrated in
wheat (Wang and Zhou, 2006), tobacco (Yamato et al., 1994), and many other plant species. Cañal et al., (1988) could demonstrate the activation of different peroxidase isozymes at different glyphosate concentrations in *Cyperus esculentus* L. plants. They identified three peroxidase species F1, F2 and F3 at pl 3.8, 4.4 and 4.8, respectively. They showed preferential expression after glyphosate treatment. Highest F1 activity was found in control plants whereas the F2 fraction was the predominant form in the plants treated with glyphosate at $10^{-2}$M and the highest F3 activity occurred in plants treated with $5 \times 10^{-3}$M glyphosate. Menezes et al., (2004) could differentiate glyphosate-tolerant transgenic soybean from non-transgenic plants by the evaluation of peroxidase activity.

SODs are also known to play important role in herbicide tolerance in several plant species. We also measured SOD activity in *V. mungo* seedlings after glyphosate treatment. As compared to other oxidative enzymes SOD activity was not significantly increased after glyphosate exposure in all genotypes. The increase in SOD activity also suggests that glyphosate is inducing oxidative stress in *V. mungo* but SOD activity is probably not playing a pivotal role in scavenging of superoxide radicals generated by ROS. A similar increased SOD activity has also been observed after herbicide treatment in several reports (Wang and Zhou, 2006; Jung et al., 2000). SOD activity is of more importance because it acts as first line of defense against oxidative damage (Gratao et al., 2005). Increased SOD activity catalyzes dismutation of $O_2^-$ to $O_2$ and $H_2O_2$ and this increase may be due to synthesis of new enzyme proteins (Moldes et al., 2008).

Several workers have earlier reported an induction of SOD activity after herbicide treatment. Matters and Scandalios, (1986) reported preferential induction of chloroplastic and cytosolic SOD in maize leaves treated with paraquat; mitochondrial SOD was not induced significantly. The differential induction of SODs suggests that different isozymic forms of the enzyme are involved in overcoming oxidative stress generated by different herbicides. Perl et al., (1993) created transgenic potato plants carrying either the cytosolic or the chloroplastic SOD genes of tomato. This study also reported that cytosolic and chloroplastic SODs are instrumental in conferring tolerance to paraquat.

The activities of SOD, POX, APX, and CAT were higher in the paraquat-resistant biotype than in the paraquat-susceptible biotype after paraquat treatment in *Erigeron canadensis* (Pyon et al., 2004). Doganlar, (2012) found that after treatment of herbicide, quizalofop-$p$-ethyl in *Lemna gibba* and *Lemna minor* SOD and POD activities were elevated in both plants at 24 h. but at 96 h, SOD activity decreased in *L. minor*.  

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Increasing diquat concentration decreased chlorophyll content while the activities of SOD, POD, and CAT, cell membrane permeability, and MDA content increased significantly while Roundup showed less change in chlorophyll content, activities of SOD, POD, and CAT increased slowly in rapeseed pod (Zhou et al., 2009). Atrazine treatment in maize roots caused significant increase in CAT, POX and SOD activities.

Žaltauskaitė and Brazaitytė, (2011) found that herbicide amidosulfuron caused increase in MDA content in Chenopodium album and Hordeum vulgare but decrease in chlorophyll and carotenoid content. Shahrtash et al., (2011) studied that herbicide paraquat caused increase in proline content, catalase activity, MDA content but decreased chlorophyll. Radetski et al., (2000) found that herbicide trichloroacetate increased activity of superoxide dismutase, catalase, peroxidase and glutathione reductase. Choi et al., (2004) studied that paraquat causes increase in POX activity, total SOD and CuZn-SOD activity but decrease in CAT activity in Rahmania glutinosa and soybean. Janicka et al., (2008) found that 48 h after treatment of herbicide haloxyfop-ethoxyethyl (HE) the SOD activity increased in the youngest part of the leaves and decreased in the oldest. CAT activity increased in the part below the spot treated with HE on 12 and 24 h after treatment. The increase in catalase activity in the wheat leaves as a result of HE treatment is the plant’s reaction to the synthesis of hydrogen peroxide and its further conversion into water and molecular oxygen. Decrease in chlorophyll content and increase in peroxidase activity after treatment of sulphinyl urea herbicide Sultan 70WG was reported by Sarsag and Unal, (2004). Herbicide roundup cause increase in antioxidants in Schefflera leaf against oxidative damage. Activity of CAT, POX and SOD was increased in leaves after roundup exposure but lipid peroxidation decreased (Abdelkader and Ahmed, 2012).

De Souza et al., (2012) reported effect of herbicide mixture (ametryn and clomazone) in Emilia coccinea and found that total chlorophyll content and carotenoids decreased with increasing doses of the herbicides. The SOD activity decreased while MDA content increased with increasing doses of herbicide. Song et al., (2007) reported accumulation of proline in chlorotoluron (phenylurea herbicide) exposed roots and leaves. Total activity of POD in roots was significantly enhanced while CAT activities were generally reduced after chlorotoluron exposure. Çavuşoğlu et al., (2011) found that glyphosate-exposure significantly reduced the germination percentage, root length and seedling weight and enhanced the lipid peroxidation and caused an increase in malondialdehyde (MDA) levels in Allium cepa. Rise in the activities of catalase,
guaiacol peroxidase, glutathione S-transferase and hydrogen peroxide was observed by treatment of 2,4,5-Trichlorophenol (Michałowicz and Duda, 2009).

Hassan and Nemat Alla, (2005) reported that herbicide fluometuron significantly reduced chlorophyll a and b contents and also caused significant inhibitions in activities of superoxide dismutase (SOD), catalase (CAT). Nemat Alla and Hassan, (2007) reported that in maize after isoproturon treatment SOD activity was significantly enhanced up to the 12th d whereas APX activity was significantly reduced after the 4th day onwards. CAT activities were similarly increased during the first 4 d but decreased from the 12th and the 8th day, respectively. Low doses increased SOD and GPX activities but high doses led to diminutions whereas CAT and APX were reduced by all doses. Badr et al., (2013) found that metosulam herbicide decreased POD activity significantly by increasing the concentration of herbicide and the duration but the activities of CAT and APX increased significantly as the herbicide treatment increased.

It is concluded that herbicide treatment induced oxidative stress in Vigna mungo and plant responded to the herbicide action via increased activity of antioxidative enzymes. The relative tolerance of few genotypes in present study might be related to elevation of antioxidative enzymes and increase in proline content.

5.3.2 Protein Content, Proline Content and MDA Content

In present investigation effect of herbicide on protein content varied according to developmental stage of plant. In seedling stage a general decrease was observed after glyphosate treatment whereas at plantlet stage (before flowering) protein content was found to be increased with the increasing herbicide concentration.

