

**Elicitor induced biochemical changes associated with nitric oxide and calcium signaling during seed germination in *Trigonella foenum-graecum* L.**

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**Botany**

**By**

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**March, 2017**

## DECLARATION

I declare that the thesis entitled "Elicitor induced biochemical changes associated with nitric oxide and calcium signaling during seed germination in *Trigonella foenum-graecum* L." has been prepared by me under the guidance of Dr. Palash Mandal, Assistant Professor of Botany Department, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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## CERTIFICATE

I certify that Saran Kumar Gupta has prepared the thesis entitled “Elicitor induced biochemical changes associated with nitric oxide and calcium signaling during seed germination in *Trigonella foenum-graecum* L.” for the award of PhD degree of the University of North Bengal, under my guidance. He has carried out the work at the Department of Botany, University of North Bengal.

He bears a good moral character and I wish him all the best in life.



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## **ABSTRACT**

Several pharmacological as well as experimental studies have suggested that consumption of foods rich in antioxidant is significantly associated with reduced risk of various disorders and human diseases, including diabetes. Some of the food types such as fruits, vegetables, sprouts as well as herbal drugs have been found to be very rich in bioactive phytochemicals. Therefore, in recent times the regular consumption of sprouts or germinated seeds, fruits and vegetables, is highly recommended as they are considered to provide long term health benefits. Free radicals are an integral part of normal physiology of a biological system, but an over production of these free radicals cause significant imbalance in the functioning of cellular antioxidant defence system and free radical formation which leads to stressful condition. Antioxidant compounds are capable of mitigating the negative effects of oxidative stress as they are efficient scavengers of the free radicals. The utilization of synthetic antioxidants is negatively perceived by nutritionists and consumers due to safety and health concern, leading to increased interest in natural sources. Fenugreek being rich source of various bioactive phytochemicals has been used for the treatment of wide spectrum of diseases and disorders since ancient times. Considering such diverse implication of fenugreek, the present study was designed aiming the enhancement in the phytochemicals especially associated with the antioxidant and antidiabetic activity as these two therapeutic properties are the major point of concern of our research work.

The elicitors of two important signalling molecules (nitric oxide and calcium) were implemented in the present research work, to investigate their role in the elicitation of therapeutic potential of fenugreek sprouts. The technique applied in the study was priming, this method has been reported be one of the important strategy in enhancement of productivity of crops. Accordingly, the seeds were pre-treated with various elicitors of nitric oxide and calcium namely, sodium nitroprusside (SNP) as an exogenous source of nitric oxide; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CP), a nitric

oxide scavenger; calcium chloride (CC) as an exogenous source of calcium ion; a calcium chelator: Ethylene glycol-bis(2-aminoethylether)-N,N,N',N, tetra acetic acid (EG); and Lanthanum chloride (LC): a calcium channel blocker. After priming, the seedlings were germinated for 3 days and the sprouts were collected at the stage of 24, 48h and 72h for experimental analysis. As a result, the present study revealed significant increase in free radical scavenging activity along with phenolics and ascorbate content by elicited sprouting in fenugreek with exogenous sources of nitric oxide and calcium ion. The sprouts pre-treated with sodium nitroprusside exhibited maximum of 26%, 50%, 37% and 7% whereas those primed with calcium chloride showed about 41%, 34%, 51% and 8.5% enhancement in the content of phenol, flavonoids, carotene and ascorbic acid content. Additionally, a significant correlated relationship between the free radical scavenging activity and the phytochemical contents was obtained, indicating these phytochemicals as efficient antioxidant agents.

Further, the sprouts were evaluated for their *in vitro* anti-diabetic activity along with quantification of alkaloids. The results suggested that the increase in the alkaloid content was found to be responsible for the enhancement in antidiabetic property of fenugreek sprouts. The negative effects of the antagonists of calcium and nitric oxide indicate that the flux of these signalling molecules within the cellular system is essential during germination phases to regulate the mechanisms responsible for the biosynthesis of antidiabetic compounds. The presence of trigonelline as a major alkaloid component was further confirmed by IR and NMR analysis.

When analyzing the time course of germination the stimulatory effect of these signal molecules was found to be most pronounced at the early phases of germination i.e. from 24h to 48h and after that the action was declined during further extension of post-germination phases. The work suggests that nitric oxide and calcium offer significant role in enhancement of therapeutic potential during the germination phase of fenugreek which may be attributed to significant elicitation of bioactive components by these signal molecules.

After successful elicitation of bioactive phytochemicals and their related therapeutic properties, the exogenous sources of nitric oxide and calcium were applied for studying their effect on the alteration of various biochemical parameters along with their antioxidant defence system during germination stages. The pre-treated seeds were exposed to saline condition and their various morphological, physiological and biochemical attributes determined. Our results suggest that priming of fenugreek seeds with exogenous source of nitric oxide and calcium enhanced the morphological and biochemical attributes along with the antioxidant defense system under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seeds which were unprimed and also those primed with the antagonists of these signalling molecules. Therefore, the enhancement in the enzymatic as well as non-enzymatic components might be due to the involvement of calcium and nitric oxide leading to tolerance towards salinity accompanied with better growth and development. Additionally, an uninterrupted influx of these signal molecules is very much essential for the better nutraceutical quality and growth of fenugreek seedlings, which can be hypothesized on the basis of the tremendous deterioration of the therapeutic potential as well as oxidative stress management status of the fenugreek seedlings revealed in the present study.

## Preface

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*Saran Kumar Gupta*  
**(SARAN KUMAR GUPTA)**

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# **CHAPTER - 1**

## **GENERAL INTRODUCTION**

Plants are considered to be an excellent source of bioactive components which have potential nutritional and therapeutic applications (Shetty and Labbe, 1997; Gupta *et al.*, 2014). Understanding the nutritional and therapeutic role of dietary intake of these plants in any form is an important scientific agenda which is essential for developing conventional foods with enhanced health benefits (Shetty, 2001).

Free radicals or reactive oxygen species are predominantly reactive and are known to reduce the concentration of molecular oxygen in the cell and create a physiological state called oxidative stress (Williams and Jeffrey, 2000). They damage the major macromolecules such as nucleic acids, proteins and membrane lipid and consequently trigger a series of aging-related problems (Halliwell *et al.*, 1992). Free radical-mediated oxidative stress is believed to be the primary cause of many disorders, such as cardiovascular diseases, brain dysfunction, cataract, diabetes mellitus, arthritis, cancer and ageing (Enayde *et al.*, 2006). In the treatment of these diseases, antioxidant therapy has gained utmost importance in the recent years.

Antioxidant compounds are capable of mitigating the negative effects of oxidative stress as they are efficient scavengers of the free radicals. The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries for the presence of potential antioxidant components (Ramma *et al.*, 2002). The food resources which are considered to be a good source of natural antioxidants has been found to be beneficial for protection against the diseases related to oxidative stress (Chinnici *et al.*, 2004).

Seeds have been the vital component of the human diet since early age (Ranal and de Santana, 2006). Cereal grains alone, which comprise 90% of all cultivated seeds, contribute up to half of the global per capita energy intake (Nonogaki *et al.*, 2010). Not surprisingly then, seed biology is one of the most extensively analyzed research areas in plant physiology (Chen *et al.*, 2002). With the seed, the independence of the next generation

plant begins. The seed, containing the embryo as the new plant in miniature, is structurally and physiologically equipped for its role as a dispersal unit and is well provided with food reserves to sustain the growing seedling until it establishes itself as a self-sufficient, autotrophic organism, because the function of a seed is to establish a new plant (Bewley, 1997). Similarly, seed sprouts have also been included in the diet as health food and it has been scientifically proved that along with being a good source of basic nutrients, sprouts also are found to possess various bioactive components with disease preventive and health promoting properties (Kurtzweil, 1999).

*Trigonella foenum-graecum* commonly known as fenugreek has been reported to possess several pharmacological and folkloric applications. Its leaves have been reported to show potential antioxidant property, antimicrobial as well as antidiabetic activity. The *in vivo* hypoglycemic activity of fenugreek seeds has been established in various animal model systems. In addition, fenugreek seeds possess potential hypocholesterolemic effect, antioxidant property and are also very effective in the treatment of diabetic disorders (Meghwal and Goswami, 2012).

The consumption of nutritional food forms is not sufficient enough for the long term survival at present era due to increasing population explosion which is creating several new forms of disease with time and the limited resources are not to be ignored. Therefore, it has become very important to develop strategies for the enhancement in the nutritional qualities of the healthy foods including sprouts to serve the rapidly increasing population with limited resources and combat wide spectrum of diseases and disorders. Additionally, the prevention and management of diseases and ailments through dietary intake becomes the most effective strategy to lead healthy life and minimum health-care costs.

The environmental factors are known to have significant impact on the morphological, biochemical attributes along with the growth and development of plants. When any of these factors exceeds the tolerance level, a stress is imposed on the plant which

influences its development along with structural, physiological and biochemical processes (Jaleel *et al.*, 2007). The increase in the salt content above optimum level, which creates salinity stress is considered one among these environmental factors which are responsible for threatening the crop productivity worldwide (Manivannan *et al.*, 2007).

The deleterious effect of salinity which affect the normal growth and development of the plant is attributed to a reduced osmotic potential, specific ion toxicity and nutrient deficiency of the substratum (Luo *et al.*, 2005; Bhattacharjee, 2008). The reduced osmotic potential due to salinity affects water availability due to the prevention of water uptake by the plants, leading to a condition known as physiological drought (Kim *et al.*, 2009). In addition, salinity is reported to induce the generation of reactive oxygen species which further leads to membrane disruption and metabolic toxicity in plant system (Mittler, 2002). To protect themselves from the oxidative stress mediated damages, plants are found to develop scavenging mechanisms from these destructive free radicals. This involves detoxification processes regulated an integrated system of non-enzymatic antioxidants such as ascorbic acid and glutathione (Sharma, 2004), and the enzymatic system which comprises of efficient antioxidants such as catalase ascorbate peroxidase, superoxide dismutase and glutathione reductase (Reshmi and Rajalakshmi, 2012).

So, not only humans, plants are also equally facing several environmental stresses which includes both natural and anthropogenic. Plants are also affected by the free radicals, which damages the physiological and biological systems. They also come across the oxidative stress mediated adverse conditions, during which their growth and development are hugely affected (Ozdener and Kuttbay, 2008). Consequently, the retardation in the growth and development might also lead to significant reduction in the nutritional quality of the plant. These free radicals by virtue of their highly unstable state, cause serious damages to the membranes and tissues which adversely affect the functioning of the plant system, thus affecting the plant productivity (Maevskaya and Nikolaeva, 2013). Therefore, there is

also a need to adopt effective strategies and techniques to protect the plant resources from such hazardous environmental stresses, in order to maintain an ecological balance in nature. Seed priming is considered as one of the pre-sowing techniques in which seeds are subjected to the low external water potential that limits hydration which does not allow the protrusion of radicle through the seed coat. This technique is known to enhance the primary development of seeds under unfavourable environmental conditions (Rozbeh *et al.*, 2011; Nasri *et al.*, 2011). The priming of seeds with various substances *viz.* water, inorganic salts, osmolytes, and hormones has been successful and reported as a cost-effective strategy to enhance tolerance under saline conditions (Joshi *et al.*, 2013).

Nitric oxide is a bioactive molecule, which functions both as a pro-oxidant as well as antioxidants in plant system (Kopyra and Gowdz, 2003). The chemical properties of nitric oxide make it a versatile signalling molecule that functions via interactions with several cellular components (Lamattina *et al.*, 2003). It is also considered as an RNS and its concentration-dependent impacts on different systems were reported to be either protective or toxic (Beligni and Lamattina, 1999). Calcium ( $\text{Ca}^{2+}$ ) is another important secondary messenger and signalling molecule which is actively involved in various physiological and developmental processes. In previous studies,  $\text{Ca}^{2+}$  has also shown a protective effect against stress by mitigation of oxidative damages and membrane stabilization (Larkindale and Knight, 2002).

However, literature suggests that reports on the role of calcium ion and nitric oxide in enhancement in the nutritional quality of fenugreek sprouts and also their effect on growth and metabolism of fenugreek during the developmental phases under salinity stress are not studied till date. Considering this fact, the present study was undertaken to investigate the role of nitric oxide and calcium ion in improving the free radical scavenging activity and antidiabetic activity along with related phenolics and also developing stress tolerance during early developmental phases through priming techniques. Also, the cross talk among these

signalling molecules, polyamines and other growth regulators were also studied in connection with the tolerance mechanism.

The results obtained will lead to a better understanding of fenugreek's responses towards salinity and the role of the two important signalling molecules, nitric oxide and calcium in the enhancement of the therapeutic potential and stress tolerance during early germination stages and also such priming techniques will be of good aid in enhancement of the nutraceutical quality of other plant species which are usually consumed in sprout form.

### **Objectives of Research**

The objectives of research work are:

1. Evaluation of antioxidant activities of the sprouts of fenugreek.
2. Phytochemical screening as well as their quantitative changes at different stages of seed germination.
3. *In-vitro* anti-diabetic activity of the sprouts of fenugreek.
4. Investigation of the effect of seed priming with different elicitors of nitric oxide and calcium signaling on morphology and various germination parameters.
5. Alteration of free-radical scavenging activity during seed germination under the influence of elicitors of nitric oxide and calcium signaling.
6. Biochemical changes during seed germination under the influence of exogenous nitric oxide and scavenger under saline condition.
7. Biochemical changes during seed germination under the influence of exogenous calcium, its chelator and channel-blocker under saline condition.
8. Interaction of calcium and nitric oxide with other growth regulators during germination under saline condition.
9. Determining the correlation between elicitor mediated alteration of biochemical metabolites and their association with activities of antioxidant enzymes.

**CHAPTER - 2**  
**REVIEW OF LITERATURE**

## **2.1 Physiological processes associated with germination**

Germination is a process during which a seed recovers from its resting stage, resumes a consistency in metabolism intensity and then completes the cellular events which leads to embryo emergence, and further prepares for subsequent growth (Nonogaki *et al.*, 2010). Germination is also simply defined as the emergence of the radicle from surrounding structural components (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006). The completion of the process of seed germination represents a key ecological and agronomic trait which determines the entry of plants in the ecosystem (Bewley & Black, 1994; Wilkinson *et al.*, 2002). Hence, the germination potential as well as the dormancy status of a seed is extremely regulated by both the internal and external factors. The process of germination is known to usually involve triphasic phenomena of water uptake. At the initial phase (Phase-I) the rate of water uptake is rapid and it continues until all matrices and cell contents are fully hydrated. In the later phase (Phase-II) limited water uptake takes place and so it is also termed as the plateau phase. During phase-II, due to less water uptake the progress of germination is slowing down. In the next phase (Phase-III), due to elongation in the embryonic axes which also indicates the accomplishment of germination process, the rate of water uptake increases. Here, the increase in the water uptake occurs due to growing of radicle and cell expansion (Bewley, 1997; Nonogaki *et al.*, 2007). After the rapid imbibition of water during initial phase there occurs a temporary membrane disturbance and solute leakage which is also believed to be recovered shortly. The recovery of membrane stabilisation indicates the synthesis of components responsible for cellular repair damages during the imbibition process (Nonogaki *et al.*, 2010). As soon as the imbibition of water takes place, the respiratory activity with the seed is triggered and oxygen consumption occurs until the radicle penetrates the surrounding structure. The cellular system of mature dry seed contains sufficient amount of Krebs's cycle enzymes and terminal oxidases to provide adequate amounts of energy in the form of ATP from oxidative phosphorylation to

carry on the metabolic activities for several hours after imbibition (Ehrenshaft and Brambl, 1990; Attucci *et al.*, 1991). The two other equally important respiratory pathways, glycolysis and the pentose phosphate pathway (PPP), are also triggered in imbibed seeds. Many seeds experience temporary oxygen deficiency due to dense internal structure during the process leading to ethanol production (Kennedy *et al.*, 1992). The glycolytic pathway predominates when mitochondrial ATP synthesis is restricted by anaerobic condition, often due to limited gaseous diffusion of oxygen by the structures surrounding the embryo. In contrast, when mitochondria become active, eventually the PPP predominates (Roberts, 1964). The reactive oxygen species (ROS) production takes place during water uptake process. A beneficial role of ROS signalling has been reported in breaking the seed dormancy (Schopfer, 2001; Oracz *et al.*, 2009) but in imbibed seeds, these free radicals are found to have adverse effect due to their involvement in the damage of the biomolecules and other cell components. The antioxidant enzymes present in the pea seeds such as catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase are found to scavenge the free radicals produced during imbibition (Wojtyla *et al.*, 2006). For the completion of the process of germination, the radicle emergence has to occur through the surrounding structure. But in some species, the constrained structure of embryo imposes mechanical barrier against emergence which may lead to physical dormancy. So, to penetrate through this barrier there requires induction of cell wall remodelling enzymes or physical weakening of the cell walls. Many researchers have proposed the weakening of endosperm barrier by various enzymes such as  $\beta$ -glucanase, endo- $\beta$ -mannase, xyloglucan endotransglycosylase, polygalacturonase, pectic methylesterase and other proteins such as expansins (Holdsworth *et al.*, 2008). The weakening of the mannan rich endosperm barrier by endo- $\beta$ -mannase enzyme has been reported in the wild type seeds of tomato (Voigt and Bewley, 1996). Though numerous studies have performed explaining different aspects of mechanisms involved during germination but still more and more updates are required to have a complete

picture of seed germination (Nonogaki *et al.*, 2010). There are also challenges for enhancement of growth performances during germination in horticultural and agronomic crops, and the technique of priming and pelleting/coating has met the need to some extent. However, success has been achieved specially for seeds planted in experimental conditions at small scale.