Biotic or abiotic stress at the germination phase causes a decrease in normal metabolic activities and consequent, reduction of growth. In this phase protein synthesis, anabolic process like photosynthesis and transport of metabolites is badly affected. As glyphosate inhibits amino acid metabolism by inhibiting shikimic acid pathway it also showed profound effect on soluble protein content. In present study glyphosate treatment prior to seed germination caused consistent decrease in protein content of treated seedlings. Highest protein content was found in control and lowest in 0.8 mM. Maximum decrease in protein content over control was observed in genotype Azad-2 whereas minimum in IPU-94-1. Glyphosate treatment to young plants showed reverse effect on protein content as it caused a general increase in protein content in all genotypes and all the treatments. Highest protein content was at 4 mM in PU-19 and Azad-2 however at 6 mM in IPU-94-1. Increase in protein content might be due to increased synthesis of
antioxidative enzymes under herbicide stress. These results are in agreement with the findings of El Tayeb and Zaki, (2009) who reported that roundup caused significant decrease in soluble proteins in all concentrations and durations in stem leaves and roots of *Vicia faba*. Reports regarding effect of glyphosate in animal systems also mention that glyphosate had significant effect on total protein (El-Shebly and El-kady, 2008) while in few reports there was increase in protein content after glyphosate treatment (Romero et al., 2011). Decrease in grain protein by treatment of isoproturon and atrazine was observed in greengram (Khan et al., 2006).

Inhibition of seed germination and decrease in protein content in root shoot axis as well as endosperm of wheat seedlings by herbicide imazethapyr was found by Rad et al., (2011). The results of impact of herbicides on protein are in line with effect of 2,4-D and isoproturon on wheat (Kumar, 2012 ) as well as of Metribuzin, butachlor and chlorimuron-ethyl on wheat and maize (Nemat Alla et al., 2008a) and of biocides in lentil (Coskun and Zihnioglu, 2002), chlorimuron ethyl in wheat (Wang and Zhou, 2006). Metosulam treatment in *Vicia faba* decreased soluble proteins and free amino acids content harshly in all organs in comparison with those of control plants after 12 and 24 h but proline content was increased (Badr et al., 2013). Hatzios and Howe, (1982) found that hexoxinone herbicide at 100 mM inhibited protein synthesis in soybean. Also, Hamed, (1990) reported that metribuzin, treatment in faba bean plants, caused a marked inhibitory effect on protein synthesis.

Pesticide endosulfan and its combination with kitazin were also found to decrease protein content in brinjal (Sammaiah et al., 2011a). Rajashekar et al., (2012) also found that pendimethalin treatment in maize seedlings reduce soluble protein content. Dowidar et al., (2010) reported decrease in total soluble protein after addition of butachlor and thiobencarb at concentrations higher than 5 and 3 ppm, respectively, in culture media in *Nostoc muscorum*. Thirumaran and Xavier, (1987) found an initial enhancement of protein followed by a sharp decrease after treatment of methyl parathion (Metacid 50) in black gram. Kumar, (2012) found that herbicides 2, 4-Dichlorophenoxy acetic acid (2, 4-D) and Isoproturon (IPU) reduce the carbohydrate and protein content gradually from lower to higher concentration of herbicides.

According to Hare and Cress, (1997) free proline accumulates in response to various types of biotic and abiotic stresses. High proline levels under stress may maintain NAD(P)+/NAD(P)H ratios at values compatible with metabolism under normal conditions. Proline is a compatible solute and functions as osmoprotectant in drought and
osmotic stress and usually proline content is increased in all types of abiotic stresses. Herbicide treatment also causes oxidative stress in plants so proline content was studied in leaves and seedlings of black gram after post-emergence spray of various concentrations of glyphosate.

Proline plays role in the osmotic adjustment (Rudulie et al., 1984; Zhang et al., 2001), protects macromolecules during dehydration (Yancey et al., 1982) and act as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989; Zhang et al., 2001). Proline accumulation in plants under abiotic stresses is a major protective mechanism. In present study, there was an enhanced level of proline biosynthesis under the glyphosate stress. Accumulation of free proline and other osmolytes during biotic and abiotic stress is well documented (Bohnert et al., 1995). Proline performs many functions under stress conditions like osmotic adjustment (Voetberg and Sharp, 1991), osmoprotection (Kavi Kishor et al., 1995), free radical scavenger and antioxidant (Sharma and Dietz, 2006), protection of macromolecules from denaturation (Vanrensburg et al., 1993). Many workers also reported increase in proline content after herbicide exposure. Huang et al., (2012) also found increase in proline content after roundup treatment in cagon grass. Fayez et al., (2011) reported increased level of proline after salicylic acid + fusilade treatments. Saladin et al., (2003a) reported accumulation of amino acid proline after soil treatment of flumioxazin in Vitis vinifera. El Tayeb and Zaki, (2009) reported significant increase in proline content after roundup treatment in Vicia faba.

MDA formation is considered the general indicator of lipid peroxidation so MDA content measurement is the most applicable method to assess lipid peroxidation, the accumulation of MDA suggests lipid peroxidation in plants (Li et al., 2012). MDA levels were significantly increased in the leaves of Vigna mungo with an increase in glyphosate concentration. Highest MDA content was found at 6 mM glyphosate treatment and maximum amount was found in PU-19 and minimum in IPU-94-1. Enhanced MDA content indicated lipid peroxidation in response to herbicide induced oxidative stress as reported by Nemat and Alla, (2005). Wang and Zhou, (2006) reported that herbicide chlorimuron-ethyl increased MDA content in leaves after 1 day exposure. Many reports are in accordance with our study (Moldes et al., 2008; Zhou et al., 2009; Çavuşoğlu, 2011; Romero et al., 2011). Moldes et al., (2008) reported that glyphosate treatment did not induce oxidative stress to any great extent due to lack of significant increase in lipid peroxidation after 72 hours of treatment. Glyphosate treatment decreased growth and pigment content of maize plants and increased lipid peroxidation, free proline and the
activity of CAT, peroxidase and glutathione-S-transferase (Sergiev et al., 2006). Shanguo et al. (2008) noticed that isoproturon and tribenuron-methyl had obvious inhibitory action on the increment of root and seedling weights. Isoproturon inhibited root activity by 65.26% and increased MDA content by 42.39%. Tribenuron-methyl (1.0 mg/ml) inhibited root activity by 32.83% and at 0.2 mg/ml, it increased MDA content by 27.41%. Zhou et al. (2009) reported that Roundup caused no obvious increase in MDA content.

5.3.3 Pigments (Chlorophyll and Carotenoids)

Effect of glyphosate treatment on photosynthetic pigments was studied in wire house experiment. Chlorophyll and carotenoids were decreased with increasing concentration of herbicides in all genotypes. Visual injury of leaves mainly consisted of chlorosis of leaf tissue caused by glyphosate treatment in black gram. Depending on dose, chlorosis was more in higher concentrations. There was dose dependent decrease in total chlorophyll, chl a, chl b and carotenoids in all the treatments and genotypes. Minimum total chlorophyll content at 6 mM was found in Azad-1 and maximum in PU-19. Similar symptoms have been reported for wheat (Deeds et al., 2006). Chlorosis is believed to be caused by destruction of chlorophyll and other accessory pigments. Chlorophyll content is proposed as a very useful parameter of herbicide toxicity and usually decreased after application of herbicides (Clua et al., 2012; Hernando et al., 1989; Reddy et al., 2001). Meschede et al., (2011) reported that the application of glyphosate and sulfumeturon methyl at higher doses were found to interfere in the carotenoid content, when compared with the control. The highest dose of glyphosate significantly reduced the content of chlorophyll and carotenoid in sugarcane. The changes observed in the chlorophyll and carotenoid levels caused by the application of the herbicides may distinctly alter the metabolism of photosynthesis by absorption and/or conversion of energy.

Decreased amount of leaf chlorophyll is an important feature of plants treated with sublethal concentrations of glyphosate (Tan et al., 2006). Glyphosate inhibits the enzymatic activity of cytochrome P450 of yeast, suggesting a similar action in plants (Xiang et al., 2005). Furthermore, glyphosate may also prevent chlorophyll synthesis by inhibiting the formation of the porphyrin precursor δ-aminolevulinic acid (ALA) (Zaidi et al., 2005). The reduction of chlorophyll content due to plants exposure to herbicides was also reported by Wang and Zhou, (2006) in wheat. Huang et al., (2012) reported significant decrease in chl a and chl b after glyphosate treatment in cogon grass. Saladin
et al., 2003(b) also reported negative impact on photosynthesis revealed by reduction in foliar chlorophyll and carotenoid contents. Jung, (2000) found that in the norflurazon treated plants of cucumber, there was a reduction in total chlorophylls and carotenoids.

Reddy et al., (2001) found that in glyphosate susceptible soyabean a single application of glyphosate at 0.28 kg/ha reduced chlorophyll content (49%), and shoot and root dry weight (50 and 57%, respectively) at 2 wk after treatment. In glyphosate-resistant, only higher dose 2.24 kg/ha reduced chlorophyll content and shoot and root dry weight. Increasing application of glyphosate on shoots significantly reduced chlorophyll concentration of the young leaves and shoots dry weight, particularly the young parts of non-glyphosate resistant soybean plants (Cakmak et al., 2009). UV-B (0.4 W m-2) irradiation and dimethoate (100 and 200 ppm) treatments, singly and in combination, declined the growth, photosynthetic pigment contents and photosynthesis (O2 evolution and CO2-fixation) of cowpea (Mishra et al., 2008). Dimethoate (100 and 200 ppm) and UV-B alone caused heavy damage on pigments and photosynthetic activity of cowpea, leading to the significant inhibition in growth.

Hernando et al., (1989) reported that glyphosate decreased cell density and photosynthetic pigment content; 1.0 mM did not allow growth in Chlorella pyrenoidosa. Glyphosate causes inhibition of chlorophyll synthesis and a decrease in carotenoids. Villanueva et al., (1985) found that after 2 weeks of foliar spray of glyphosate (at 0.1, 1.0, 5.0, and 10.0 mM) growth was inhibited, and chlorosis and leaf apex necrosis were observed. Plant height was reduced, leaf fresh weight was decreased by 40%, and leaf dry weight was slightly affected. Chlorophyll and carotenoid levels were decreased by 52 and 54%, respectively, following glyphosate treatment.

Glyphosate caused significant reduction in the chlorophyll and carotenoids content of purple nutsedge leaves (Abu-Irmaileh and Jordan, 1978). Ralph, (2000) found that photosynthetic pigments chl-a and chl-b were significantly reduced by glyphosate but there was no significant effect on carotenoid concentration. Alvi et al., (2003) found that increasing concentrations of atrazine decrease the contents of chlorophyll a, chlorophyll b and carbohydrates, which reflects its effectiveness as a photosynthetic inhibitor. Potassium and protein contents were decreased significantly at 10 to 100 ppm atrazine. Chen et al., (2012a) reported combined effects of UV-B radiation and herbicides glyphosate, MCPA and DCMU in forming cyanobacteria, Anabaena sp. and Microcystis viridis. Glyphosate and MCPA significantly decreased the contents of Chl-a and carotenoid contents in irradiated cells. The addition of GPS and MCPA decreased
SOD activity, and increased DNA damage, ROS generation and MDA content. Romero et al. (2011) found that glyphosate increased protein and malondialdehyde (MDA) content and CAT and SOD activity. There was reduction in carotenones, total chlorophyll, and chla/b ratio on increasing concentrations of glyphosate in tolerant strain of *Chlorella kessleri*. Chlorophylls a and b and carotenoids of plant leaves decreased significantly with increasing concentration of metosulam in *Vicia faba* (Badr et al., 2013). Reduction of pigment content in the presence of herbicide occurred either by initiation of pigment degradation or by inhibition or biosynthesis of either chlorophylls or carotenoids (Nakajima et al., 1996). Herbicide atrazine and glyphosate were also found to reduce pigment content in algae (Ahmed et al., 2000; Fawzy, 2008).

### 5.4 Effect of Glyphosate on Plant Tissue Culture

In vitro culture for selection of stress tolerant genotypes had been widely used in many crop plants for various abiotic stress like salinity, drought or/and osmotic stress, heavy metal stress and other stress caused by other agrochemicals and pesticides (Table 2.1). But there are very few reports regarding in vitro screening of herbicide tolerant genotypes in any pulse crop, especially in *Vigna mungo*. Two best callusing media (A) and (C) were chosen from several combinations of PGRs to screen tolerant genotypes on the basis of performance of six genotypes on herbicide supplemented callus media. In callus media (A) among four explants used for callusing cotyledonary node was most superior followed by hypocotyl, epicotyl and shoot apex. Genotypes that showed callusing response at higher concentration 0.2 mM viz. PU-19, PU-35, IPU-94-1 and Azad-1 were treated as more tolerant than those genotypes that did not showed response viz. PU-31 and Azad-2. Callus fresh weight (CFW) after 30 DAI was also used as an important parameter to assess the relative tolerance of genotypes for herbicide. CFW was decreased in all explants of all genotypes with increase in herbicide. Among explants, maximum and minimum CFW was obtained in CN and shoot apex respectively. At 0.2 mM maximum CFW was found in IPU-94-1 followed by PU-19, Azad-1 and PU-35.

In callus media (C) good callusing responses was found for all explants like leaf segments, cotyledonary node (CN) and hypocotyl but CN were best for callusing in this combination too. Here only IPU-94-1 showed callusing response at 0.4 mM whereas at 0.2 mM maximum percent callus response was maximum in PU-19 followed by IPU-94-1 and Azad-1. CFW at 0.2 mM was highest in Azad-1 and PU-19. Thus on the basis of callus response in both media PU-19, IPU-94-1 and Azad-1 were treated as tolerant as compared to other three genotypes.
In present study herbicide glyphosate showed an inhibitory effect on callus and shoot culture of all *Vigna mungo* genotypes and on the basis of their overall performance under herbicide stress they were categorized under tolerant and sensitive ones. When herbicides were incorporated in tissue culture media it produced inhibitory effect on percent response, shoot number per explant, shoot length, callus growth and callus fresh weight in all genotypes but genotypic variation was observed for morphogenetic response. Percent response, shoot number per explant, shoot length, callus growth and callus fresh weight were decreased in a dose dependent manner in all explants, genotypes, and treatments in shoot media (D), (H), (I). In shoot media-(D) cultures at 0.05 mM, maximum percent shoot response was recorded in Azad-1 while minimum in PU-35 whereas shoot number per explant was highest in genotype IPU-94-1 and lowest in PU-19.