## **2.2 Elicitation and the role of elicitors in plant system**

Elicitation is considered as an effective strategy to enhance the synthesis of wide spectrum of bioactive secondary metabolites. The factors applied for elicitation are known as elicitors. Elicitors are responsible for inducing physiological alteration in the plant system which leads to enhancement in the bioactive components (Duenas *et al.*, 2015). Elicitors may be categorised into biotic elicitors: one with biological origin and other abiotic elicitors: not associated with the biological origin, the hormones are also considered as the elicitors (Baenas *et al.*, 2014).

The elicitor signal transduction is an important subject of investigation. In response to the physiological alteration caused by these elicitors, plants activate an array of defense mechanisms, as during pathogen infections or environmental stimuli, thus affecting the plant metabolism and further enhancing the biosynthesis of bioactive components (Zhao *et al.*, 2005). These defense mechanisms includes, hypersensitive responses, production of reactive oxygen species, structural barrier such as lignification, induction of antioxidant enzymes and pathogenesis related proteins, alteration in ion fluxes and also regulation of gene expression associated with various secondary metabolite biosynthetic pathways (Garcia-Brugger *et al.*, 2006; Ferrari, 2010). The mode of elicitation may be of two types namely, pre-harvest elicitation and the postharvest elicitation. The former includes, seed priming (Cho *et al.*, 2008), soaking seeds in aqueous solution of elicitor, or applying exogenous spray method over the leaves (Baenas *et al.*, 2014) or in a hydroponic system (Wei *et al.*, 2011). A daily elicitation with 10  $\mu$ M of methyl jasmonate by exogenous spraying resulted in 31%, 23%

and 22% enhancement in flavonoid, phenolic and glucosinolates content, respectively, in 7 days old broccoli sprouts (Perez-Balibrea *et al.*, 2011). During postharvest elicitation the elicitors are applied on the harvested products such as foods and vegetables for enhancement in the quality composition. It was observed that ultraviolet irradiation resulted in the significant increase in the antioxidant activity of the grapes (Crupi *et al.*, 2014). Also, an elevation of 35% and 52% was reported in the phenolic content and antioxidant potential of strawberry fruits when elicited with exogenous application of methyl jasmonate for one week (De la Pena Moreno *et al.*, 2010).

Unfortunately, during seed germination subsequent reduction in the antioxidative potential of the sprouts has been observed due to decrease in the phenolics which are known to be potent antioxidant agents (Swieca *et al.*, 2012). Nevertheless, successful attempts have been made through various elicitation processes regarding the improvement of antioxidant potential of sprouts along with the related phenolics. In this context, elicitation of phenylpropanoid pathways by UV irradiation have resulted a significant increase in the antioxidant property of broad beans sprouts (Shetty *et al.*, 2002). Later, Randhir *et al.* (2004) have implemented the biotic elicitors such as oregano extract, fish protein hydrolysate and lactoferrin and enhanced the antioxidant potential of mung bean sprouts. An increase in the composition of different antioxidant molecules such as polyphenols, ascorbic acid and  $\beta$ -carotene as well as increased digestibility and solubility of proteins has been obtained in chick pea (Khattak *et al.*, 2007). Also, the elicitation technique of high pressure condition led to an increase in antioxidative activity in the sprouts of *Vigna sinensis* (Doblado, 2007). These elicitors are not only known for the enhancement of the nutraceutical properties of fruits, vegetables and sprouts; moreover, they also play vital role in many physiological processes such as germination, breaking of seed dormancy, and also providing tolerance towards different environmental stress conditions. The role of phytohormones: gibberellins, ethylene and brassinosteroids in promoting the process of seed germination by breaking the

seed dormancy are well known (Kucera *et al.*, 2005). Another important plant hormone which has been successfully utilized as a potent elicitor molecule is salicylic acid. It has also been implemented through varied methods of application (soaking, adding to the hydroponic solution, or spraying solution) and has been shown to protect various plant species against abiotic stresses by inducing stress tolerance mechanisms (Horvath *et al.*, 2007).

Polyamines are polycationic, low molecular weight and nitrogenous growth regulators present ubiquitously in all living system. The most commonly available free polyamines in plants are di-amine putrescine, tri-amine spermidine and tetra-amine spermine. These regulator molecules are also known to enhance the tolerance level in several plants towards the environmental stresses such as drought, salinity, chilling and potassium deficiency (Martin-Tanguy, 2001; Takahashi and Kakehi, 2010; Alcazar *et al.*, 2010).

Different studies have suggested additive or synergistic responses after combination of these elicitors; also different signal transduction pathways are associated with the environmental stresses and elicitors. Consequently, these associated pathways might antagonize or harmonize with each other, leading to negative or additive interactions, respectively (Zhang *et al.*, 2002; Cho *et al.*, 2008; Cevallos-Casals and Cisneros-Zevallos, 2010). Furthermore, it is also observed that the nature, dosage and time of treatment strongly affect the intensity of the plant responses.

### **2.3 Importance of Nitric oxide signalling in plant system**

Nitric oxide (NO) is an important signalling molecule, which has been known to participate in wide spectrum of regulatory functions in almost all stages of plant development (Wilson *et al.*, 2008; Sirova *et al.*, 2011). In the year 1975, the emission of NO from plants was first observed by Klepper in soybean plants treated with herbicides (Klepper, 1979). Plants not only react to the atmospheric or soil NO, but they are also able to generate NO via reduction of apoplastic nitrite (Bethke *et al.*, 2004) or by carotenoids in presence of light (Cooney *et*

*al.*, 1994). The major production of NO in plants, however, is probably carried through the action of NAD(P)H-dependent nitrate reductase enzyme (Dean and Harper, 1988) which is also considered as an endogenous source of NO in plant system (Yamasaki *et al.*, 1999).

The synthesis of NO in animals is carried out by the enzyme nitric oxide synthase (NOS) via deamination of L-Arginine. But, there are no such genes in plant system including *Arabidopsis thaliana* that are homological to NOS genes of animals (Gupta *et al.*, 2011). Among the photosynthetic members, only *Ostreococcus tauri*, an unicellular green algae was found to possess a NOS having a homology of only 45% with the human NOS (Foresi *et al.*, 2010). At present several pathways involved in NO synthesis in plant system are known, also some are assumed which are given in Figure 2.1 (Mamaeva *et al.*, 2015). The biosynthetic pathways leading to the production of NO in plants might be either oxidative or reductive. The oxidative pathway is carried out by NOS like enzyme which also includes synthesis from polyamines. The reductive pathway is mediated by enzymes such as nitrate reductase (NR) and nitrite-NO reductase (Ni-NOR). Furthermore, this pathway includes xanthine oxidoreductase (XOR) in peroxisomes and cytochrome *c* oxidase (COX) that synthesizes NO from nitrite in mitochondria (Mamaeva *et al.*, 2015).

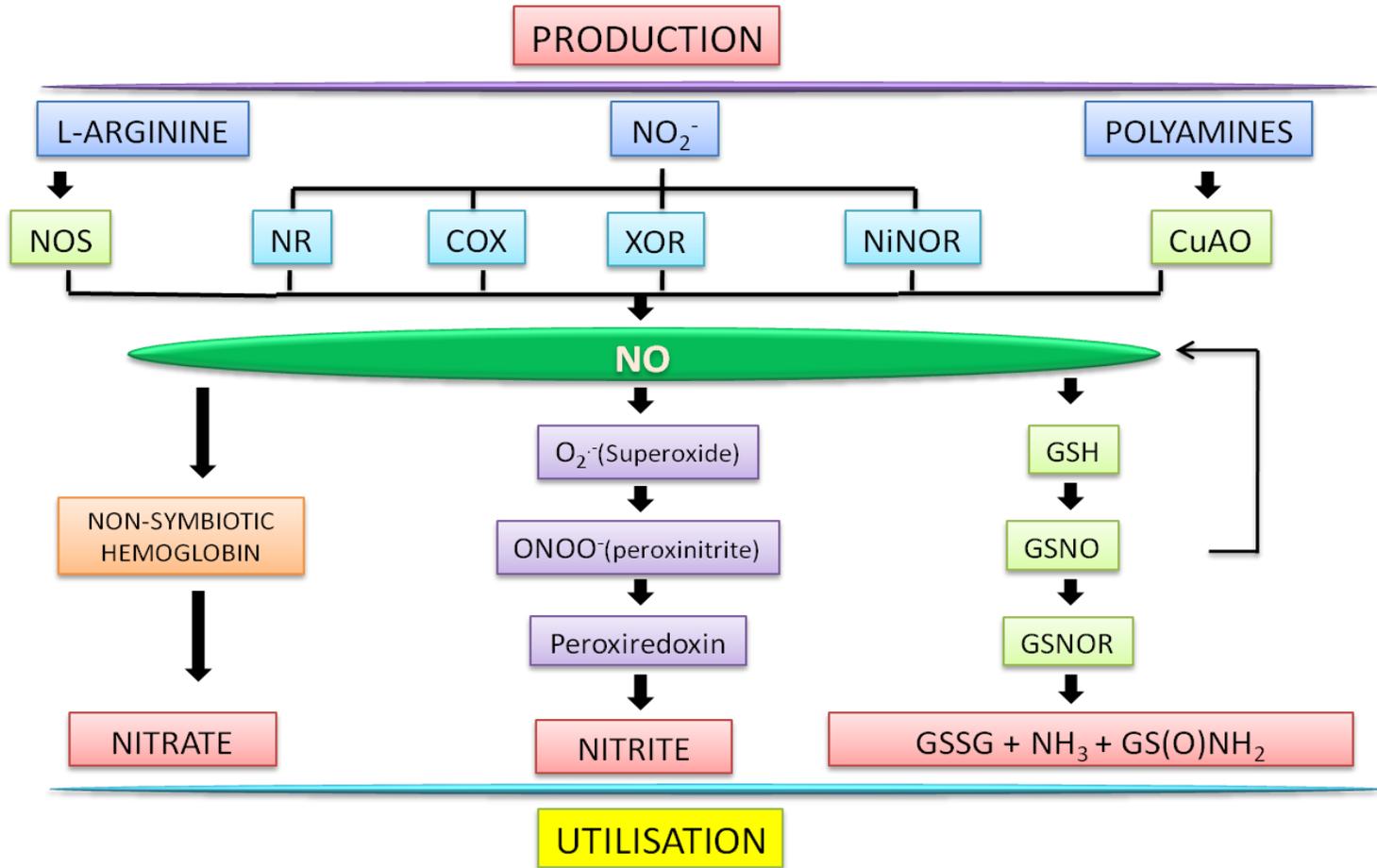
The application of exogenous NO to plants or cell cultures has revealed valuable information about the influence of this molecule on various physiological and biochemical processes. The summary of the functions NO associated with various physiological, biochemical and molecular processes is given in Figure 2.2 (del Rio *et al.*, 2004). The earlier reports suggest that NO can mediate the biological effects of signalling molecules such as phytohormones. The biosynthesis of NO has been found to be induced by cytokinin in different plants and hence the possibility of involvement of NO in the cytokinin-induced programmed cell death process is proposed by Neill *et al.*, (2003). Likewise, it has been demonstrated that NO synthesis in cucumber roots is induced by auxin (Pagnussat *et al.*, 2003). Additionally, the interaction between both the gaseous molecules NO and ethylene in

the maturation and senescence of plant tissues has been reported during plant development (Lamattina *et al.*, 2003).

The identification of the NO synthesis enzymes and the discovery of regulatory role of NO in the activity of specific proteins within subcellular compartments provided significant understanding of NO signalling at the molecular level (Hanafy *et al.*, 2001; Kone *et al.*, 2003; Stuehr *et al.*, 2004). Over the past decade, considerable progress has been made in understanding the mechanism of NO signalling in plants. NO modulates the activity of most proteins through nitrosylation and tyrosine nitration mechanism. The post translational modifications via nitrosylation as well as S-nitrosylation have been resulted in regulation of several plant proteins *in vitro* also *in vivo* to some extent. The proteins which are the targets of NO include haemoglobin, cytochrome c oxidase, metacaspase 9, glyceraldehyde-3-phosphate dehydrogenase, and methionine adenosyltransferase (Besson-Bard *et al.*, 2008). Endogenous NO has been found to function as a calcium ion-mobilizing messenger by inducing the rise in cytosolic Ca<sup>2+</sup> concentrations. The rise in cytosolic Ca<sup>2+</sup> concentration further aid NO to modulate the protein kinases and channels involved in the signalling cascade, thus regulates important physiological process such as stomatal closure, adventitious root formation also the expression of defense genes (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2006). In *Arabidopsis*, it was demonstrated that the production of NO by elicitors such as lipopolysaccharides is regulated by Ca<sup>2+</sup> influx mediated by the cyclic nucleotide-gated channel (Ali *et al.*, 2007).

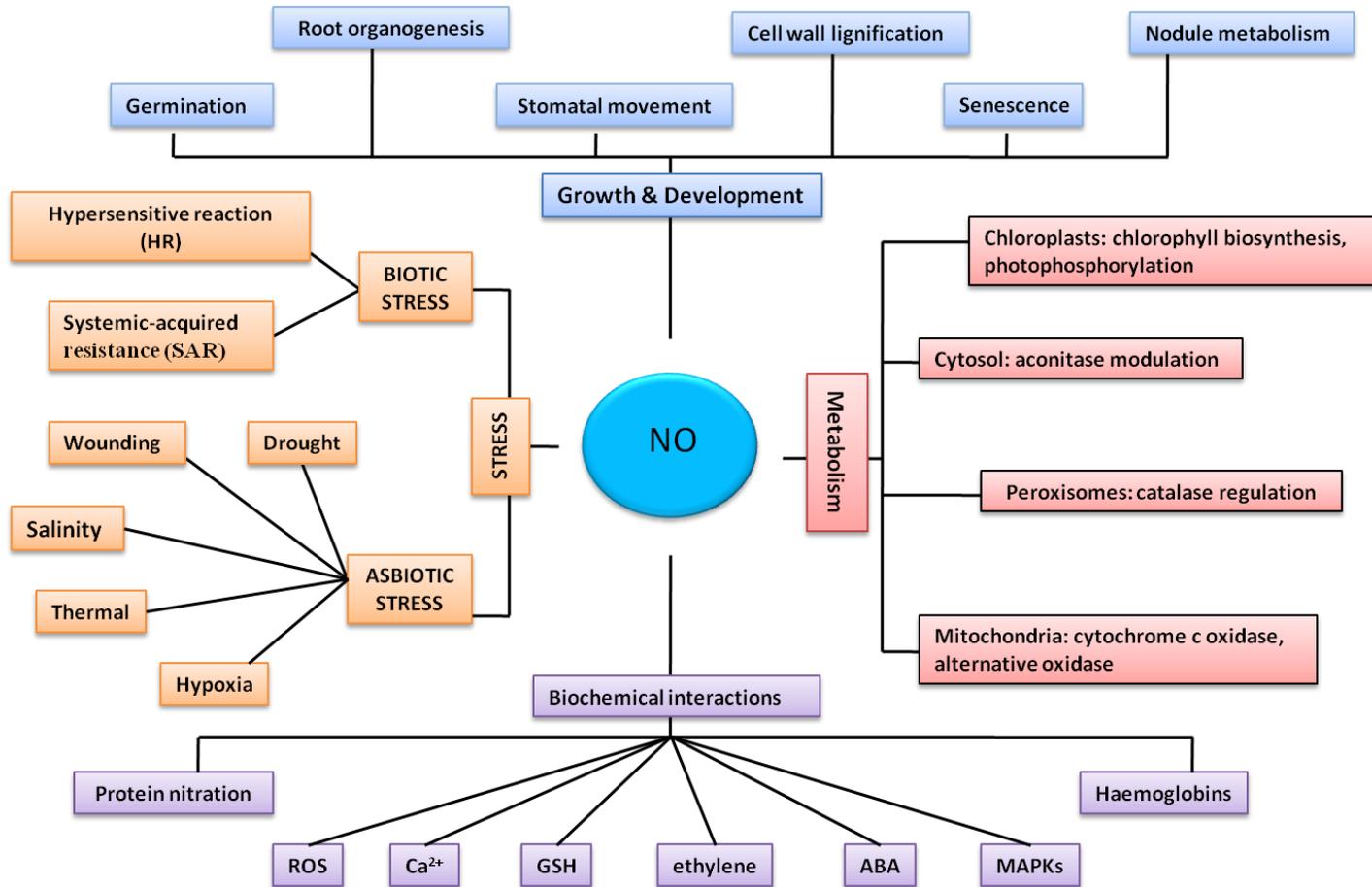
#### **2.4 Role of Calcium signalling in plant system**

The calcium ion has been well established as a second messenger in several plant signalling pathways, conveying a wide range of stimuli to appropriate physiological responses. Ca<sup>2+</sup> signals are considered as a core regulator of cell physiology and cellular responses of plants to the environment. Many extracellular and environmental signals including both abiotic and



**Figure 2.1:** Pathways involved in synthesis and utilization of NO in plant system (modified from Mamaeva *et al.*, 2015)

NOS: Nitric oxide synthase; NR: Nitrate reductase; COX: Cytochrome oxidase; XOR: Xanthine oxidoreductase; CuAO: Cu-amine oxidase; NiNOR: Nitrite-NO reductase; GSH: Reduced glutathione; GSNO: S-nitrosoglutathione; GSNOR: S-nitrosoglutathione reductase; GSSG: oxidized glutathione; GS(O)NH<sub>2</sub>: Glutathione sulfinamide