Similar inhibition has been reported in chickpea with using Endosulfan (Saxena and Beg, 1988) and with Rogor (Rao and Naidu, 1989) incorporated in growth medium. Tolerance of wheat genotypes to the sulfonylurea herbicide metsulfuron-methyl was studied using *in vitro* culture by Kondic-Spika et al., (2009). The study recommended that fresh callus weight was the best for screening tolerant from sensitive genotypes. Presence of metsulfuron-methyl in the medium had an inhibitory effect on callus growth in all wheat genotypes. Validity of the tolerance level expressed by FCW was confirmed at the level of whole plants in *in situ* experiment. Effect of agrochemicals on callus cultures of plants were studied by many researchers like difenzoquat and atrazine (Bozorgipour and Snape, 1991), sulphonylurea (Kondic and Šesek, 1998; Kondic-Špika et al., 2007) and dicamba (Kilinc, 2004.) Other stresses like osmotic stress also effect relative callus growth rate and shoot induction. With the increase in PEG concentration relative callus growth rate and shoot induction decreased (Aazami et al., 2010). Sammaiah et al., (2011b) reported that the growth of brinjal callus with regard to fresh and dry weight and percentage of callus induction for three pesticides Rogor, Endosulfan and Kitazin were inversely correlated with increasing concentrations. Koch et al., (2009) reported screening of herbicide resistant populations by spraying sugarcane seedlings with 0-1.5 l/ha Arsenal. They also found that sugarcane callus cells exposed to 0.042μM imazapyr cause 50% inhibition of plantlet regeneration. Kilinc, (2004) found that dicamba effect callus induction rate and callus fresh weight in wheat genotypes. He suggested that usage of the dicamba concentrations higher or lower than 5mg l⁻¹ decreased the callus induction rate and increase in dicamba concentration from 5 to 10 mg l⁻¹
decreased the callus weight. Bird, (1993) reported increase in percent branch reduction by herbicides 2,4-D, atrazine and glyphosate in vitro cultures of Myriophyllum spicatum. Herbicide atrazine reduced fresh weight gain and this reduction was less at lower trifluralin concentrations in carrot callus tissue (Sloan and Camper, 1981).

Nedev et al., (2003) screened maize genotypes for herbicide resistance by in vitro selection by adding Stomp 330 (pendimetalin) by culture of immature maize embryos. Pendimetalin concentrations of 0.26%, 0.10% and 0.05% showed a negative effect on callus induction and reduced in vitro growth rates. Herbicide (Stomp 330) resistant maize calli were obtained using the method of in vitro selection of mutant cells. The resistance persisted at a concentration of 0.01%.

Fresh weight of cultured cells of tobacco was found to decrease in presence of glyphosate by Dyer et al., (1988). Baillie et al., (1993) also found that increasing chlorosulfuron concentration in barley callus culture reduce growth and quality of callus. Root and plant dry weight of in vitro selected lines were also decreased by chlorosulfuron. In vitro culture of barley callus has been used to produce plants with increased glyphosate tolerance. Callus from immature embryos of barley Hordeum vulgare L. were cultured on Murashige and Skoog medium with 10^{-6}, 10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, or 10^{-2}M glyphosate for one, four or thirty months. Plants were regenerated from callus maintained in glyphosate medium at 10^{-6}, 10^{-5} or 10^{-4}M for four months, at 10^{-5} or 5×10^{-4}M for one month and at 10^{-5}M for thirty months. The progeny of each regenerated plant was analyzed for response to glyphosate. Some progenies showed increased tolerance to glyphosate (Escorial et al., 1996).

Atrazine-resistant plants were regenerated from cells selected in green cultures of Nicotiana plumbaginifolia (Cseplo et al., 1985). In another study, cotyledonary node plus epicotyl explants of soybean were cultured on atrazine-containing medium. Some explants yielded organogenic shoots from which plants were regenerated. Some of these plants displayed resistance to atrazine (Wrather and Freytag, 1991). Calli of Nicotiana debneyi displaying very high levels of tolerance to amitrole were selected by stepwise exposure to increasing concentrations. Regenerated plants displayed tolerance as calli (Swartzberg et al., 1985). Amitrole tolerance at the whole plant level was reported in tobacco plants regenerated from cell lines selected in vitro (Chaleff, 1986b). A similar result was achieved with maize (Anderson et al., 1987). Chen et al., (2012b) developed protocol of in vitro selection and greenhouse screening for glyphosate-tolerant variants in manilagrass. Newly subcultured calli of more than 5 years’ old were transferred to
selection medium containing 2 mM glyphosate. After two rounds of selection, survived calli were transferred to regeneration medium without glyphosate. Regenerated plantlets were then transferred to regeneration medium containing 0.5 mM glyphosate to select tolerant plantlets. Fully developed plantlets were transferred to a greenhouse and then subjected to greenhouse screening by foliar spraying with 0.05 % glyphosate solution. Six glyphosate-tolerant plantlets were obtained and proliferated for determination of SOD-tolerance using morphological and physiological measurements. Fourteen days after foliar application with 0.1 % glyphosate, only TP5 showed enhanced SOD-tolerance, higher chlorophyll a content and catalase activity.

Harms et al., (1991) developed procedure for the rapid and direct selection of herbicide-resistant mutant plants. In this procedure adventitious shoot formation from suitable explants, such as leaf discs, on a shoot-inducing culture medium containing a toxic herbicide concentration. Resistant green shoots were thus isolated from tobacco leaf explants cultured on medium containing 100 μgl⁻¹ primisulfuron. Their acetohydroxyacid synthase (AHAS) enzyme activity was less inhibited by sulfonylurea herbicides than that of unselected, sensitive wild type plants.

Herbicide tolerant callus and suspensions cell culture of rice were selected by Wei, (1998). Rice callus was induced from immature seeds on basal MS solid medium supplemented with 10 μM 2, 4-D and finely dispersed cell suspension cultures were initiated from the callus using B5 basal liquid medium consisting of 10 μM 2, 4-D. During the stressing and selecting stages, 2, 4-D was observed to be taken up by the rice callus and suspension cells. In the selection and toxicity studies, 2, 4-D-tolerant callus cultivar Puteh Perak was selected in MS solid media at 400, 600 and 800 μM 2, 4-D concentration while tolerant cell-suspension in basal B5 liquid media containing 200 and 400 μM 2, 4-D. Instantaneous and gradual stressing method both were used for herbicide tolerant callus and suspensions cell culture. Sunflower genotypes differed regarding their response of callus viability in presence of herbicide glyphosate and callus viability decreased with increasing herbicide concentration (Raducanu, 2004). Reduction in callus fresh weight in rice variety KDML 105 by 10⁻⁴M glyphosate were also reported by Chaum et al., (1999).