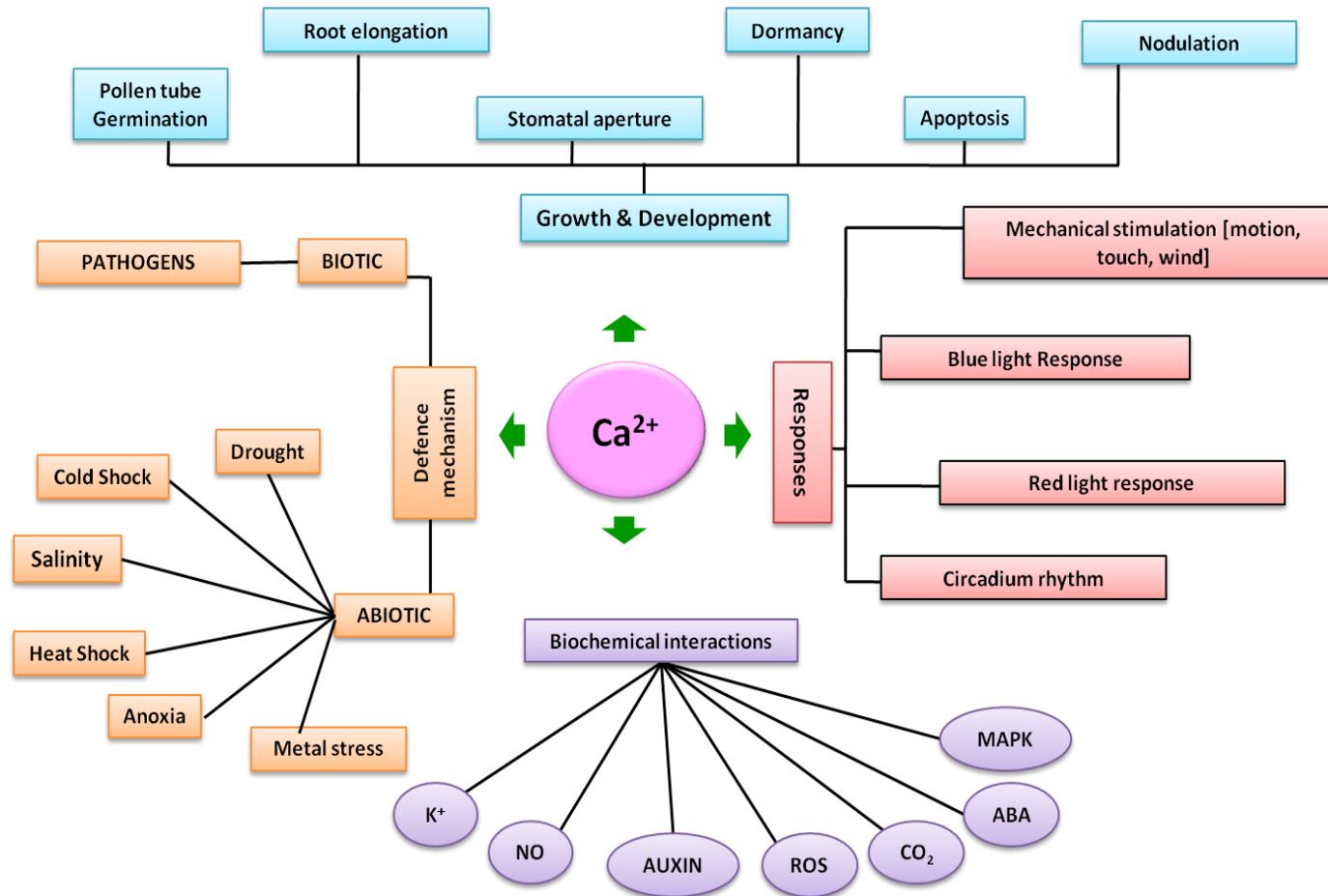


**Figure 2.2:** Functions of Nitric oxide associated with various physiological, biochemical and molecular processes (modified from del Rio *et al.*, 2004)

biotic factors, elicit change in the cellular level of calcium, termed as calcium signatures (Lecourieux *et al.*, 2006). This “Ca<sup>2+</sup> signatures” represent a central mechanistic principle for stimulus-specific information in the cell system. The channels, pumps, and carrier proteins serve as the mechanistic basis for generation of Ca<sup>2+</sup> signals by modulating the flux of calcium ions among the subcellular compartments, cell and its extracellular environment (Dodd *et al.*, 2010).

The disorders due to Ca-deficiency in plants have been considered to be very much harmful in horticulture sector commercially (Poovaiah, 1986). Some of the diseases caused due to deficiency of calcium in plants are tipburn and brown heart in leafy vegetables, blossom end rot of tomato fruit, empty pod in peanut also structural weakness in cell wall. The Ca-deficiency generally occurs when there is unavailability of sufficient calcium in the developing tissues due to failure of calcium mobilization by phloem. On the other hand, presence of excess calcium in the substratum also creates a cytotoxic environment for plants. The excessive calcium reduces the germination rate of the seeds and also retards the plant growth rates (Poovaiah, 1986; White and Broadley 2003). The other functions of calcium ion in the plant systems are elucidated in Figure 2.3.

Since the presence of higher calcium ion concentration is cytotoxic, a submicromolar level of calcium ion is maintained by Ca<sup>2+</sup>ATPases and H<sup>+</sup>/Ca<sup>2+</sup> antiporters in unstimulated cells (Sze *et al.*, 2000; Hirschi, 2001). These proteins maintain this optimum level by fluxing the extra cytosolic Ca<sup>2+</sup> either to the apoplast or the lumen of vacuole or endoplasmic reticulum (Sanders *et al.*, 2002). There are other class of proteins which change their conformation or catalytic activity upon binding with the calcium ion and hence regulate the calcium signals. Also it has been reported that specific sensors and signals of calcium ion signatures regulated cellular responses to specific biotic and abiotic stimuli (White, 2000). The proteins responsible for the perception and decoding of Ca<sup>2+</sup> signals are present in the cytosol and nucleus of the plant cell. Several calcium sensors with different Ca<sup>2+</sup>-binding



**Figure 2.3:** Involvement of  $\text{Ca}^{2+}$  signal in various physiological, biochemical and molecular processes in plant system (modified from White and Broadley, 2003; Leucourieux *et al.*, 2006)

characteristics, subcellular localizations and signalling interactions comprises a toolkit that helps in decoding the information within  $\text{Ca}^{2+}$  signatures in the form of spikes or oscillations (Dodd *et al.*, 2010; Batistic and Kudla, 2012). Further these sensor proteins accordingly carry the processing of this information into respective alterations in cell function. Conceptually, plant  $\text{Ca}^{2+}$  sensor proteins that are functionally signalling components have been classified into sensor relays and sensor responders (Sanders *et al.*, 2002). The sensor responder proteins which include  $\text{Ca}^{2+}$ -dependent protein kinases (CDPK) combine both sensing function and responding function, regulated by calcium-binding proteins that often cause conformational changes (e.g., protein kinase activity) within a single protein. Consequently, these kinases mediate the information encoded in  $\text{Ca}^{2+}$  signals into phosphorylation events of specific target proteins. In contrast, sensor relay proteins such as calmodulin (CaM) and calmodulin like protein (CML) also contain multiple calcium-binding domains and undergo conformational changes with  $\text{Ca}^{2+}$  signals but lacking the enzymatic function. Therefore, these proteins have to interact with other target proteins and regulate their activity for transduction of  $\text{Ca}^{2+}$  signal, which means they must undergo  $\text{Ca}^{2+}$ -dependent protein-protein interactions (Luan *et al.*, 2002). The calcineurin B-like (CBL) protein are another family of sensor proteins which lack the enzymatic activity hence belong to sensor relay proteins. However, their specific interaction is with a family of protein kinases designated as CBL-interacting protein kinases (CIPKs), so, CBL–CIPK complexes are considered as bimolecular sensor responders (Hashimoto and Kudla, 2011). CaM is highly conserved in all eukaryotic members, whereas CML, CDPK and CBL proteins have been found to be present only in plant system (Batistic and Kudla, 2009). The specific binding of  $\text{Ca}^{2+}$  with Calmodulin7 (Cam7) results in direct interaction and regulation, while other calmodulins are likely to mediate gene regulation via interacting with other transcriptional (co)regulators. Metabolic and biosynthetic processes such as brassinosteroid synthesis are important targets of direct  $\text{Ca}^{2+}$ -dependent modulation (Du and Poovaiah,

2005), but on the other hand  $\text{Ca}^{2+}$ -dependent phosphorylation and gene regulation provides the major cellular currencies for transduction of specific  $\text{Ca}^{2+}$  signals into targeted downstream responses (Harper and Harmon, 2005).

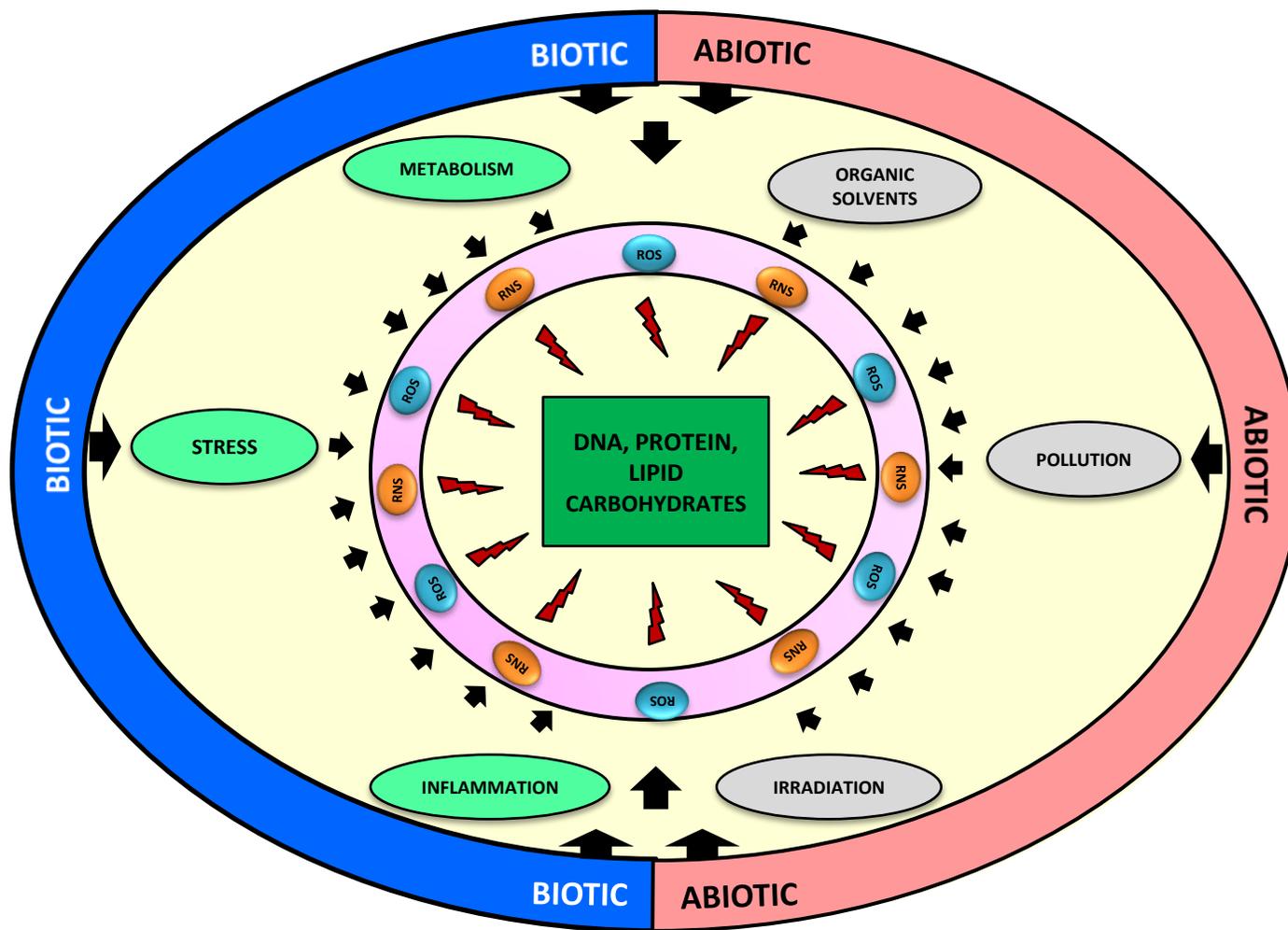
## 2.5 Concept of free radicals

A free radical is defined as a molecular species which is capable of independent existence and possesses an unpaired electron in its outermost atomic orbital. This unpaired electron results in presence of certain common properties that are shared by most of the radicals. These free radicals are highly unstable as well as highly reactive. They have the capability to either donate an electron or accept an electron from other molecules, therefore altering their native properties (Cheeseman and Slater, 1993; Lobo *et al.*, 2010). The free radicals generated from oxygen are called reactive oxygen species (ROS) and those from nitrogen are termed as reactive nitrogen species (RNS). ROS includes various forms of activated oxygen molecules, such as superoxide ( $\text{O}_2^{\cdot -}$ ), hydroxyl ( $\cdot\text{OH}$ ) and peroxy ( $\text{ROO}\cdot$ ), as well as non-free radicals hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and singlet oxygen ( $^1\text{O}_2$ ). Likewise, RNS includes nitric oxide ( $\text{NO}\cdot$ ) and nitrogen dioxide ( $\text{NO}_2\cdot$ ) and free radicals such as nitrous acid ( $\text{HNO}_2$ ) as peroxy nitrite ( $\text{ONOO}^-$ ) (Halliwell, 1994; Chanda and Dave, 2009). These free radicals are generated under normal physiological conditions but become harmful when not being eliminated from the cellular systems. In fact, such imbalance between the production and elimination of reactive oxygen species in the cell system leads to a condition known as oxidative stress. After excessive accumulation, they attack vital biomolecules leading to cell damage and homeostatic disruption. The major targets of these free radicals are lipids, nucleic acids, proteins and carbohydrates (Aruoma, 1994). The formation of free radical is a consequence of both enzymatic and non-enzymatic reactions which occurs continuously in the cell system. Enzymatic reactions include those phenomena involved in the phagocytosis, respiratory chain, synthesis of prostaglandin also in the cytochrome P450 system (Lui *et al.*,

1999; Lobo *et al.*, 2010). Free radicals can also be produced in non-enzymatic reactions between oxygen and organic compounds as well as those initiated by ionizing reactions. In the cellular system, free radicals can be derived from two sources either endogenous sources such as nutrient metabolism, ageing process etc or exogenous sources which include tobacco smoking, radiation ionization, pollution, organic solvents, etc (Buyukokuroglu *et al.*, 2001). The various sources of free radical formation are illustrated in Figure 2.4.

## **2.6 Oxidative stress and their impact on human health**

When oxygen traps single electron, it becomes very unstable and reactive and results in generation of destructive chain reactions against various biomolecules. The high degree of toxic effect of oxygen is associated with its high capacity of generating free radicals and thus, destroying major biological molecules (Valko *et al.*, 2006). Oxidative stress may also occur in tissues damaged by trauma, infection, heat injury and toxins. These injured tissues rapidly increases the synthesis of radical generating enzymes, activates phagocytes, causes release of free iron and copper ions and disruption of the respiratory chains (Rao *et al.*, 2006). A role of oxidative stress has been postulated in many conditions resulted from the damages caused such as membrane lipid peroxidation, protein oxidation and DNA damage (Lobo *et al.*, 2010). Protein containing amino acids such as methionine, histidine, arginine, and cysteine are found to be most vulnerable to oxidation reaction. Protein oxidation leads to alteration of signal transduction mechanism, enzyme activity and proteolysis susceptibility, which leads to ageing (Freeman and Crapo, 1982). Lipid peroxidation is a damaging process which involves a source of secondary free radicals, which acts as second messenger or may directly react with other biomolecules causing biochemical lesions. Lipid peroxidation usually occurs on sites of cell membranes with polyunsaturated fatty acid and it further proceeds with radical chain reaction (Lovell *et al.*, 1995). It has also been reported previously that especially in ageing and cancerous disorders, DNA is considered as a major



**Figure 2.4 :** Sources of free radical formation (modified from Buyukokuroglu *et al.*, 2001)

target (Woo *et al.*, 1998). These deleterious effects of oxidative stress has been significantly attributed to numerous diseases including neurological disorders (Alzheimer's disease, Parkinson's disease, memory loss and depression), muscular dystrophy, cardiovascular disease, inflammatory diseases (arthritis, glomerulonephritis, vasculitis, adult respiratory diseases syndrome), gastric ulcers, hypertension, diabetic disorders and carcinogenesis (Chanda and Dave, 2009; Lobo *et al.*, 2010).

## **2.7 Prevention of human diseases through dietary intake of sprouts**

Considering the rapid increase in risk factors of human to several fatal diseases, a global trend has been set towards the use of natural substance dietary sources as therapeutic antioxidants. It has been suggested that there lies an inverse relationship between the dietary intake of antioxidant rich food and incidence of human diseases. The use of natural antioxidants would be promising alternative for those synthetic antioxidants in respect of cost, compatibility and side effects inside the human system (Lobo *et al.*, 2010).

Sprouts are gaining significant commercial importance due to its enriched nutritional and antioxidant value, also sprouting causes removal of some anti-nutrients and thus make them safer for consumption (Laila and Murtaza, 2014). Sprouts mainly originate from the members of Leguminosae family and there are different plants which are consumed as sprouts, such as alfalfa, broccoli, mung bean, radish, soybean and fenugreek (Yang *et al.*, 2013). It has been widely accepted that sprouts provide higher nutritional value than seeds and their production is simple and cost effective, thus, they are highly preferred as health food (Bodi *et al.*, 2013).

During the course of germination the complex molecules are converted to their simpler forms such as polysaccharides degrade into oligo and monosaccharides, fats into free fatty acids and proteins into oligopeptides and free amino acids. Since, they enhance the efficiency of catabolic enzymes therefore germination can be considered as a kind of pre-digestion mechanism. While sprouting the reduction in the anti-nutritive factors such as

trypsin inhibitor, tannin and phytic acid takes place along with increase in bioactive compounds with health-maintaining impact and phytochemical properties (glucosinolates, enzymes, natural antioxidants). Thus, the method of sprouting can lead to the development of functional foods having positive impact on the human health (Sangronis and Machado, 2007).

Several research works have shown that different food forms rich in phytochemicals possess health promoting and diverse diseases and disorders preventive properties (Saxena *et al.*, 2013). The phenolic components have drawn progressive attention as potential agents for prevention and treatment of several oxidative stress mediated diseases (Gan *et al.*, 2010). Several studies have been conducted regarding the comparative analysis of antioxidant activity, antioxidant enzyme activity and related phenolic content in seeds and sprouts of various plants. Furthermore, it was observed that sprouting of soybean, mungbean and cowpea resulted in enhanced nutritional value of seeds, in terms of phenolics in a natural way (Chon, 2013).

Likewise, numerous reports have been documented till date suggesting sprouts as a potential anti-diabetic functional food (Laila and Murtaza, 2014). Sprouts including mung bean sprouts (Yao *et al.*, 2008; Yeap *et al.*, 2012), broccoli sprouts (Bahadoran *et al.*, 2012), buckwheat sprouts (Watanabe and Ayugase, 2010), and chickpea sprouts (Mao *et al.*, 2008) have been shown to exhibit a potential antidiabetic activity under *in vivo* conditions. The presence of some health-protecting bioactive components at much higher concentration in the sprout than in the developed plant has also been reported (Fernandez-Orozco *et al.*, 2008). These phytochemicals have significant antigenotoxic effect against H<sub>2</sub>O<sub>2</sub> induced DNA damage; additionally, in those people who consumed cabbage and leguminous sprouts compared to the control diet, reduction in the risk of cancer was observed. The methanolic extract of the radish sprout was shown to exhibit potential antioxidant activity attributed to

components with efficient radical-scavenging capacity such as flavonoids and esters of sinapic acid (Takaya *et al.*, 2003).

The sprouts of five lentil varieties, when analysed for amino acid composition, it was found that the amino acid content was enhanced significantly in sprouts with asparagine being highest in concentration than in seeds (Rozaan *et al.*, 2001). Urbano *et al.* (2005) fed some rats with raw green pea and sprouted green pea for six days; interestingly the nitrogen balance, protein efficiency ratio and utilizable carbohydrate index were found to be significantly higher in those rats consuming sprouted green pea. A good amount of organic phosphates, a mixture of polyphenols, enzymes and reducing glucosides were found in the wheat sprouts by Amici *et al.* (2008). Also, the phenolic derivatives present in the sprouts were identified as gallic acid, catechin, epicatechin, epigallocatechin-3-gallate and epigallocatechin (Amici *et al.*, 2008). Furthermore, they claimed that the extract of the wheat sprouts were responsible for inhibition cancer cell growth along with enhancement of intracellular oxidative proteins. Similarly, broccoli sprouts were found to hinder the growth of various types of cancerous cells such as lung cancer, urinary bladder cancer, prostate cancer cells, skin tumour, ovarian cancer and breast cancer (Abdulah *et al.*, 2009; Zhang *et al.*, 2006; Wang and Shan, 2012).

Therefore, sprouts might be an affordable and efficient solution to the burden of diseases and disorders if included in our daily diet and further implemented in pharmaceutical preparations.

## **2.8 Phytotherapeutic potency of *Trigonella foenum-graecum***

Fenugreek (*Trigonella foenum-graecum*) is a leguminous herbaceous plant cultivated as semi-arid crop. It is a member of Fabaceae family and is given several names in different languages; for example, Methi (Hindi), Koroha (Japanese), Fenugrec (French), Fieno greco (Italian), Pazhitnik (Russian), Hulba (Arabian), Alholva (Spanish) and K'u-Tou (China)

(Srinivasan, 2006). India is major producer of fenugreek, also one of the main consumers for its culinary and medicinal uses. The seeds of fenugreek are used in the form of spice for seasoning and flavouring agent also making soups in comparatively larger quantities (Srinivasan, 2006; Meghwal and Goswami, 2012).

The taxonomic position of fenugreek according to scientific classification of plant kingdom is given below:

Kingdom: Plantae

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Trigonella*

Species: *foenum-graecum*

The fenugreek (*Trigonella foenum-graecum*) seed usually sprouts within three days under favourable environmental conditions. Seedling grows erect, semi-erect or branched based on its variety and attains a height of 30 to 60 cm. The plant develops with compound pinnate, trifoliate leaves, axillary white to yellow flowers, and 3-15 cm long thin pointed hoop-like beaked pods. Each pod is said to bear 10-20 oblong greenish-brown seeds within its unique hooplike grooves. Fenugreek is a self pollinating annual crop and being a leguminous plant it is an efficient atmospheric nitrogen fixer (Srinivasan, 2006).

The specific epithet "foenum-graecum" in latin means "Greek hay" indicating its use as a forage crop also the plant is believed to be native to the Mediterranean region. However, at present it is grown all over the world as a spice crop including United States of America, parts of Europe, Canada, northern Africa, Australia, Argentina, west and south Asia; India being the leading producer as reported by Prajapati *et al.*, 2014.

Historical records indicate that almost all parts of fenugreek plants especially seeds and leaves were used for treatment of several diseases and disorders, such as treating mouth ulcers, abdominal pain, curing baldness, treatment of cardiovascular and hepatic disorders,

arthritis, gastric problems, spleen and liver enlargement, kidney ailments, anorexia among several others, in the subcontinent of India, Greece, Arab and China (Prajapati *et al.*, 2014). The seeds of fenugreek are small in size, golden yellow in colour, rigid and exist in four-faced stone shape and biologically it is endospermic in nature (Altuntas *et al.*, 2005). They are considered as the most important and useful part among the whole plant body. The taste of seeds is bitter due to the presence of saponin and alkaloids but their bitterness is minimized and flavour can be enhanced by roasting. The whole seed or powder is used as spices in pickles and vegetable dishes (Jani *et al.*, 2009). The leaves and tender stem are also edible; they are enriched with several nutritional components such as calcium, magnesium, sodium, potassium, zinc, vitamin C, carotene, nicotinic acid and riboflavin (Srinivasan, 2006).

The volatile oils from fenugreek, which are present in about 6-8%, have active odorous compounds and bitter taste. The unsaponifiable part of oil contains the lactation-stimulating factor due to which fenugreek is used to increase lactation in women and cattle traditionally. Further, oil is also used for cosmetic industries in manufacturing perfumes, and is reported to possess potential insect and pest repellent property (Srinivasan, 2006; Meghwal and Goswami, 2012).

Fibre in the diet provides health benefits by replacing calories, suppressing appetite, and controlling excess weight-gain. It is reported that near about 50% dry weight of fenugreek seeds comprises of edible dietary fibre, which believed to be highest among the natural sources of fibers (Madar and Shomer, 1990).

The dietary fibre of fenugreek is known to reduce the risk of alimentary disorders such as diverticulosis, hemorrhoids, and anal fissures (Raju *et al.*, 2001). Also, it is suggested that consumption of 30g fenugreek dietary fibre a day results in weight loss without the side effects of dieting. Furthermore, the fibre of fenugreek has been implemented in preparation of various food items such as chapatti, chips and wafers, which

has further fortified the nutritional value of these food stuffs as reviewed by Srinivasan, (2006).

Due to presence of numerous bioactive components, fenugreek has been considered as an important source of medicinal values. Fenugreek seeds contain protein rich in lysine and tryptophan; it is a good source of phenol and flavonoids hence exhibit potential antioxidant activity (Randhir *et al.*, 2004). The supplementation of fenugreek powder resulted in enhancement of antioxidant status along with reduced lipid peroxidation phenomena (Madar, 1984, Anuradha and Ravikumar, 2001). Compounds such as saponins, coumarin, nicotinic acid, sapogenins, and trigonelline, present in fenugreek are thought to account for several therapeutic effects (Billaud and Adrian, 2001). Trigonelline and sapogenins present in fenugreek are reported show potential hypoglycemic effects. Thus, the most important documented medical use of fenugreek is to control blood sugar in both insulin-dependent and noninsulin-dependent diabetics (Sharma *et al.*, 1996; Bordia *et al.*, 1997). The steroidal saponins such as diosgenin, tigogenin and neotigogenin are claimed to inhibit cholesterol absorption as well as its synthesis, thus exerting potential role in prevention of arteriosclerosis. It has been clinically demonstrated that fenugreek consumption leads to significant reduction in human serum total cholesterol, triglycerides and LDL cholesterol (Sowmya and Rajyalakshmi, 1999; Prasana, 2000). Diosgenin extracted from fenugreek are used as a precursor molecule for the synthesis of steroidal hormones such as cortisone and progesterone. It has also been found to be potentially implemented in treatment of cancer (Aggarwal and Shishodia, 2006).

## **2.9 Mechanism of antioxidant action**

The word “antioxidant” has been an important topic of research and equally popular in modern society due to its health benefits. The dictionary definition of this term is rather straightforward: “a substance that opposes oxidation or inhibits reactions promoted by

oxygen or peroxides". A more biologically relevant definition of antioxidants would be "synthetic or natural substances that are capable of combating the damaging effects of oxidation in cell system" as reviewed by Huang *et al.*, (2005).

Early research based on the role of antioxidants in biology focused on their capability to prevent the oxidation of unsaturated fats, which is considered as major cause of rancidity (German, 1999). Accordingly, the antioxidant activity of a substance was determined simply by measuring the oxidation rate of fat kept in a closed container with oxygen. The possible mechanism of antioxidant action was first observed when it was recognized that a substance which gets oxidized itself possess antioxidative activity as reviewed by Lobo *et al.*, (2010). The exploration of the mechanism involved in the prevention of lipid peroxidation by vitamin E led to the identification of antioxidants as reducing agents which actually prevent oxidation by scavenging ROS (Wolf, 2005). The antioxidant molecule regulates the level of free radicals in a cell system. There are two principle mechanisms of action which have been proposed for the removal of free radicals by antioxidants (Rice Evans and Diplock, 1993). The first mode involves the chain breaking mechanism in which the primary antioxidant molecule donates an electron to the free radicals present in the system and stabilises them. The other mechanism involves removal of ROS/RNS initiators by quenching chain initiating catalyst. Antioxidants may also neutralise these free radicals by other mechanisms including metal ion chelation, co-antioxidants or regulation of gene expression (Krinsky, 1992).

The mode of action of antioxidant in the regulation of free radicals level is mainly of two types: enzymatic and non enzymatic. Accordingly, the antioxidants are classified into two groups: enzymatic and non enzymatic (Nimse and Pal, 2015). The enzymatic antioxidants include important enzymes such as catalase, peroxidase, superoxide dismutase and glutathione reductase. These antioxidants are primarily involved in the first line of defence i.e. suppression of formation of free radical in the cellular system. They also

minimize the levels of lipid hydro-peroxides and  $H_2O_2$ , hence play important role in the prevention of lipid peroxidation and further maintaining the integrity and function of the cell membranes. On the other hand, non enzymatic antioxidant system mainly includes vitamins and phenolic compounds. These are involved in the second line of defence that means they act as free radical scavenger and inhibit the chain initiation or disrupt the chain propagation reactions (Krinsky, 1992; Lobo *et al.*, 2010). There are other factors which play role of antioxidants such as proteolytic enzymes which include proteases and peptidases; they prevent the accumulation of oxidized proteins by degrading and removing from the cellular system (Niki, 1993).

### **2.10 Synthetic antioxidants**

The synthetic antioxidants are usually applied for preservation of food stuffs which are rich in lipids. They are chemically synthesized due to their unavailability in nature. The mode of action of both natural and synthetic antioxidants are similar, hence their efficiencies are difficult to compare. The activity of synthetic antioxidants depends on various factors such as their chemical structure, solubility, polarity as well as their redox potential (Pokorny, 2007). These synthetic antioxidants act in different forms namely, free radical terminators, chelating agents and singlet oxygen scavengers. The compound which acts as free radical terminator comprises of those which are implemented in food preservation due to their capability to prevent lipid peroxidation by termination of free radical chains. Some synthetic antioxidants which act as chain terminators are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and octyl gallate (OG). Those which are potential oxygen scavengers are glucose oxidase and ascorbyl palmitate, they function as reducing agents. The chelating agents, quercetin, tannins, phytates, and ethylene diaminetetracetic acid prevent lipid peroxidation by chelating the heavy metals such as iron and copper which are responsible for oxidation of lipids (Leopoldini *et al.*, 2006; Pokorny,

2007; Venkatesh and Sood, 2011 ). Synthetic antioxidants are more preferred over other natural antioxidants, because the synthetic forms are more liposoluble. Natural antioxidants are generally more polar than synthetic ones, except for carotenes. Therefore, natural antioxidants are not sufficiently soluble in the lipid phase, which limits their efficiency in prevention of peroxidation of bulk lipids. The synthetic antioxidants which are used in food are believed to exhibit similar effects as those used as dietary supplements in the biological systems (Evans, 1997; Brul and Coote, 1999). Though the synthetic antioxidants exhibit similar efficiency of that of the natural ones but due to their negative effects they are avoided. There are few synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG) currently permitted to be used as food preservatives. The amount of synthetic antioxidant permissible in food stuffs is limited to 0.02% of total antioxidants (Pokorny, 2007). The intake of synthetic antioxidants at recommended level may cause no harm to health but long term consumption might cause modification of acute toxicity of several carcinogenic and mutagenic chemicals thus leading to chronic adverse effects (Pokorny, 2007; Venkatesh and Sood, 2011).

### **2.11 Secondary metabolites as a rich source of antioxidant**

The plant metabolites are classified into two different categories namely primary metabolites and secondary metabolites. The substances produced through primary metabolic processes are called primary metabolites which include amino acids, sugars, fatty acids and nucleic acids. These primary metabolites are involved in the growth and maintenance of cells (Kliebenstein and Osbourn, 2012). On the other hand, secondary metabolites are derived from these primary metabolites through various biosynthetic pathways. These secondary metabolites are organic in nature and are indirectly involved in the growth and development of plants. They are basically involved in the defense mechanism of plant against various

adverse environmental conditions. They are capable of removing excess free radicals from the cellular system, help in modulating immune system along with killing of pathogenic microbes and preventing herbivory (Kasote *et al.*, 2015).

Till date, several thousand types of secondary metabolites have been reported to be identified and isolated in plant system. Due to their potential bioactivity such as antioxidant, antimicrobial and anti-carcinogenic property, they are used for pharmaceutical or agricultural purposes including manufacturing of natural antibiotics (Stone & Williams, 1992; Korkina, 2007).

Secondary metabolites are further divided into three large groups, they are the nitrogen containing alkaloids, and other two without nitrogen are terpenoids and phenolics (Patra *et al.*, 2013). Alkaloids are heterocyclic, nitrogen containing compound which are accumulated in near about 20% of plant species which mainly include indole, tropane and purine alkaloids having diverse clinical properties (Ziegler and Facchin, 2008). They usually possess potential bioactivity and are usually bitter in taste. The tropane alkaloids such as atropine and scopolamine present in *Atropa belladonna*, *Datura* spp and *Hyoscyamus niger* are reported to possess anticholinergic activity and are used to reduce smooth muscle spasms and pain. Many other such as vinblastine and vincristine has antitumor properties; quinine has antipyretics and antimalarial properties and reserpine is used to treat high blood pressure (Grynkiewicz and Gadzikowska, 2008; Reyburn *et al.*, 2009). However, under *in vitro* antioxidant measurement assay conditions, the radical scavenging potential of alkaloids is reportedly moderate to nonexistent.

Terpenoids comprise another large family of secondary metabolites, consisting of over 40,000 different compounds (Aharoni *et al.*, 2005). Monoterpenes, sesquiterpenes and diterpenes have been found to possess notable antioxidant activity in different *in vitro* assays. However, most of these activities have no physiological relevance (Baratta *et al.*, 1998). Tetraterpenes and carotenoids have been shown to possess potent antioxidant activity

within both *in vivo* and *in vitro* studies (Palozza and Krinsky, 1992). Among all secondary metabolites, phenolic antioxidants appear to be the most important due to their promising antioxidant activity in both *in vivo* and *in vitro* systems. Plant phenolics are generally classified into five groups which comprises of phenolic acids, flavonoids, lignans, stilbenes and tannins (Blokhina *et al.*, 2003). Phenolic compounds generally contain one or more aromatic rings along with one or more hydroxyl groups with them. It is assumed that the degree of antioxidant property of phenolics depends upon the number of free hydroxyls present and conjugation of side chains to the aromatic rings of the molecule (Morgan *et al.*, 1997). The major phenolic compounds; flavonoids and phenolic acids are biosynthetically derived from amino acids phenylalanine or tyrosine via Shikimate pathway (Dewick, 2009). The compounds belonging to these two classes of secondary metabolites are found to exhibit best antioxidant activity. The stable delocalization system in flavonoid comprises of aromatic and heterocyclic rings as well as unsaturated bonds helps to delocalize the free radicals present in cellular system. Various flavonoids such as quercetin and catechins are reported to be better antioxidants than other the antioxidant components such as vitamin C and vitamin E (Rice-Evans *et al.*, 1997). Flavonoids and phenylpropanoids are also found to act as H<sub>2</sub>O<sub>2</sub> scavengers by being oxidized by peroxidase enzymes (Sakihama *et al.*, 2002; Michalak, 2006). Under experimental conditions, the antioxidant property of the plant phenolics is usually attributed to ability to donate electrons, reducing power and chelating ability of heavy metal ions (Rice-Evans *et al.*, 1997). As their defence strategy, plants synthesize several forms of secondary metabolites that play important roles in ROS metabolism and prevent oxidative damages. These metabolites are also found to play important role in providing plants' adaptability to environmental fluctuations. Secondary metabolites provide both passive and active resistance to living beings. In passive resistance, these metabolites are readily available and help the concerned molecules to fight against

stress whereas in active resistance, metabolites itself are produced in higher amount in response to specific stressors (Korkina, 2007).

However, the secondary metabolites have numerous scientifically proven pharmaceutical properties for human health as well as for defense mechanism for but many of these effects are still unknown which are yet to be investigated.