Seeds of fourteen tomato (Lycopersicon esculentum Mill.) cultivars were germinated under 0, 25, 50, 75, 100 mM NaCl. According to their germination response, six cultivars were selected as: salt-tolerant (Pascal and Tnshet Star), moderately salt-tolerant (Imperial and Tnshet Crystal) and salt sensitive (Pahuja and Queen). Explants
from hypocotyls of these cultivars were selected for callus induction. About 0.2 g of callus was grown under previous salt levels for four weeks. Callus relative growth rate (RGR), fresh and dry weights, proline, Na\(^+\) and K\(^+\) contents were evaluated. Significant differences were found among cultivars. Tnshet Star had the highest callus growth, while Pahuja had the lowest under salt levels during the incubation period; high salinity resulted in lowering callus fresh and dry weights of callus as well as reducing its RGR (Mohammed et al., 2006).

In stepwise selection and procedure we aimed to screen tolerant genotype and select callus tolerant to higher dose of glyphosate. Stepwise selection method was also used by Tong et al., (2010) in *Gossypium hirsutum* L. to obtain glyphosate-tolerant upland cotton mutant from the embryogenic calli. The calli were transferred to the selection medium and multi-step selection pressure process was performed until the calli could proliferate in the presence of 20 mM glyphosate. The regenerated plants from the glyphosate-tolerant calli were analysed for glyphosate response, tolerance of progenies and shikimate accumulation on whole-plant basis.
6.0 Conclusions

The present study was carried out with the objective of investigating *in vitro* methods that can be used for screening of herbicide tolerance in *Vigna mungo* genotypes. During the course of investigation effect of glyphosate was seen on growth and morphological parameters of seedlings and young plants, callus induction, callus growth, multiple shoots and shoot bud induction and root proliferation in different seedling explants in six *V. mungo* genotypes. Effect of glyphosate on biochemical parameters like pigments, protein content, proline content and MDA content were also studied in *in vitro* grown seedlings and field grown plants. Activity of antioxidative enzymes viz. catalase, peroxidase and SOD were also studied.

The present investigation clearly demonstrated that glyphosate significantly affected morphological and biochemical parameters of seedlings and young plants of *Vigna mungo* genotypes. The effect of the herbicide was evaluated both on petridish raised seedlings as well as in *in vitro* grown seedlings. Glyphosate concentrations ranging from 0.1 mM (*in vitro* germination) to 10 mM (petri dish germination) reduced germination, survival, root length, shoot length and fresh weight and dry weight of seedlings in all the genotypes in a dose dependent manner which showed adverse impact of glyphosate application on non target crop black gram. Among all the parameters used for screening of herbicide tolerant genotypes, germination percentage, survival percentage and seedling root length were found best. On the basis of germination percentage at 10 mM, from higher to lower GP, genotypes could be arranged as Azad-1>PU-19>PU-35>IPU-941 >PU-31=Azad-2. In *in vitro* germination at highest glyphosate concentration (0.8mM) from higher to lower GP, genotypes could be arranged as PU-19>IPU-94-1>Azad-1>PU-35>Azad-2>PU-31. EC$_{50}$ for germination percentage was different in different genotypes. It was 0.8 mM for PU-19, IPU-94-1 and Azad-1, 0.4 mM for PU-35 and PU-31 and 6mM for Azad-2. Survival percentage calculated for petri dish grown seedlings at highest glyphosate concentration (10 mM) was higher in PU-19, Azad-1 and IPU-94-1 than PU-35, PU-31 and Azad-2. Survival percentage for *in vitro* grown seedlings at highest glyphosate concentration (0.8 mM) was highest in PU-19 and lowest in PU-31. These findings clearly indicate that PU-19, Azad-1 and IPU-94-1 were better able to withstand higher glyphosate doses as compared to other genotypes.
Percent reduction in root length in petri dish grown seedlings with respect to control in highest dose (10 mM) was PU-19 (91.96), PU-35 (93.87), IPU-94-1 (90.91), PU-31 (94.58), Azad-1 (91.77) and Azad-2 (95.21). Percent reduction (%) with respect to control in highest dose 0.8 mM was PU-19 (87.92), PU-35 (89.26), IPU-94-1 (86.67), PU-31 (93.39), Azad-1 (88.73) and Azad-2 (89.45). This clearly indicates the relative tolerance among genotypes and we can conclude that the genotypes with less inhibition at highest glyphosate concentration can better tolerate the herbicide stress. Almost similar was observed in in vitro grown seedlings.

Effect of glyphosate on in vitro response of various explants like hypocotyl, epicotyl, cotyledonary node, shoot apex and leaf segments were also assessed during study and it was observed that glyphosate negatively affects callus induction, callus growth, induction of multiple shoots, shoot buds and roots. With the increase in glyphosate concentration there was gradual decrease in percent callus response and callus fresh weight in both combinations (callus media A and C) tested for callusing in all explants of six genotypes. Three shoot media D, H and I were used to assess effect of herbicide on shoot induction and shoot growth. In all shoot media percent shoot response, number of shoots per explant and shoot length was decreased with the increasing herbicide concentration in all explants of six genotypes. Similar response was observed for rooting in root media. On the basis of over all in vitro response of explants of genotypes in herbicide supplemented media IPU-94-1, Azad-1 and PU-19 were found tolerant, PU-35 was moderately tolerant and Azad-2 and PU-31 were sensitive for high concentration of herbicide.

Three tolerant and better performing genotypes were subjected to gradual and shock treatment of herbicide and on the basis of percent of surviving cultures and callus tolerance index, IPU-94-1 and PU-19 was more tolerant as compared to Azad-1. On the basis of callus growth and percent of surviving cultures it was concluded that shock treatment of herbicide stress caused more inhibition on callus growth as compared to gradual treatment in all three genotypes.

In vivo response of IPU-94-1, Azad-1 and PU-19 to herbicide was also evaluated in field under wire house conditions. After post emergence spray of herbicide to young plants, effect of herbicide was observed on survival percentage, plant height, number of nodes, internodal length, number of pods, pod length, number of seeds per pod, number of seeds per plant. All the growth and yield parameters were found to decrease with the increasing concentration of herbicide. Among biochemical parameters activity of
catalase, peroxidase and SOD, proline content, total protein content and MDA content were increased while total chlorophyll, chl-a, chl-b and carotenoids were decreased with the increase in herbicide dose. Thus on the basis of response of three genotypes in wire house, it was concluded PU-19 can better tolerate the herbicide toxicity as compared to IPU-94-1 and Azad-1.