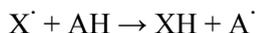
## **2.12 Assessment of antioxidant activity**

The terms “antioxidant activity” and “antioxidant capacity” indicates different meanings; the antioxidant activity indicates the kinetics of a reaction involving an antioxidant and the free radical it reduces or scavenges, while antioxidant capacity is the measurement of efficiency of an antioxidant in the thermodynamic conversion of an oxidant. The antioxidant activity or capacity of antioxidant molecules depend on its chemical nature and free radicals, concentration of free radicals as well as their sources. Therefore, evaluation of single assay is not sufficient for the proper determination of antioxidant potential of an extract because the mode of action of different antioxidants varies (Pokorny, 2007; Apak *et al.*, 2013).

The major factors that form the basis for the selection of more reliable method are biological relevance as well as method of quantification. The antioxidant capacity estimated by any of the assay of *in vitro* method should closely reflect during *in vivo* system analysis also. A good method should be capable of assessing different types of antioxidants or their mixture and should provide an accurate value. Furthermore, a good method should be able to distinguish the antioxidant compounds with different reaction kinetics (Prior *et al.*, 2005).

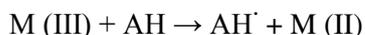
Several *in vitro* assays and methods have been introduced for the measurement of antioxidant potential of bioactive components either as pure compound or in extract form. A basic classification of antioxidant assays is done on the basis of the mode of action of antioxidants and accordingly they are classified in two major categories namely, hydrogen atom transfer (HAT) based assay and electron transfer (ET) based assay (Chanda and Dave, 2009).

The hydrogen atom transfer based assays determine the capability of a compound to scavenge free radicals by the mechanism involving hydrogen atom donation to the unstable free radicals. The hydrogen atom transfer based assays are usually based upon following mode of reaction:



The HAT based reactions are usually rapid and complete within few seconds or minutes, also they are solvent and pH independent (Prior *et al.*, 2005). Some of the assays which involve HAT based reactions are Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and  $\beta$ -carotene bleaching assay (Huang *et al.*, 2005).

The electron transfer based assays determine the potential of an antioxidant compound to reduce any unstable compounds including metals, carbonyls, and radicals by transfer of single electron. The electron transfer based assays are usually based upon following mode of reaction:



The ET based reactions are usually slow and takes place for long duration, consequently measurement of antioxidant capacity is based on percent decrease in product rather than kinetics. Additionally, these reactions are pH dependent (Sartor *et al.*, 1999). The assays which involve ET based reactions are trolox equivalent antioxidant capacity (TEAC),  $\alpha, \alpha$ -diphenyl- $\beta$ -picryl-hydrazyl radical scavenging assay (DPPH), Ferric reducing antioxidant power (FRAP), Superoxide anion radical scavenging assay, Nitric oxide radical scavenging assay, Hydroxyl radical scavenging assay and total phenol assay (Huang *et al.*, 2005). Some of the assays implemented for the determination of antioxidant potential of a sample are discussed here.

DPPH is a simplest and stable free radical that possesses the capacity to accept an electron to attain a stable diamagnetic molecule. The purple chromogenic solution of DPPH

radical is reduced by antioxidant thus changing the colour to the corresponding yellowish hydrazine. The DPPH solution loses its original colour when the concentration of antioxidant compound is increased in the reaction mixture (Calliste *et al.*, 2001; Calliste *et al.*, 2010). The scavenging of the DPPH radicals is monitored spectrophotometrically by recording the decrease in absorbance at 517 nm. Since DPPH is soluble in organic solvent (methanol), therefore the evaluation of hydrophilic antioxidant is not possible by this method.

Another important method of determining the antioxidant activity involves scavenging of another free radical 2, 2'-azinobis (3-ethyl-benzothiazoline 6- sulfonate (ABTS) which is water as well as organic soluble. In this method the cation ABTS<sup>•+</sup> radical is generated by oxidation of ABTS by reacting with persulfate overnight. The resultant bluish green chromogenic solution is reacted with the test sample for estimating its antioxidant potential. A decrease of the intensity of chromogenic solution as well as ABTS<sup>•+</sup> concentration is said to be linearly dependent on the concentration of antioxidant (Stratil *et al.*, 2006). Although wavelengths such as 415 and 645 nm have been used for recording the change in absorbance of reaction mixture (Prior *et al.*, 2005), but the wavelength of 734 nm has been generally preferred due to minimal interference of plant pigments. This method can be implemented for evaluating the antioxidant potential of both lipophilic and hydrophilic compounds.

Another assay that is used for the measurement of antioxidant activity is ferric reducing antioxidant power (FRAP); this reaction measures the reduction of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) to a colored product at wavelength of 700 nm (Benzie and Strain, 1996). The reaction detects compounds with redox potentials so this assay is a reasonable method for determining the redox status in cellular system. The reducing power is considered to be associated with the extent of hydroxylation and conjugation in phenolic components (Pulido *et al.*, 2000). The FRAP mechanism is totally based on electron transfer,

so in combination with other assays it can be useful in distinguishing dominant mechanisms with different antioxidants (Cao *et al.*, 1997).

For classical antioxidant activity, an assay based on a HAT is preferred over ET mechanism because of the predominant presence of peroxy radical in lipid oxidation of biological systems. However, it is also important to perform the assays for other radical sources such as peroxynitrite, superoxide, and hydroxyl because they are equally active in the biological system (Prior *et al.*, 2005; Salazar *et al.*, 2008).

Some of the problems often overlooked during these analytic studies included sample homogeneity, identification of critical control points, extraction efficiency, and failure to adhere to better control procedures. Other factors which should be taken under consideration for better results are critical handling, identification of interferences and solution for their elimination, storage and statistical analysis (Prior *et al.*, 2005).

### **2.13 Relation between antioxidants and antidiabetic activity**

Diabetes mellitus has been ranked as the fourth most common disease responsible for mortality. According to the International Diabetes Federation, the increase in the number of diabetic patients is so rapid that the figure may reach up to 552 million around the world by the year 2030 (Whiting *et al.*, 2011). It is reported that most of the diabetic disorders are characterized by hyperglycemia, pathway-selective insulin resistance, lack of insulin action, atherosclerosis and development of diabetes-specific pathology in renal glomerulus and peripheral nerve (Giacco and Brownlee, 2010).

Hyperglycemia causes tissue damage complications due to activation of five major mechanisms: Increased polyol pathway flux of glucose and other sugars, increase in the intracellular production of advanced glycation end-products (AGEs), enhancement in the expression of the AGEs receptor and its activating ligands, over expression of protein kinase C (PKC) isoforms and the hexosamine pathway. Several reports have suggested that all

these five mechanisms are activated by overproduction of reactive oxygen species specially superoxide by mitochondrial electron transport chain (Du *et al.*, 2000; Nishikawa *et al.*, 2000). Superoxide is the initial reactive oxygen species formed by mitochondria, which is further converted to other more reactive free radicals that can cause cellular damages in various ways (Wallace, 1992).

During electron transfer through Complexes I, III and IV normally the protons are pumped outwards into intermembrane space, generating a proton gradient that further drives ATP synthase (Complex V). But during diabetic condition due to high intracellular glucose concentration, more glucose-derived pyruvate is being oxidized in the TCA cycle, eventually increasing influx of electron donors (NADH and FADH<sub>2</sub>) into electron transport chain. Consequently, the voltage gradient across the mitochondrial membrane increases until a critical threshold is attained. At this stage, electron transfer inside complex III is blocked, compelling the electrons to back up to coenzyme Q, which generates superoxide by donating the electrons to molecular oxygen (Trumpower, 1990).

The major antioxidant enzymes that regulate the process of removal of free radicals are superoxide dismutase (SOD), glutathione peroxidase and catalase. When overproduction of ROS or chronic hyperglycemia occurs, the activity of these enzymes is found to be suppressed thus leading activation of oxidative stress pathways (Ceriello, 2006). This indicates that the antioxidant enzyme system plays vital role in prevention of oxidative stress mediated diabetic complications.

The mitochondrial superoxide dismutase converts this oxygen free radical to H<sub>2</sub>O<sub>2</sub>, which is further converted to H<sub>2</sub>O and O<sub>2</sub> by the action of other enzymes, peroxidase or catalase (Johansen *et al.*, 2005; Shen *et al.*, 2006). GSH peroxidase which is located in the mitochondria catalyzes the degradation process of H<sub>2</sub>O<sub>2</sub> by reduction, where two reduced glutathione (GSH) molecules are oxidized to glutathione disulfide (GSSG). Catalase, another antioxidant enzyme localized in peroxisomes, detoxifies H<sub>2</sub>O<sub>2</sub> that diffuses to the

cytosol from the mitochondria, by converting it into water and molecular oxygen (Johansen *et al.*, 2005). In the diabetic heart, over expression of MnSOD or catalase has been observed which is believed to protect cardiac mitochondria from oxidative damage and improves respiration (Ye *et al.*, 2004; Shen *et al.*, 2006).

Besides, antioxidant enzymes there are non enzymatic antioxidant such as vitamin A, C, E and polyphenols which help in removal of these free radicals causing diabetic disorders (Evans *et al.*, 2002). Vitamin C plays a vital role in scavenging of ROS and RNS by being oxidized itself. The oxidized forms of vitamin C namely, ascorbic radical and dehydroascorbic radical are regenerated by glutathione, NADH or NADPH. In addition, vitamin C is capable of reducing the oxidized antioxidant compounds such as vitamin E and glutathione (Garcia-Bailo *et al.*, 2011). Vitamin E is a fat-soluble antioxidant component which scavenges the lipid hydroperoxides thus preventing the membrane damages. Furthermore, together with vitamin C it helps in regeneration of glutathione by interacting with lipoic acid (Evans *et al.*, 2002). Since oxidative stress is responsible for the progression of diabetes and its complications, amelioration of oxidative status, by antioxidant non-enzymatic defences has been proposed by several authors. Several clinical observational trials have particularly studied and also proved the correlation between vitamin E status in plasma and/or diet and type 2 diabetes incidence as well as diabetic complications (Mayer-Davis *et al.*, 2002).

Numerous conventional drugs are reported to be used for the management of hyperglycemia; however, most of them were found to be unsuccessful in providing long-term solution. Some of the synthetic antidiabetic components such as metformin, sulfonylureas, biguanides and insulin have found to be used in diabetes treatment but moreover they have serious adverse side effects (Patel *et al.*, 2012). Among the known bioactive compounds and phytochemicals, polyphenols have gained much attention because of their potential hypoglycaemic property with minimal side effects (Bahadoran *et al.*,

2013). The beneficial health effects of the polyphenols have led to an upsurge interest in these natural compounds among the researchers during the past decade. Several investigations and clinical trials have successfully demonstrated the potential role of plant polyphenols in diabetes management (Pandey and Rizvi, 2009).

The rising trend in the prevalence of diabetes and its complications all over the world suggests that the available medical treatments are not sufficient enough; therefore, the implementation of supplementary or complimentary treatments in the form of functional foods and their nutraceuticals might provide significant enhancement in the diabetic management strategies (Sabu and Kuttan, 2002; Tag *et al.*, 2012).

#### **2.14 Salinity and its effects on plant system**

Salinity is considered as one of the major factor affecting the crop production throughout the world. Salinity either in water or soil represents one of the major abiotic stresses especially in arid and semi arid regions, which can severely limit the agricultural production (Shanon, 1998). High concentration of salt creates ion imbalance and hyperosmotic stress in plant system which consequently leads to oxidative damages. Such drastic changes in plant system cause retardation of growth, molecular damages, membrane disruption and even death. For the plant to be tolerant to salinity stress: their homeostasis must be re-established along with detoxification mechanism must be boosted parallely (Zhu, 2001).

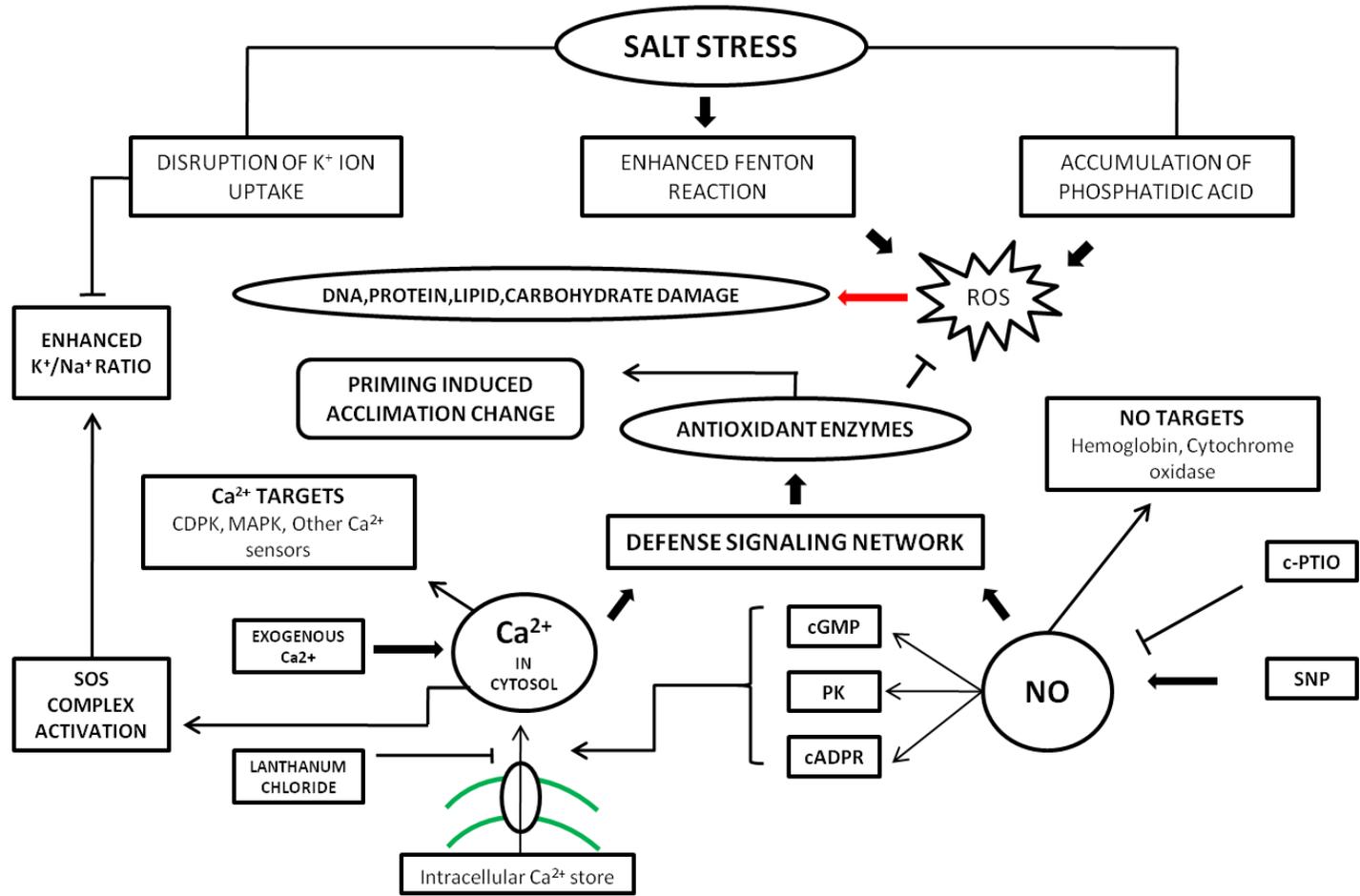
The two major consequences of salinity on plant system are osmotic stress and ionic toxicity; these physical conditions affect all other physiological, biochemical and developmental processes in plants (Yadav *et al.*, 2011). High salt content in the substratum creates rise in osmotic pressure of the substratum thus, affecting the water uptake capacity of plants. Furthermore, decrease in the turgor pressure of the plant cells cause closing of stomata which leads to reduced carbon fixation but increase in ROS production. These highly reactive and unstable free radicals disrupt various cellular processes by damaging the

major biomolecules like lipids, proteins, and nucleic acids (Parida and Das, 2005). Ionic toxicity is the physiological state in which the equilibrium of ions is disturbed which causes perturbation in cellular metabolism and processes. High concentration of sodium ions at the surface of the root disrupts plant nutrition by inhibiting both  $K^+$  uptake and enzymatic activities within the cell (Aslam *et al.*, 2011). Potassium is an important nutrient which regulates huge number of enzymes activities associated with various major pathways (Kader and Lindberg, 2010); on the other hand, sodium ions inhibit the activity of enzymes.  $Na^+$  is a cation almost similar to  $K^+$ , for this reason  $Na^+$  can cross the cell membrane without much disturbance (Parida and Das, 2005). As suggested by Rodriguez-Navarro, (2000) optimum concentration of  $K^+$  required is 100-200mM in the cytosol and the concentration of cytosolic  $Na^+$  excess of 10mM creates stress environment in the system. The oxidative stress induced by salinity can retard plant growth as major part of energy is wasted on conserving water and improving ionic balance (Kader and Lindberg, 2010).

Nitric oxide and calcium both are considered as highly versatile signalling molecules. Various literatures have reported the significant involvement of both of these molecules in wide range of physiological and developmental processes in plants. Additionally, these molecules have found to mitigate the adverse effect of varied environmental stresses including salinity which has been schematically depicted in Figure 2.5.