In conclusion, germination percentage, survival percentage and root length bioassay, percent callus/shoot response and reduction in callus fresh weight can be utilized for the selection of herbicide tolerant genotypes in *Vigna mungo* as well as other crop plants. Among *in vitro* methods used for screening, *in vitro* germination method and gradual stress treatment methods were found better for screening of herbicide tolerant genotypes than other methods in *Vigna mungo*. Glyphosate application caused oxidative stress and elevation of antioxidant defense system in non target crop black gram. Genotypes PU-19, IPU-94-1 and Azad-1 can better combat the oxidative stress than PU-31, PU-35 and Azad-2 induced by glyphosate application which may be utilized in *Vigna mungo* improvement programme as well as they can be used for study of molecular basis of herbicide resistance in these genotypes in contrast to susceptible genotypes.
Herbicides are expected to protect crop plants from weed competition without harming the crop plants. Glyphosate (N-[Phosphonomethyl] glycine) is a non selective systemic herbicide used for control of annual and perennial plants including grasses, sedges, broad leaved weeds and woody plants. But broad spectrum herbicides like glyphosate can also damage non target plants. Black gram is one such non target crop and therefore, it becomes necessary to select ideal black gram genotypes, which may be tolerant to herbicide stress and produce substantial yield under stress conditions.

In present study an attempt has been made to investigate *in vitro* methods that can be used to screen herbicide tolerant genotypes of black gram (*Vigna mungo* L. Hepper).

Black gram is a valuable field crop, both in terms of economic returns and in its contribution to total farm income as a leguminous rotation crop. It is a member of the Asiatic *Vigna* crop group. It is a warm season annual pulse grown mostly as an opportunity crop in rotation with cereals. Black gram's main advantages are that, being a legume, it does not require nitrogen fertilizer application, and has a relatively short (90-120 days) growth duration. It is an important food legume crop of the Indian subcontinent comprising of India, Burma, Bangladesh, and Sri Lanka. Its seeds are highly nutritious with protein (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins. In India black gram is very popularly grown in Andhra Pradesh, Bihar, Madhya Pradesh, Maharashtra, Uttar Pradesh, West Bengal, Punjab, Haryana, Tamil Nadu and Karnataka. Black gram is considered to have been domesticated in India from its wild ancestral form *V.mungo var. silvestris*. Black gram is a specialized food crop used primarily in South Asia for dhal production. Seeds are used in preparation of many south Indian dishes, e.g. dosa, idli, vada etc. It is cultivated mainly for forage, silage and edible seeds. It is also used as a green manure crop and, in some parts of the world, as a fodder crop.

Glyphosate is a post-emergent, systemic and non-selective herbicide used in both agricultural and non-agricultural areas. It is used to kill all plant types including grasses, perennials, and woody plants. There are different forms of glyphosate used as herbicides: glyphosate trimethylsulfonium salt, glyphosate-isopropylammonium salt and glyphosate-sesquiodium. It is sold under various trade names by different companies, some of which
are Accord®, Fallow Master®, Glyphomax®, Honcho®, MirageR®, Pondmaster®, Protocol®, Roundup Ultra®, Roundup Pro®, Silhouette®, Touchdown®, etc. It is mainly absorbed into the plant through the leaves and then transported throughout the plant where it acts on the plant’s enzyme system. It acts as a potent inhibitor of shikimic acid pathway for biosynthesis of aromatic amino acids. It is a competitive inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase with respect to phosphoenolpyruvate (PEP) and noncompetitive with respect to shikimate-3-phosphate (S3P).

Investigations regarding herbicide tolerance in Vigna mungo are very few and to the best of our knowledge there is also no report on in vitro screening for herbicide tolerance in black gram caused by glyphosate herbicide. The objective of the present work was therefore to investigate in vitro methods of screening for herbicide tolerant black gram genotypes. Genotypes under study are high yielding and widely used in North India specially Uttar Pradesh where glyphosate is used on large extent to eradicate weed populations.

Abiotic stress caused by herbicides has become a major limiting factor for crop productivity in present scenario. To combat this problem screening of herbicide tolerant genotypes became an efficient approach which needs to rely on appropriate methodology for screening of various genotypes. Many methodologies have been proposed by different researchers time to time to obtain herbicide tolerance in important crops. These methods can be classified in two groups: in vitro and in vivo methods.

Seeds of six genotypes PU-19, PU-35, IPU-94-1, PU-31, Azad-1 and Azad-2 were grown in petri dishes after 24 hour treatment of herbicide glyphosate for 15 days. The data was taken on various seedling growth parameters. Seeds were also grown in herbicide supplemented MS basal media (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM) for 15 days and seedling growth parameters were calculated. Biochemical parameters like free proline and total protein content, activity of antioxidative enzymes like catalase, peroxidase and superoxide dismutase were also estimated. In another screening method various seedling explants of six genotypes viz. epicotyl, hypocotyl, cotyledonary node, shoot apex and leaf segments were obtained from in vitro grown seedlings and inoculated on MS media augmented with PGRs and various concentrations of herbicide (0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM). Effect of herbicide on callusing, shooting and rooting response was analyzed in different genotypes of black gram after 30 and 45 days after inoculation. In callus culture screening, calluses were
subjected to shock and gradual treatment of herbicide and response were analyzed in terms of percent of surviving cultures and tolerance index. Seeds of three better performing genotypes were also grown in wire house and post emergence treatment of glyphosate (control, 1 mM, 2 mM, 4 mM, 6 mM and 8 mM) was given at three leaved stage of seedlings. Effect of herbicide on various morphological and physiological parameters was assessed.

**Effect of herbicide on seedling growth parameters, antioxidative enzymes and other biochemical parameters**

In petri dish and *in vitro* seed germination experiment it was observed that glyphosate treatment reduce germination percentage, survival percentage, root length, shoot length, fresh weight and dry weight in all the six genotypes but genotypes behaved differentially for the herbicide. In petri dish grown seedlings all the growth parameters were decreased in dose dependent manner. In control maximum germination percentage were obtained in IPU-94-1 and minimum in PU-31 and Azad-2. At highest dose (10 mM) maximum percent decrease over control in germination percentage in PU-35 and Azad-2 while minimum in Azad-1. Survival percentage at 10 mM was maximum in PU-19 and minimum in Azad-2 and PU-31. Seedling root length was most sensitive for herbicide among all the parameters studied. Highest root length was observed in control and maximum in PU-19 and minimum in Azad-2 and at 10 mM PU-19 and Azad-2 had highest and lowest root length respectively. The fresh weight of control seedlings and also at 10 mM was highest in PU-19 and minimum in Azad-2. Maximum and minimum reduction of fresh weight at 10 mM was observed in PU-31 and PU-35. The dry weight of control seedlings and at 10 mM was highest in PU-19 and minimum in Azad-2. The maximum seedling tolerance index was observed in control and minimum in 10 mM.

As compared to peridish germination method, *in vitro* germination in MS media supplemented with herbicide showed clear relative tolerance among genotypes for the herbicide glyphosate and it could be considered as better and more sensitive method of screening for herbicide tolerance among various cultivars growing naturally.

Catalase activity increased with the increasing herbicide concentration in all genotypes. It was lowest in control and highest in 0.8 mM. In control, activity was highest in Azad-1 and lowest in PU-35. At 0.8 mM maximum activity was in PU-19 while lowest in PU-31. It was assumed that to overcome the oxidative stress caused by glyphosate, catalases might be playing a pivotal role in *Vigna mungo* genotypes. The protective role of catalase in PU-31 and Azad-1 was not sufficient enough to protect the
plants from herbicide injury as shown by survival percentage and seedling root length in PU-19. The induced catalase activity in PU-19 and IPU-94-1 might be playing a supportive role causing less damaging symptoms at higher doses.