### **2.15 NO treatment and its effect on the therapeutic potential and oxidative stress management in plant system under salinity stress**

Nitric oxide as a bioactive molecule has been known to exhibit both pro-oxidant as well as antioxidant property in plants (Beligni and Lamattina, 2002; Delledonne *et al.*, 2002). Nitric oxide induces a set of plant defense genes, which includes the two important enzymes of phenylpropanoid pathway namely, phenylalanine ammonia lyase and chalcone synthase



**Figure 2.5:** Interplay of Nitric oxide and Calcium ion and their role in alleviation of oxidative stress mediated damages under salinity stress

(Crawford and Guo, 2005). Since, secondary metabolites have both nutritional and physiological significance; various strategies have been introduced by several workers for the enhancement of secondary metabolite production. Considering the above facts, NO oxide has been successfully implemented by various researchers in the elicitation of secondary metabolites in many plants. The adventitious roots of *Echinacea purpurea* when treated with sodium nitroprusside (SNP) at concentration of 100  $\mu$ M exhibited enhancement in the accumulation of caffeic acid derivatives, flavonoids and phenolics. Additionally, NO treatment induced the antioxidant defense enzymes along with decrease in free radicals and lipid peroxidation (Wu *et al.*, 2007). Later, El-Beltagi *et al.* (2015) treated the cell suspension culture of *Gingko biloba* with various concentrations of SNP, a potent NO donor; the content of secondary metabolites along with other parameters were analysed at time interval of 12h, 48h and 72h after NO treatments. It was found that the 500 $\mu$ M SNP treatment resulted in significant increase in the production of secondary metabolite contents which included saponins, phenolics and flavonoids. Further, Manivannan *et al.*, (2016) studied the effect of different elicitors including methyl jasmonate, salicylic acid and SNP on the cell suspension culture of *Scrophularia kakudensis*. The study demonstrated that along with other elicitors applied SNP treatment resulted in increased production of phenols and flavonoids in the cell suspension culture of *Scrophularia kakudensis* which is considered as an important medicinal plant.

Furthermore, numerous evidences have been provided regarding the active involvement of NO in alleviation of salinity stress in plant system. Most of the cellular damages caused by salinity are usually associated with ROS mediated oxidative stress. NO is said to possess considerable capacity to regulate oxidative stress mediated damages along with the level and toxicity of ROS.

The properties of NO which makes it capable to exert a protective function against oxidative stress mediated damages as suggested by Yadav (2010) are given below:

- i. It reacts with lipid radicals and stops the propagation of lipid oxidation.
- ii. Scavenging the superoxide anion and formation of peroxynitrite which is toxic for plant, later neutralized by ascorbate and glutathione.
- iii. Involvement in the activation of antioxidant enzymes.

The lupin seeds when subjected to SNP treatment showed better germination under saline stress as well as heavy metal stress (lead and cadmium) suggesting involvement of NO in auxin signalling pathway (Kopyra and Gowdz, 2003). Later, Zheng *et al.* (2009) demonstrated that pre-soaking of wheat with SNP for 20h prior to germination resulted in increased germination rate, radicle weight under 300mM NaCl. Additionally, decrease in Na<sup>+</sup> concentration but increase in K<sup>+</sup> concentration in the seeds were observed thereby indicating role of NO in maintaining a balance between K<sup>+</sup> and Na<sup>+</sup> during germination under salt stress. A significant enhancement in the activity of antioxidant enzymes was observed accompanied with reduced level of malondialdehyde (MDA), H<sub>2</sub>O<sub>2</sub> and superoxide anions. The pre-treatment of citrus root with exogenous SNP for a duration of 48h exhibited induction of primary antioxidant responses in the leaves of citrus subjected to salinity stress. The study revealed that SNP pre-treatment enhanced the activity of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) also prevented the NaCl-dependent protein oxidation (Tanou *et al.*, 2009). Zheng *et al.* (2009) claimed in their study that NO treatment effectively contributed to better accumulation of ferritin, a protein active in chelation of excess of ferrous ion present in the cellular system of barley plant subjected to salt stress. Exogenous application of NO through different modes have reported to be effective in regulating the functioning of photosynthetic pigments (Ruan *et al.*, 2002), improving salt tolerance by modulating proton pump activity in maize (Zhang *et al.*, 2006), regulating osmoregulation and proline metabolism in tobacco (Ke *et al.*, 2013) and prevention of mitochondria oxidative damage (Zheng *et al.*, 2009). Furthermore, various evidences have been provided

by research workers about the protective effect of NO during other stress conditions besides salinity; alleviating the negative effects of UV radiations in wheat seedlings (Yang *et al.*, 2013); mitigating the oxidative injuries under heavy metal stress in lupin seeds (Kopyra and Gowdz, 2003); modulating the metabolism of biochemicals during osmotic stress (Ke *et al.*, 2013). Other beneficial effects of NO donor reported are regulation of seed germination in *Senna macranthera* (da Silva *et al.*, 2015); maintenance of optimum Na<sup>+</sup>/K<sup>+</sup> ratio in cotton seedlings (Dong *et al.*, 2014); enhancement in the enzymes involved in nitrogen metabolism namely nitrate reductase and nitrite reductase in tomato (Manai *et al.*, 2014), also reduction of lipid peroxidation, hydrogen peroxide and superoxide anions; elevation in the activity of major antioxidant enzymes accompanied with increase in the accumulation of biochemicals such as proline, glutathione and sugars under salinity stress in numerous plant system (Hayat *et al.*, 2012; Dong *et al.*, 2014; da Silva *et al.*, 2015; Hameed *et al.*, 2015; Ahmad *et al.*, 2016).

#### **2.16 Application of Calcium and its effect on the therapeutic potential and oxidative stress management in plant system under salinity stress**

Calcium is considered as multifunctional element in plants besides as a nutrient, it is involved in several physiological processes like maintenance of membrane integrity, cell wall structure, regulating the activity of key enzymes and phytohormones interaction (Barker and Pilbeam, 2007). Additionally, it plays vital role in signalling network as a secondary messenger under varied environmental conditions (Tuteja, 2009; Batistic and Kudla, 2012). By virtue of this property it is capable of ameliorating the adverse effects of abiotic stresses including chilling, thermal, drought, heavy metals and salinity (Ma *et al.*, 2005; Shao *et al.*, 2008; Siddiqui *et al.*, 2011; Zehra *et al.*, 2012).

Many authors have suggested the beneficial role of calcium ion in the alleviation of the adverse effects of abiotic stress conditions. Therefore, the maintenance of optimum

supply of calcium in saline soil is considered as an important factor in preventing the severity of specific ion toxicities, in those crops which are susceptible to salinity stress injury (Grattan and Grieve, 1999). In their study Hasegawa *et al.*, (2000) have suggested that during salt stress plants are able to tolerate high saline concentration by inducing the signal transduction cascade involving calcium ion. Thus, when exposed to stress conditions including salinity, plants increase the cytosolic  $\text{Ca}^{2+}$  accumulation to combat the oxidative damages. Although the basic mechanism involved has remained unexplained still now, prevailing models for  $\text{Ca}^{2+}$  functioning include both membrane stabilisation and signalling significance. Considering the potential role of calcium ion in overcoming the negative impacts of several stresses, it has been implemented in various modes in order to provide stress tolerance to plants. Jaleel *et al.*, (2007) demonstrated that when *Catharanthus roseus* plants were supplemented with calcium chloride under drought condition, calcium ion provided osmoprotection to the plants along with increase in glycine betaine accumulation and indole alkaloid content in the shoot and roots of the plant. Also, a significant enhancement in the activity of antioxidant enzymes namely superoxide dismutase, catalase and peroxidase was reported in the same plant subjected to salinity stress (Jaleel *et al.*, 2007). According to Khan *et al.*, (2010) when calcium chloride was applied to linseed in combination with gibberellic acid proved more effective in ameliorating the negative effects of NaCl stress. It was found that the electrolyte leakage of membranes was reduced considerably with decrease in the accumulation of lipid peroxides and hydrogen peroxide. Later Sharma and Dhanda, (2015) suggested the protective role of calcium chloride treatment in *Vigna radiata* in which it was found that the presence of calcium ion helped in maintenance of photosynthetic pigments under salt stress. Similarly, calcium was found to maintain the rate of photosynthesis in *Zoysia japonica* under drought stress by reducing the damage of photosynthetic pigment (Xu *et al.*, 2013); increasing the germination rate and growth of forest trees under simulated acid rain (Liu *et al.*, 2011); involvement in the

enhancement of chilling stress in *Stylosanthes guianensis* by interacting with abscisic acid (Zhou and Ghou, 2009); the application of calcium in the culture medium was found to activate the accumulation of flavonol in *Polygonum hydropiper* (Nakao *et al.*, 1999); also increase in the activity of antioxidant enzymes, regulation of biochemical metabolism and maintenance of membrane integrity by calcium has been reported in plant system under various stress conditions (Jaleel *et al.*, 2007; Zhou and Ghou, 2009; Khan *et al.*, 2010; Xu *et al.*, 2013; Sharma and Dhanda, 2015). Besides these, calcium chloride treatment of fruits and vegetables has resulted in significant increase in their shelf life. The application of calcium chloride reduced post harvest decay in apples, strawberries, oranges and pineapples; increased vitamin C content and also improved the firmness and quality of fruits (Sams *et al.*, 1993; Garcia *et al.*, 1996; Vilas Boas *et al.*, 1998, Gonclaves, 2000). Furthermore, it has been reported that calcium helps in reducing sporulation and growth of pathogens in fruits and vegetables (Conway *et al.*, 1994).

### **2.17 Polyamines and their role in the physiology, antioxidant and biochemical attributes of plant system under salinity stress**

Polyamines are considered as a new class of growth regulators which includes spermidine: a triamine; spermine: a tetramine and their obligate precursor putrescine: a diamine, which are actively involved in the regulation of development and physiological processes in plants (Kusano *et al.*, 2007). These are small ubiquitous polycations involved in the processes associated with the growth and development of plant and are well known for exhibiting anti-stress effects attributed to their acid neutralizing and antioxidant properties, as well as membrane and cell wall stabilizing abilities (Liu *et al.*, 2007; Zhao and Yang, 2008). Furthermore, it has been suggested that these factors play important role in controlling the defense responses of plants towards diverse environmental stresses, such as heavy metal toxicity, drought, chilling stress, and salinity (Gill and Tuteja, 2010). The simplicity in their

structure, their universal distribution in the cellular compartments, and active involvement in physiological activities ranging from structural stabilization of major macromolecules to cellular membranes prove them to be an attractive group of metabolites to regulate a multitude of biological functions. The accumulation of polyamines in the cell is reported to sequester extra nitrogen thus minimizing ammonia toxicity and also helps in balancing the total nitrogen distribution into the concerned pathways (Moschou *et al.*, 2012; Guo *et al.*, 2014).

In their review Minocha *et al.* (2014) have precisely summarized the various probable roles of polyamines in providing the tolerance to plants against stress conditions. These include: (i) serving as compatible osmolytes along with proline, glycinebetaine and GABA; (ii) interactions with macromolecules like DNA, RNA, and cellular membranes and their stabilization; (iii) scavenging of free radicals along with enhancement in the production of antioxidant enzymes and metabolites; (iv) acting as signal molecules in ABA-regulated stress response pathway; (v) regulations of ion channels; (vi) involvement in programmed cell death (Alcazar *et al.*, 2006; Alet *et al.*, 2011; Hussain *et al.*, 2011; Gupta *et al.*, 2013; Shi and Chan, 2014). Exogenous application of polyamines in several plants has reported beneficial effects of these metabolites in regulating the stress responses under wide range of environmental conditions. In their study, Tang and Newton, (2005) have demonstrated the effect of application of putrescine, spermine and spermidine on the callus culture and plantlets of Virginia pine under salt stress. The study revealed that among the three polyamines, putrescine was found to enhance the activity of antioxidant enzymes, inhibiting the activity of acid phosphatase along with considerable reduction in lipid peroxidation of plant tissues. Likewise, exogenous application of spermidine for 7 days in rice plant increased the yield, calcium content and resulted in higher  $K^+/Na^+$  ratio than control plants under saline conditions (Saleethong *et al.*, 2013). Furthermore, foliar spray of putrescine in wheat under drought stress significantly increased the photosynthetic attributes, and

accumulation of essential biochemicals thus resulting in stress tolerance and increase in yield as reported by Gupta *et al.* (2012). Similarly, several authors have claimed the involvement of these polyamines in boosting the tolerance level of numerous types of plants under varied stress conditions (Wimalasekera *et al.*, 2011; Shi *et al.*, 2013; Tanou *et al.*, 2014)

The available literature reviewed above revealed that *Trigonella foenum-graecum* is a plant of great medicinal value which has been serving the mankind since ancient times. Being rich source of bioactive components makes it better alternative for the treatment of wide spectrum of disease and disorders in human beings, thus, avoiding the synthetic sources and their side effects. Though almost all the plant parts of fenugreek have been reported to possess potential therapeutic value however, works on the sprouts of fenugreek has been yet to be updated. Inclusion of sprouts in the daily diet is believed be more beneficial due to the loss of anti nutritive factors during the process of germination and also the metabolites are usually stored in simpler form which are easily digestible. Several techniques and strategies have been introduced by the researchers regarding the enhancement in the bioactive components of various plants among which the priming technique has been considered as one of the effective strategy. Calcium ion and nitric oxide have not only been implemented for the enhancement of these bioactive components but, these signalling molecules have also been successfully used in the development of stress tolerance in plants under wide range of environmental stresses. Therefore, this study have been carried out to explore the role of these two important signalling molecules in enhancing the stress tolerance level of fenugreek towards salt stress along with improvement in the therapeutic properties of the fenugreek in sprouting stage through priming technique.

## **CHAPTER - 3**

### **ALTERATION IN FREE RADICAL SCAVENGING ACTIVITY OF FENUGREEK SPROUTS BY PRIMING WITH NITRIC OXIDE AND CALCIUM ELICITORS**

### 3.1 INTRODUCTION

Several pharmacological as well as experimental studies have suggested that consumption of foods rich in antioxidant is significantly associated with reduced risk of various disorders and human diseases, including diabetes (Arts and Hollman, 2005). Some of the food types such as fruits, vegetables, sprouts as well as herbal drugs have been found to be very rich in bioactive compounds such as polyphenols, vitamins C and E,  $\beta$ -carotene etc, which possess potential antioxidant activity. Therefore, in recent times the regular consumption of sprouts or germinated seeds, fruits and vegetables, is highly recommended as they are considered to provide long term health benefits (Randhir *et al.*, 2004; Gupta *et al.*, 2014)

Free radicals or reactive oxygen species, such as superoxide anion ( $O^{2-}$ ), hydroxyl radical ( $OH^{\cdot}$ ) and peroxy radical ( $ROO^{\cdot}$ ), are highly reactive and are known to reduce the concentration of molecular oxygen in the cell and creates oxidative stress in the cellular system (Williams and Jeffrey, 2000). They are an integral part of normal physiology of a biological system, but an over production of these free radicals cause significant imbalance in the functioning of cellular antioxidant defence system and free radical formation which leads to stressful condition (Bramley 2000; Wang 2003; Cavas and Yurdakoc 2005). Since, these free radicals are highly reactive, they readily react with the essential biomolecules such as nucleic acids, proteins and membrane lipid and disturb their normal state, thus, triggering a series of disease and disorders both in plant and animal system (Halliwell *et al.*, 1992). Free radical-mediated oxidative stress is believed to be the primary cause of many disorders in human, such as cardiovascular diseases, Alzheimer's disease, Parkinson's disease, cataract, diabetes mellitus, atherosclerosis, cancer and ageing (Halliwell, 2000; Metodiewa and Koska, 2000; Young and Woodside, 2001; Heinecke, 2003). In the treatment of these diseases, antioxidant therapy has gained utmost importance in the recent years (Enayde *et al.*, 2006).

The level of free radicals and their negative effects can be mitigated by either natural or synthetic antioxidants (Larson, 1995; Gazzani *et al.*, 1998; Velioglu *et al.*, 1998). The utilization of synthetic antioxidants is negatively perceived by nutritionists and consumers due to safety and health concern (Sultana *et al.*, 2007), leading to increased interest in natural sources (Rafat *et al.*, 2011). Antioxidant compounds play vital role in protecting cells against destructive chemical compounds such as free radicals and reactive oxygen species (ROS) that are continuously produced during cell metabolism and their concentration increases under stress conditions (Hassimotto *et al.*, 2005).

Antioxidant compounds are capable of mitigating the negative effects of oxidative stress as they are efficient scavengers of the free radicals. The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries for the presence of potential antioxidant components (Ramma *et al.*, 2002). The fruits and vegetables are considered to be a good source of natural antioxidants and have been found to be beneficial for protection against the diseases related to oxidative stress (Chinnici *et al.*, 2004). Flavonoids and polyphenols exist widely in plants and are considered as important dietary antioxidants, which are responsible for the prevention of oxidative damage in biological system (Hassimotto *et al.*, 2005; Andarwulan *et al.*, 2010) which will be discussed in next chapter.

*Trigonella foenum-graecum* commonly known as fenugreek, being rich in antioxidants and phytochemicals has been traditionally used as a food, forage and medicinal plant (Petropoulos, 2002; Basch *et al.*, 2003). The pharmacological and folkloric uses of different plant parts of fenugreek have been reported by different researchers. Its seeds have been reported to have anti-diabetic (Xue *et al.*, 2007; Karim *et al.*, 2011), anti-cancerous (Shabbeer *et al.*, 2009), anti-inflammatory (Subhashini *et al.*, 2011) and antioxidant activity (Kaviarasan *et al.*, 2007). Its leaves have been reported to possess potential antibacterial activity (Yasmin *et al.*, 2009), anti-diabetic (Mitra and Bhattacharya, 2006) and antioxidant

property (Gowda *et al.*, 2010). Randhir *et al.* (2004) have also reported about the presence of potential antioxidant activity in the sprouts of fenugreek.

In recent times seed sprouting is gaining more significance commercially because it enhances the nutritional value of the seeds. A large number of chemical modifications occur to mobilize the stored carbohydrates and protein reserve into the germinating sprout (Chang and Harrold, 1988). Sprouting also removes some anti-nutritive factors such as enzyme inhibitors from the seed that make sprouts safe for consumption. Sprouting in fenugreek is known to enhance its soluble protein and fibre content along with reduced phytic, tannic acid and trypsin inhibitors (Mansour and El-Adawy, 1994).

Nitric oxide is a short-lived bioactive molecule (Zhang *et al.*, 2006), which is considered to function as pro-oxidant as well as antioxidant in plants (Kopyra and Gwozdz, 2003). Nitric oxide molecule is now recognized as an important signalling molecule and reported to be involved in various key physiological processes like abiotic stress tolerance (Ruan *et al.*, 2002), plant defense mechanism (Delledonne *et al.*, 2002), germination (Sarath *et al.*, 2006), growth and development of plants (Pagnussat *et al.*, 2002) etc. Calcium ion ( $\text{Ca}^{2+}$ ) is another important secondary messenger and signalling molecule which is actively involved in various physiological and developmental processes (Lecourieux *et al.*, 2006; Dodd *et al.*, 2010).

However, the literature suggests that reports on the role of calcium ion as well as nitric oxide molecule and the effect of their exogenous application on the antioxidant activity of fenugreek sprouts during early developmental phases is not studied yet. Therefore, a study on the influence of calcium ion and nitric oxide priming on antioxidant activity may provide insight to their role in regulating the mechanism involved. Considering this fact, the present study was designed to investigate the effect of priming with exogenous sources of calcium ion and nitric oxide on the free radical scavenging activity of fenugreek sprouts.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Elicitation process and germination

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of sodium nitroprusside (SNP) as an exogenous source of nitric oxide; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CP), a nitric oxide scavenger; calcium chloride (CC) as an exogenous source of calcium ion; a calcium chelator: Ethylene glycol-bis(2-aminoethylether)-N,N,N',N, tetra acetic acid (EG); and Lanthanum chloride (LC): a calcium channel blocker. The concentration of different priming agents used were: 5mM, 10mM, 20mM, 40mM and 80mM of SNP; 100µM of CP; SNP 5mM + 100µM CP, 10mM + 100µM CP, 20mM + 100µM CP, 40mM + 100µM CP, 80mM + 100µM CP, 120mM + 100µM CP, 1mM, 2mM, 5mM and 10mM each of CC, EG and LC; 1mM CC + 500µM EG, 2mM CC + 500µM EG, 1mM CC + 500µM LC and 2mM CC + 500µM LC. For control set, seeds were primed with normal water and kept in a rotary shaker along with the treated seeds. After priming for 24 h, the seeds were washed thrice with sterile water moisture dried and kept in the seed germinator for germination for 72 h.

**Table: 3.1:** The different treatment composition and their unique abbreviation used in the present study

Treatment Composition	Abbreviation
Control	T0
SNP 10mM	Tn1
SNP 20mM	Tn2
SNP 40mM	Tn3
SNP 80mM	Tn4
SNP 120mM	Tn5
SNP 10mM + CP 100µM	Tn6
SNP 20mM + CP 100µM	Tn7
SNP 40mM + CP 100µM	Tn8

SNP 80mM + CP 100 $\mu$ M	Tn9
SNP 120mM + CP 100 $\mu$ M	Tn10
CP 100 $\mu$ M	Tn11
CC 1mM	Tc1
CC 2mM	Tc2
CC 5mM	Tc3
CC 10mM	Tc4
EG 1mM	Tc5
EG 2mM	Tc6
EG 5mM	Tc7
EG 10mM	Tc8
CC 1mM + 500 $\mu$ M EG	Tc9
CC 2mM + 500 $\mu$ M EG	Tc10
LC 1mM	Tc11
LC 2mM	Tc12
LC 5mM	Tc13
LC 10mM	Tc14
CC 1mM + 500 $\mu$ M LC	Tc15
CC 2mM + 500 $\mu$ M LC	Tc16

### 3.2.2 Preparation of methanolic extract

The fenugreek sprouts of 3 different stages: 24h, 48h & 72h were crushed in mortar-pestle and individually processed through soxhlet extraction apparatus with methanol for duration of 8h. The refluxed samples were separated from the residues by filtering through Whatman No. 1 filter paper and the extract was concentrated to a definite concentration of 1g/ml using a vacuum rotary evaporator at 50°C. The obtained methanolic extracts were stored in brown bottles and kept in refrigerator for further experimental analysis.

### 3.2.3 Animal material

Goat liver, required for anti-lipid peroxidation assay, was collected from slaughter house instantly after slay and the experiment was conducted within an hour of collection.

### 3.2.4 DPPH based free radical scavenging activity (DPPH)

The radical scavenging activity of the sprout extracts were measured by DPPH method (Blois, 1958). The reaction mixture contained 1.8 ml of 0.1mM DPPH and 0.2 ml of methanolic extracts. The reaction mixture was vortexed and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. A reaction mixture without test sample was considered as control.

Radical scavenging activity was expressed as percent inhibition from the given formula:

$$\text{Percentage inhibition of DPPH radical} = [(A_0 - A_1)/A_0] \times 100.$$

Where,  $A_0$ : absorbance of the control and  $A_1$ : absorbance of the extract or standard. Then percentage inhibitions were plotted against concentration and from the graph,  $IC_{50}$  value was calculated.  $IC_{50}$  value is the concentration of extract required for 50% scavenging of free radicals in a reaction mixture.

### 3.2.5 ABTS<sup>+</sup> radical cation(s) decolorization assay

The spectrophotometric analysis of ABTS<sup>+</sup> radical cation(s) scavenging activity was determined according to Re *et al.* (1999) method with some modification. This method is based on the ability of antioxidants to quench the ABTS<sup>+</sup> radical cation, a blue/green chromophore with characteristic absorption at 734 nm. The ABTS<sup>+</sup> was obtained by reacting 7 mM ABTS<sup>+</sup> radical cation(s) in H<sub>2</sub>O with 2.45 mM potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), stored in the dark at room temperature for 6 h. Before usage, the ABTS<sup>+</sup> solution was diluted to get an absorbance of  $0.750 \pm 0.025$  at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 ml of ABTS<sup>+</sup> solution was added to 1 ml of the methanolic extract. After 30 min,

percentage inhibition at 734 nm was calculated for each concentration, relative to a blank absorbance. Solvent blanks were run in each assay.

The ABTS<sup>+</sup> scavenging was calculated using the following formula:

$$\text{ABTS}^+ \text{ scavenging effect (\%)} = (1 - A_s/A_c) \times 100$$

Where  $A_c$  is the initial concentration of the ABTS<sup>+</sup> radical cation(s) and  $A_s$  is absorbance of the remaining concentration of ABTS<sup>+</sup> radical cation(s) in presence of the extract.

### **3.2.6 Determination of metal chelating activity**

The chelating activity of the extracts for ferrous ions was estimated according to the method of Dinis *et al.*, (1994) with slight modification. To 0.4 ml of sample extract, 1.6 ml of methanol was added and mixed with 0.04 ml of FeCl<sub>2</sub> (2 mM). After 30s of incubation, 0.8 ml of ferrozine (5 mM) was added. After 10 min at room temperature, the absorbance of the Fe<sup>2+</sup>-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe<sup>2+</sup> was calculated as

$$\text{Chelating rate (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where  $A_0$  was the absorbance of the control (blank, without extract) and  $A_1$  was the absorbance in presence of the extract.

### **3.2.7 Determination of reducing power activity**

The ferric reducing antioxidant power of methanolic extracts was determined by the standard method (Oyaizu, 1986). Different concentrations of 1 ml of methanolic extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (K<sub>3</sub>Fe(CN)<sub>6</sub>) (2.5 ml, 1%). The mixture was incubated at 50°C for 20 minutes. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 minutes at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water

(2.5 ml) and FeCl<sub>3</sub> (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using ultraviolet-visible spectrophotometer.

### **3.2.8 Determination of superoxide anions scavenging activity**

The superoxide anions generated by phenazine methosulphate (PMS) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), were detected by the reaction with nitro-blue tetrazolium (NBT) (Nishikimi *et al.*, 1972). Reaction mixture contained 1 ml sample extract, 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4) and 1 ml of NADH solution (936 µM). Finally, the reaction was initiated by addition of 100 µl PMS solution (120 µM) to the mixture. The reaction mixture was incubated for 5 min at 25° C for and absorbance was recorded at 560 nm. Percentage inhibition and IC<sub>50</sub> value was calculated.

### **3.2.9 β-Carotene-linoleate bleaching assay**

The antioxidant activity was evaluated based on the β- carotene bleaching protective method developed by Velioglu *et al.* (1998) β-Carotene (0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were transferred into a round bottomed flask. Chloroform was totally evaporated at room temperature under vacuum at reduced pressure using a rotary evaporator. After evaporation, 50 ml of distilled water was added to the mixture, and then shaken vigorously to form an emulsion. 2 ml of emulsion was then added to 0.2 ml of methanolic extract or methanol (as control) into test tubes and immediately placed in a water bath at 50°C. The absorbance was read at 30 min intervals for 2 h at 470 nm. Degradation rate (DR) was calculated according to first order kinetics, based on equation (Al-Saikhan *et al.*, 1995);

$$\ln a/b \times 1/t = DR_{\text{sample}} \text{ or } DR_{\text{standard}}$$

where  $\ln$  is natural log,  $a$  is the initial absorbance (470 nm) at time 0,  $b$  is the absorbance (470 nm) at 30, 60, 90 or 120 min and  $t$  is the initial absorbance (470 nm) at time 0.

Antioxidant activity (AA) was expressed as percent of inhibition relative to the control, using the following formula:

$$AA (\%) = \frac{DR_{control} - DR_{sample \text{ or standard}}}{DR_{control}} \times 100$$

### 3.2.10 Anti-lipid peroxidation assay

The anti-lipid peroxidation activity of the sample extracts was determined by the method suggested Bauchet and Barrier (1998) with slight modification using the goat liver homogenate. 0.1 ml of sample extract was reacted with 2.8 ml of 10% goat liver homogenate and 0.1ml of 50 mM hydrated ferrous sulphate. This mixture was incubated at 37°C for 30 min. 1 ml of reaction mixture was taken with 2 ml of reagent composed of 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) in acetic acid (50%) for terminating the reaction. Then the mixture was kept in a water bath for 1 hour at 100°C and then centrifuged at 10,000 rpm for 5 min. The OD value of the supernatant was taken at 535 nm. Anti-lipid peroxidation (ALP) % was calculated by using the following formula:

$$ALP \text{ percent} = \frac{\text{Abs. of Fe}^{2+} \text{ induced peroxidation} - \text{abs. of sample}}{\text{Abs. of Fe}^{2+} \text{ induced peroxidation} - \text{abs. of control}} \times 100.$$

### 3.2.11 Statistical analysis

The data were pooled in triplicate and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes of fenugreek sprouts of different experimental set. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Perugia, Italy);  $p < 0.05$  was considered significant in all cases. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the  $IC_{50}$  values of

antioxidants and their standard error of estimates (SEE). Correlation coefficient analysis was conducted using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) to identify the relationship patterns between different antioxidant activities.

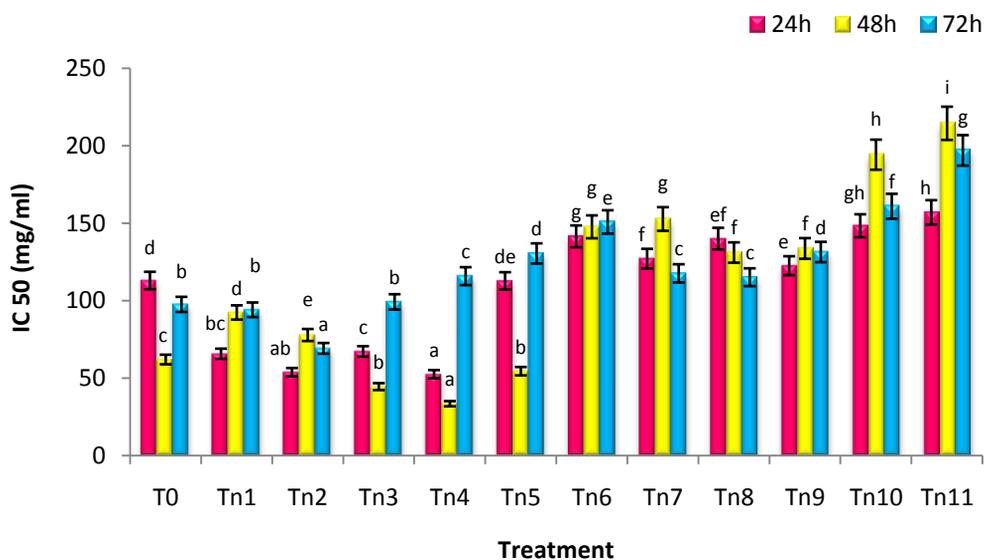
### **3.3 RESULTS AND DISCUSSION**

Antioxidant compounds are very much effective in inhibiting the processes involved in peroxidation such as: neutralizing existing free radicals in the system, inhibiting the peroxidation catalysis by metal ions and also through breaking the lipid-radical chain reactions (Cervato *et al.*, 2000). Therefore, the evaluation of the antioxidant activity of a sample is an important parameter for assessing its nutritional value (Rice- Evans *et al.*, 1996; Pellegrini *et al.*, 1999; Scalfi *et al.*, 2000). The antioxidant activity or capacity of antioxidant molecules depend on its chemical nature and free radicals, concentration of free radicals as well as their sources. Consequently, evaluation of single assay is not sufficient for the proper determination of antioxidant potential of an extract because the mode of action of different antioxidants vary widely (Pokorny, 2007; Apak *et al.*, 2013). The antioxidant activity of the sprouts was measured in terms of their free radicals such as DPPH, ABTS<sup>+</sup>, and superoxide scavenging potential; metal chelation, lipid peroxidation, reducing power and beta-carotene protective activity.

DPPH being the relatively stable radical has been widely used for the evaluation of antiradical activity (Brand-Williams *et al.*, 1995). The assay is based on the reduction of alcoholic DPPH solution by hydrogen donating antioxidant components. The remaining amount DPPH radical in the reaction mixture after incubation period corresponds inversely to the free radical scavenging potential of antioxidant substance (Blois, 1958). As a result, the DPPH assay revealed the antioxidant activity in elicited fenugreek sprout extract was significantly improved over control. Among the different treatments applied for nitric oxide elicitors, 80mM of sodium nitroprusside exhibited the best IC<sub>50</sub> value of 52.62 mg/ml in

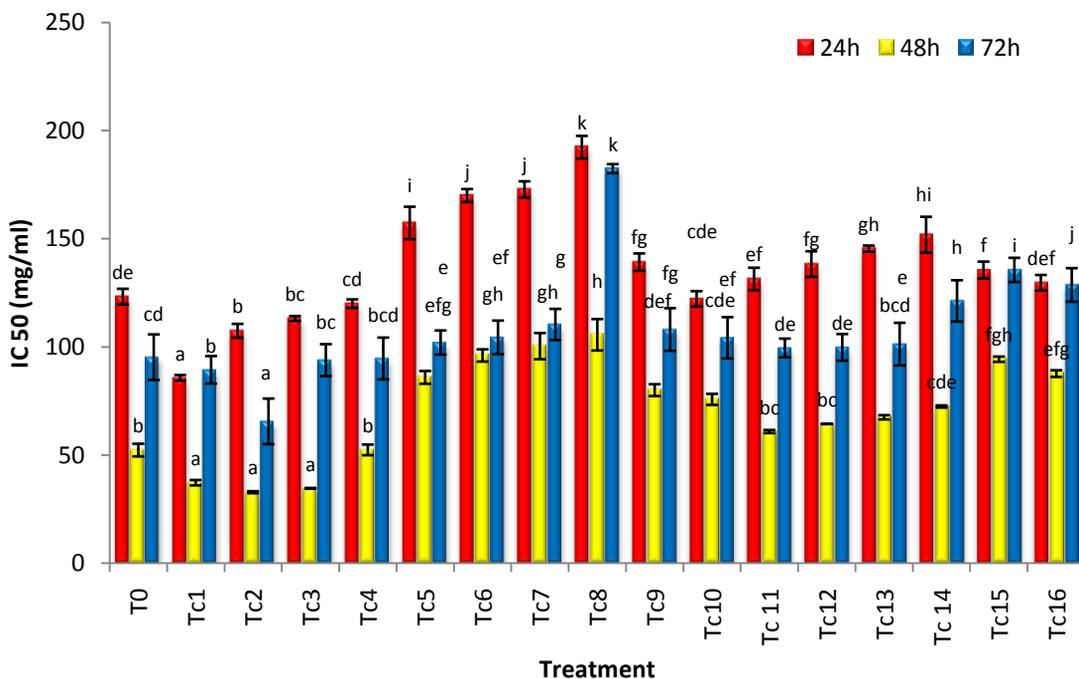
comparison to that of control set of 113.04 mg/ml at 24 hours stage. Later on day 2, the best antioxidant activity was again exhibited by sprouts pre-treated by 80mM of sodium nitroprusside. At 72 hours of germination it was found that the sprouts pre-treated with 20mM of sodium nitroprusside showed best activity (Figure 3.1). During all 3 days of germination after priming, it was found that supply of exogenous nitric oxide resulted in enhancement of antioxidant activity of fenugreek sprouts. Likewise, the priming with different elicitors of calcium it was found that pre-treatment of seeds with exogenous calcium resulted in significant increase in DPPH radical scavenging activity (Figure 3.2). The sprout extracts were also evaluated for scavenging ability of ABTS<sup>+</sup> radical and similar trend of result was observed (Figure 3.3 & 3.4). It was revealed that higher concentration of SNP resulted in decrease in the antiradical activity of sprouts thus suggesting implementation of optimum dose. Also, the application of antagonist of nitric oxide, i.e. CP and calcium, i.e. EG and LC negatively affected the activity of sprouts. The significant increase in the decolourization of DPPH and ABTS<sup>+</sup> solution by pre-treated fenugreek sprouts with respect to control extract indicates probable involvement of nitric oxide and Ca<sup>2+</sup> in the synthesis of antioxidant factors.