Like catalase, peroxidase activity was also increased with increase in herbicide concentration in all genotypes. It was lowest in control seedlings and among genotypes it was maximum in PU-19 and minimum in PU-35. The peroxidase activity was highest at 0.8 mM, highest in IPU-94-1, and lowest in Azad-1.

As compared to other oxidative enzymes SOD activity was not significantly increased after glyphosate exposure. From control to 0.6 mM it was increased but after that decrease was observed except in PU-19, Azad-1 and Azad-2 where it increased. The SOD activity at 0.8 mM was highest in PU-19 and lowest in PU-35. The increase in SOD activity also suggests that glyphosate is inducing oxidative stress in V. mungo.

Glyphosate caused decrease in protein content of in vitro grown seedlings in all the treatment and in all the genotypes. Maximum protein content was in control and value was highest for Azad-1 followed by IPU-94-1 and PU-35 while lowest for Azad-2. Maximum percent reduction over control at 0.8 mM was in Azad-2 while minimum was obtained in IPU-94-1.

**Effect of herbicide on in vitro response of explants on callusing, shoot and root media**

Callusing response was observed in various explants in callus media (A-NAA-0.5+BAP-1.0 mg/l) and (C-NAA-1.5+BAP-1.0 mg/l) supplemented with herbicide. Cotyledonary node was most superior for callus induction followed by hypocotyl, leaf segments, epicotyl and shoot apex. In callus media (A) for cotyledonary explants, in control maximum callus response was found in IPU-94-1 and minimum in Azad-2. In control highest CFW was recorded in PU-19 and lowest in PU-31 whereas at 0.2 mM lowest CFW was found in PU-35 and highest in IPU-94-1. Reduction in callus fresh weight (RCFW) appears to be a good screening parameter for assessment of tolerant genotypes in black gram. Percent reduction in callus fresh weight in comparison of control increased in a dose dependent manner. At 0.4 mM complete reduction was observed as no callus formed even after 30 days in any genotype. Genotype PU-31 and Azad-2 showed 100% reduction in 0.2 mM whereas IPU-94-1 scored least reduction in CFW.

In hypocotyl explants, callusing was observed up to 0.05 mM in all genotype but in 0.1 mM only Azad-1 and Azad-2 showed callusing while at 0.2 mM PU-19 and Azad-
showed some callusing. Percent callus response and callus growth decreased with increase in glyphosate concentration. In control, genotypes PU-35, IPU-94-1, PU-31 and Azad-1 showed higher callus response than PU-19 and Azad-2. At 0.1 mM more callusing and CFW was found in PU-19 than Azad-2 and Azad-1. As compared to other explants CFW was very less in shoot apex. In control, genotype with maximum CFW was IPU-94-1 and minimum in PU-19. In 0.05 mM highest CFW was again in IPU-94-1 and lowest in Azad-2. Effect of glyphosate were also studied on multiple shoot and shoot buds induction using three shoot media: D (NAA-1.0+BAP-1.5+Kn-0.5 mg/l), H (NAA-0.5+ BAP-2.0+ Kn-1.0 mg/l), I (NAA-2.0+BAP-2.0+Kn-1.0 mg/l). In media –(D) among the four seedling explants cotyledonary node and shoot apex showed better positive response for shoot formation than epicotyl and hypocotyl in control as well as herbicide media in all the six genotypes. In epicotyl shoot was not observed in any treatment as well as genotype whereas cotyledonary node showed good shooting and callusing in control as well as stress media. There was progressive decline in percent shoot response, shoot number per explant and shoot length as glyphosate concentration increased in all genotypes but response varied with the genotype. At higher glyphosate concentration 0.1 mM shooting was observed in PU-35, PU-31 and IPU-94-1 while only IPU-94-1 showed shooting at 0.2 mM. At 0.05 mM highest percent shoot response was recorded in Azad-1 while lowest at PU-35. In control, highest number of shoots per explant was observed and among genotypes it was highest in genotype IPU-94-1 while lowest in PU-19. At 0.05 mM concentration of herbicide maximum number of shoots per explant was recorded in Azad-2 while minimum in PU-31 at the same herbicide concentration longest shoots were recorded in Azad-1 and shortest in PU-35. Hypocotyl showed callusing and shooting and shoot bud formation was recorded only in control in genotypes PU-35, PU-31 and Azad-1.

In shoot media (H) best shooting response was observed in cotyledonary explant followed by shoot apex and hypocotyl in which directly shoots and shoot buds formed with callusing. As glyphosate concentration increased, there was progressive reduction in % shoot response, shoot number and shoot length in all genotypes. Shooting was observed in control as well as herbicide media of 0.01 mM, 0.05 mM while at 0.1 mM response was not observed in PU-35 and PU-31. At 0.2 mM response was observed only in PU-19 and Azad-1. No response was observed at 0.4 mM in any explant and genotype. Highest percent shoot response was found in control and it was highest in
Azad-1 and lowest in PU-31. In control highest number of shoots per explant was recorded in Azad-1 followed by PU-31, Azad-2, PU-19, PU-35, IPU-94-1. At highest stress level (0.4 mM glyphosate) highest number of shoots per explant was recorded in Azad-1 followed by IPU-94-1, PU-19, Azad-2, PU-35. In media (H) hypocotyl explants also showed good response for shooting in control as well as herbicide treated media. At 0.05 mM shoots were not formed except in Azad-1. Azad-1 also showed maximum response at 0.01 mM while minimum was found in PU-35 and PU-31. Shooting response in shoot apex explants was observed at 0.05 mM in all genotypes. In 0.1 mM, genotype PU-35 and IPU-94-1 showed shoot response while in 0.2 mM only genotype IPU-94-1 showed response.

In shoot media (I) cotyledonary and shoot apex showed multiple shoots, shoot buds and shoots with callus in control and herbicide treated media but cotyledonary node were found superior for callusing as well as shooting. For cotyledonary node explants genotype PU-35 showed maximum percent shooting response and genotype IPU-94-1 showed minimum response in control. At 0.1 mM where all genotypes showed organogenesis, highest and lowest response was observed in PU-19 and Azad-2 respectively. In control, maximum number of shoots was found in PU-19 and minimum in PU-35 and Azad-2 while in 0.1 mM maximum shoot number were again noticed in PU-19 and minimum in Azad-2. At 0.2 mM longest shoots were recorded in PU-19 followed by IPU-94-1 and Azad-1 while shortest shoots were formed in genotype PU-31 and Azad-2.

For shoot apex explants in control greatest percent response was recorded in Azad-2 followed by PU-19, Azad-1, IPU-94-1 and lowest in PU-31. At 0.1 mM genotype PU-19 showed highest regeneration response followed by PU-35, Azad-1 while genotype Azad-2 showed minimum response. Maximum number of shoots in control was found in genotype IPU-94-1 while minimum in Azad-1 and PU-19. At 0.1 mM genotype PU-19 and IPU-94-1 showed maximum shoot number and Azad-1 and Azad-2 showed least shoot number.

Effect of glyphosate on root formation, root number and root length was analyzed using root media (MS media supplemented with NAA+BAP-3.0+1.0mg/l and glyphosate). Hypocotyl and cotyledonary node explants showed better rooting response than leaf segments and shoot apex. For leaf explants, in control percent root response
was maximum in IPU-94-1 followed by Azad-1 and PU-35 and minimum in PU-19. At 0.01 mM, percent root response was decreased with respect to control in all genotypes except in PU-19 where it was increased up to 80.00%. Maximum number of roots per explant was found in control and among genotypes highest in PU-19 and minimum in Azad-2. In cotyledonary node explants at 0.01 mM only PU-19, PU-35 and Azad-1 showed rooting in cotyledonary node explants while at 0.05 mM PU-19, IPU-94-1 and Azad-2 showed response and maximum value was found in IPU-94-1. Number of roots per explant and root length did not showed definite pattern of decrease on increasing herbicide concentrations like other parameters in other responses like callus fresh weight, shoot number and shoot length. Usually longest roots were in control but in some genotypes PU-35 and Azad-1 average root length was slightly increased in 0.01 mM.

Hypocotyl explants were found best for rooting and callusing in PGR combination of NAA (3.0 mg/l)+BAP (1.0mg/l). Rooting were observed in all genotypes in control, 0.01 mM (except Azad-2), 0.05 mM (except IPU-94-1), 0.1 mM (except Azad-2) and at 0.2 mM in PU-19, PU-35 and Azad-1.

**Gradual and shock stress treatment of callus cultures of selected tolerant genotypes**

In gradual treatment glyphosate treatment significantly inhibited callus growth as observed by consistent decrease in fresh weight with the increasing concentration of herbicide. At 0.01 mM significant (p < 0.05) decrease in fresh weight was observed except in IPU-94-1. However at next herbicide level 0.05 mM, all genotypes showed significant reduction in fresh weight. Maximum fresh weight was observed in PU-19 (1.61±0.09g) whereas IPU-94-1 showed minimum fresh weight (1.48±0.06g). 0.4 mM glyphosate was found to be lethal to all genotypes. When cultures were gradually subcultured on media with higher concentration of glyphosate, inhibition was observed in all growth parameters but it was less than shock treatment. At highest stress level 0.4 mM maximum percentage of surviving cultures were found in PU-19. Tolerance index was also highest for IPU-94-1. In direct shock treatment at highest stress level (0.4 mM) maximum percentage of surviving cultures was observed in IPU-94-1while minimum value obtained in PU-19 and in Azad-1 there was no cultures survived at this concentration. Among genotypes maximum callus fresh weight at 0.4 mM was found in IPU-94-1 with minimum 86.74% reduction over control while Azad-1 showed 100% reduction in fresh weight. These results showed that IPU-94-1 and PU-19 shows higher tolerance than Azad-1.
**In vivo assessment of effect of herbicide and tolerance in Vigna mungo genotypes**

Glyphosate treatment showed adverse impact on survival percentage and plant height, number of nodes per plant, internodal length, pods per plant, pod length and seeds per pod. Consequently glyphosate also reduced yield as reflected by a gradual decrease in the growth and yield in all the three black gram genotypes and it was decreased in dose dependent manner. At highest concentration, 6 mM, genotype PU-19 showed maximum survival percentage and height whereas Azad-1 showed minimum. On the basis of overall morphological and yield parameters it was concluded that high doses of herbicide glyphosate reduce crop yield in black gram genotypes PU-19 and IPU-94-1 were more tolerant than Azad-1.

The influence of glyphosate was also seen on leaf pigments. The pigment content was measured in the leaves of treated plants. It was seen that glyphosate severely affected pigment content. The reduction in pigment content suggested that glyphosate disturbed pigment metabolism in plants. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids registered a decrease after glyphosate treatment. Chl a content was highly reduced in PU-19 as compared to IPU-94-1 and Azad-1. In case of Chl b, Azad-1 showed more decrease as compared to IPU-94-1 and Azad-1.

Activity of antioxidative enzymes was also evaluated in three genotypes after glyphosate treatment. Catalase, peroxidase and superoxide dismutase activity were increased with increase in herbicide concentration. At 6 mM glyphosate dose catalase activity was maximum in PU-19 followed by IPU-94-1 and Azad-1. Peroxidase activity also significantly increased like catalase with the increasing herbicide concentration (1 mM and 2 mM) and then it was decreased but in case of PU-19 it consistently increased up to 4 mM and then decreased. At 6 mM highest activity was obtained in Azad-1 whereas lowest activity was shown by IPU-94-1. After the glyphosate treatment, SOD activity was found to increase significantly in all the three varieties from 2 mM to 6 mM except in IPU-94-1 where it decreased sharply at 6 mM.

Post emergent treatment of glyphosate caused a general increase in total protein content as compared to control in all treatments and genotypes. At 6 mM minimum protein content was found in PU-19 (6.20±0.09 mg/g FW) and maximum in IPU-94-1.
To assess effect of herbicide glyphosate on lipid membranes of leaves, malondialdehyde content was analysed in leaves of three *Vigna mungo* genotypes. MDA was found to increase significantly in 4 mM and 6 mM in all the three genotypes. MDA content at 6 mM was maximum in PU-19, followed by IPU-94-1, and lowest in Azad-1. The increase in MDA content indicates that glyphosate caused lipid membrane damage in *V. mungo*.

After the glyphosate treatment, proline was usually found to increase in all treatments and genotypes but significant increase was found only at 4 mM and 6 mM. Proline content at 6 mM was maximum in Azad-1 and minimum in PU-19.

In conclusion germination percentage, survival percentage, root length, seedling tolerance index, activity of catalase, peroxidase, and SOD, callus fresh weight, and callus tolerance index are the few important parameters that can be utilized for the screening of herbicide tolerant genotypes in *Vigna mungo* as well as other crop plants. Among various *in vitro* methods used in present study, *in vitro* seed germination method and gradual stress treatment of herbicide to calli gave more accurate and precise findings about relative tolerance of cultivars for the herbicide.

Glyphosate application cause oxidative stress and elevation of antioxidant defense system in non target crop black gram. Genotypes PU-19, IPU-94-1 and Azad-1 can better combat the oxidative stress than PU-31, PU-35 and Azad-2 induced by glyphosate application. Selected genotypes and *in vitro* methods used in present study may be utilized in *Vigna mungo* and other crop improvement programme as well as for the study of molecular basis of herbicide tolerance in these tolerant genotypes in contrast to susceptible genotypes.

Herbicide treatment caused decrease in germination percentage, survival percentage, root length, shoot length, fresh weight and dry weight of seedlings in all the genotypes and in both germination screening methods. Genotypes showed differential response towards glyphosate toxicity at its higher doses.


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