The reducing capacity of a biological compound acts as a significant indicator of its antioxidant potential. In the reducing power assay, the antioxidant components convert the oxidized form of iron (Fe<sup>+3</sup>) from ferric to ferrous (Fe<sup>+2</sup>) chloride (Moein *et al.*, 2008). The reducing ability of the sprouts was determined with ascorbic acid equivalent. Higher ascorbic acid equivalent value indicates higher reducing capacity of samples. It was observed that the sprouts pre-treated with SNP (10mM and 20mM) showed enhanced reducing activity at 24h stage, SNP (20mM and 80mM) at 48h stage and SNP (20mM and 40mM) at 72h stage (Figure 3.5) when compared to that of control sets. For calcium elicitors enhancement in reducing power was found in sprouts pre-treated with CC (1mM and 2mM)



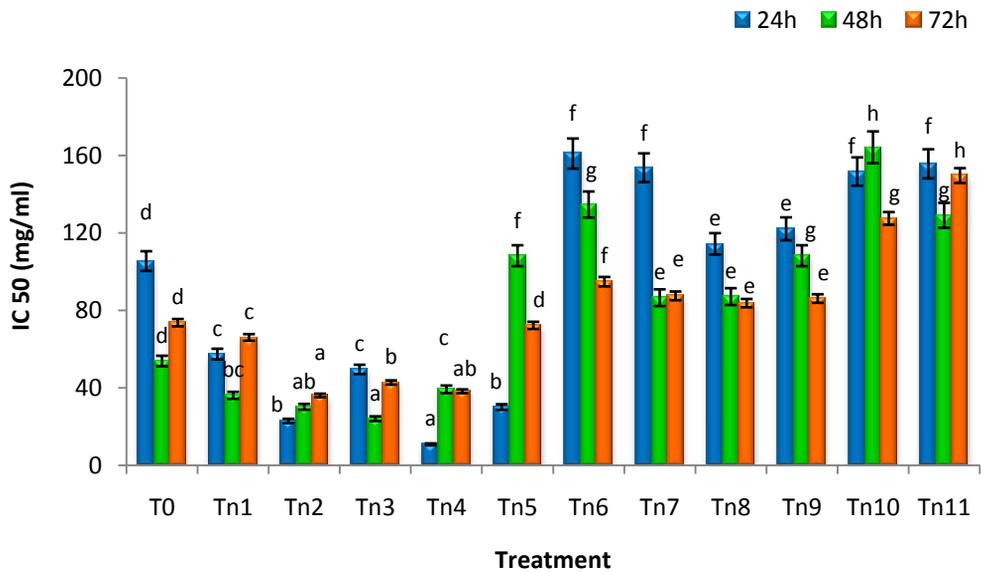
**Figure 3.1:** DPPH free radical scavenging activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



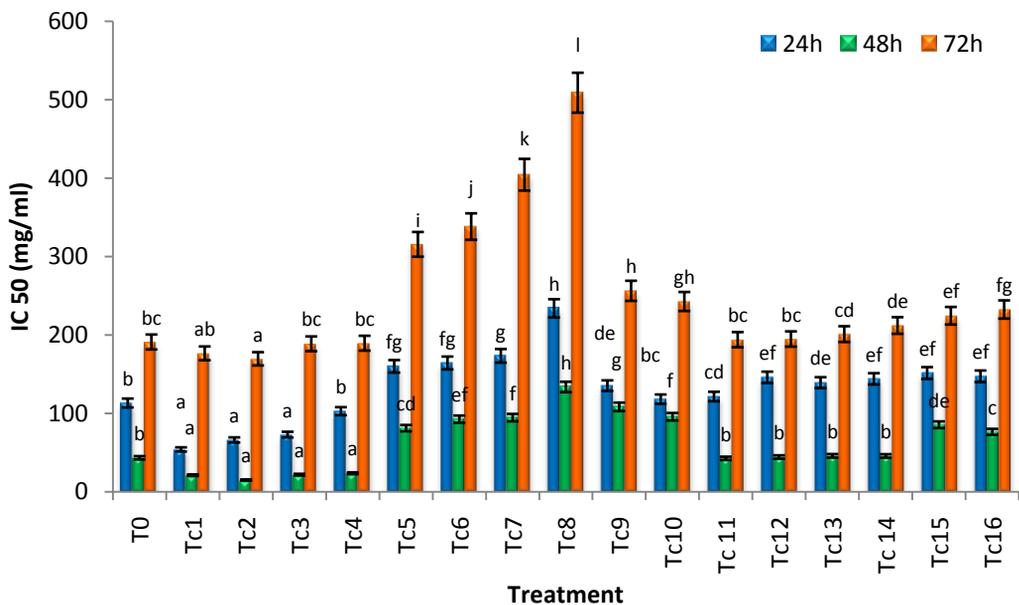
**Figure 3.2:** DPPH scavenging activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



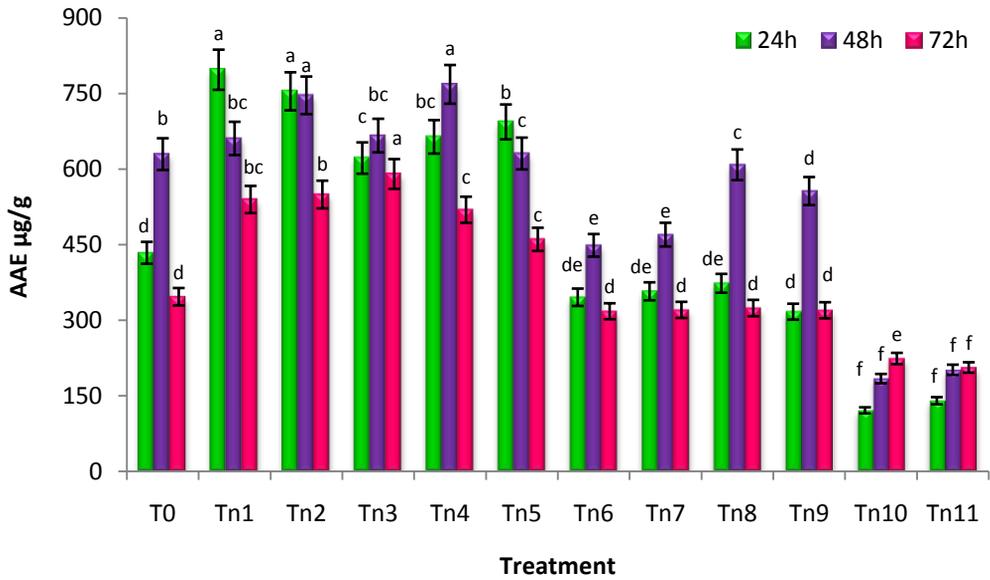
**Figure 3.3:** ABTS scavenging activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

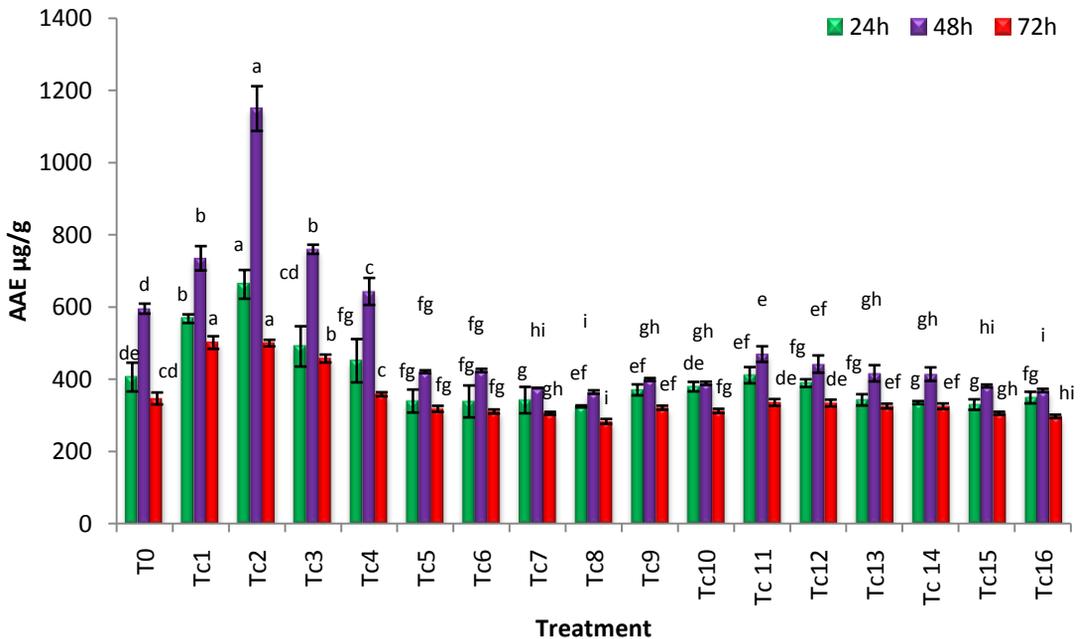


**Figure 3.4:** ABTS scavenging activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 3.5:** Reducing power activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination  
 Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 3.6:** Reducing power activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination  
 Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

at 24h stage, CC (2mM and 5mM) at 48h stage and CC (1mM and 2mM) at 72h stage (Figure 3.6). Among all the treatments SNP 20mM and CC 2mM were found to be most consistent throughout 3 days of germination.

Another important antioxidative mechanism involves the chelation of transition of metal ions such as copper and ferrous ions, which prevent the participation of these metal ions in Fenton and Haber–Weiss reactions further inhibiting the generation of highly reactive hydroxyl radicals (Ademiluyi and Oboh, 2008). The antioxidant components which are efficient in ferrous ion chelation helps in mobilization of iron present in tissues by forming soluble, stable complexes that are excreted along with the faeces and urine (Shinar and Rachmilewitz, 1990; Hebbel *et al.*, 1990). Hence chelation therapy has been implemented in reducing iron-related complications such as thalassemia which is characterized by iron overload in vital body organs (Rund and Rachmilewitz, 2005; Loukopoulos, 2005). In case of metal chelating assay the sprouts germinated from seeds primed with SNP-20mM, SNP-40mM SNP-10mM showed enhancement among nitric oxide treatment at 24h, 48h and 72h stage respectively (Figure 3.7). On the other hand, among calcium elicitors; CC-1mM and CC-2mM primed sprouts showed enhanced activity than control though CC-1mM being the most consistent (Figure 3.8).

Superoxide is considered as one of the most destructive radicals because it acts as a precursor for other major ROS, like hydrogen peroxide, hydroxyl and singlet oxygen (Kannat *et al.*, 2007). Superoxide along with these free radicals are capable of damaging almost all the molecules found in biological system and hence considered as a destructive group in the free radical pathology (Rao *et al.*, 2010). Therefore, scavenging or removal of such harmful molecule is very much essential. Our result indicated that the extract obtained from the sprouts pre-treated with exogenous nitric oxide and calcium were much more efficient in scavenging the superoxide radicals (Figure 3.9 & 3.10).

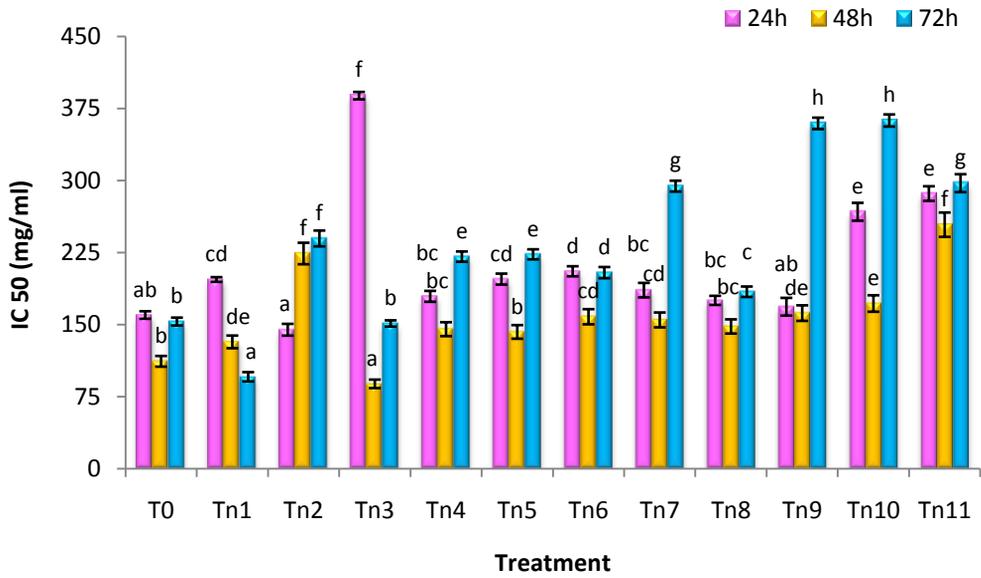
The  $\beta$ -carotene bleaching assay estimates the ability of the extract to function at a lipid water interface to inhibit the bleaching of  $\beta$ -carotene by  $H_2O_2$  catalyzed oxidation (Siddhuraju and Becker, 2003). The linoleic acid free radical, formed upon the release of a hydrogen atom from its diallylic methylene groups, targets the highly unsaturated beta-carotene molecules for degradation. As beta-carotene molecules get oxidized in this model system, the compound loses its chromophore and characteristic orange colour, in the absence of antioxidant compound, which is spectrophotometrically measured (Wang *et al.*, 2009). From the assay it was established that the sprouts subject to priming with SNP does not showed any enhancement in the activity during initial stage that is 24h but priming with SNP-20mM resulted in enhancement of BCB activity both at 48h and 72h of germination (Figure 3.11). In case of calcium elicitors, CC-1mM and CC-2mM were found to enhance BCB activity in fenugreek sprouts while CC-1mM being the most consistent throughout 72h (Figure 3.12).

In biological systems, malondialdehyde (MDA) a very reactive species takes part in cross-linking of DNA with proteins hence damaging the cells (Kumarappan *et al.*, 2012). MDA, being a major product of peroxidation phenomena is used to study the anti-lipid peroxidation activity by means of reaction with TBA at acidic conditions and high temperature. Lipid peroxidation is the oxidative degradation of lipids through peroxide formation which induces severe damage to adjacent bio-molecules in cells causing cell death. Thus, the inhibition of such destructive processes is very much important for the protection of the living system (Yang *et al.*, 2008). The sprouts pre-treated with elicitors of nitric oxide: SNP-10mM and SNP-20mM; SNP- 10mM and SNP-80mM; SNP-20mM and SNP-40mM showed enhancement in the anti-lipid peroxidation activity at 24h, 48h and 72h stage respectively (Figure 3.13). On the other hand, among calcium elicitors; CC-1mM primed sprouts showed enhanced activity than control throughout 72h of germination

(Figure 3.14) thus indicating most suitable priming agent for preventing lipid peroxidation in biological system.

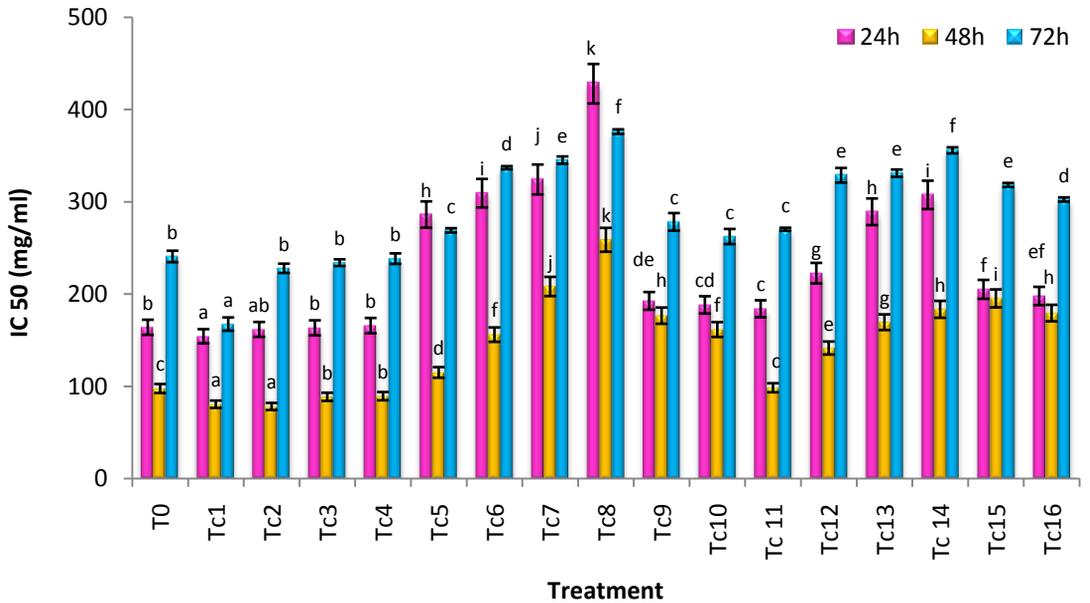
Overall it was observed that both elicitors SNP and CC were efficient in enhancing the free radical scavenging activity of fenugreek sprouts (Figure 3.15 to Figure 3.21); however when applied at lower concentration but priming at higher concentration the activity were reduced. The mode of action of antioxidant components, which helps in defence mechanisms in all stages of plant, depends on growth and developmental phases (Howard *et al.*, 1994; Deepa *et al.*, 2007). Accordingly, in present study it was found that generally the antioxidant activity of fenugreek sprouts increased during 48h of germination in comparison to 24h stage but later the activity was found to decline towards the stage of 72h of germination. Highest antioxidant activity was observed during 48h stage confirming that the molecules responsible for antioxidant activity were elicited appropriately at this stage. Such significant variation in the free radical scavenging activities among different stages of fenugreek sprouts indicates that the potential efficacy of antioxidants varies considerably during developmental phases. Also the decrease in the activity of the sprouts under the influence of the antagonists of these signal molecules provides a possibility of the involvement of these molecules in the synthesis of the components responsible for antioxidant activity of fenugreek sprouts.

The results achieved with Pearson's correlation revealed that the scavenging capacity of nitric oxide primed fenugreek sprouts for DPPH and ABTS<sup>+</sup> radicals are closely associated (Table 3.2), indicating involvement of similar mode of action of these two radical scavenging activities. Furthermore, DPPH showed significant association with other activities such as metal chelation, reducing power and  $\beta$ -carotene protective activity (Table 3.2).



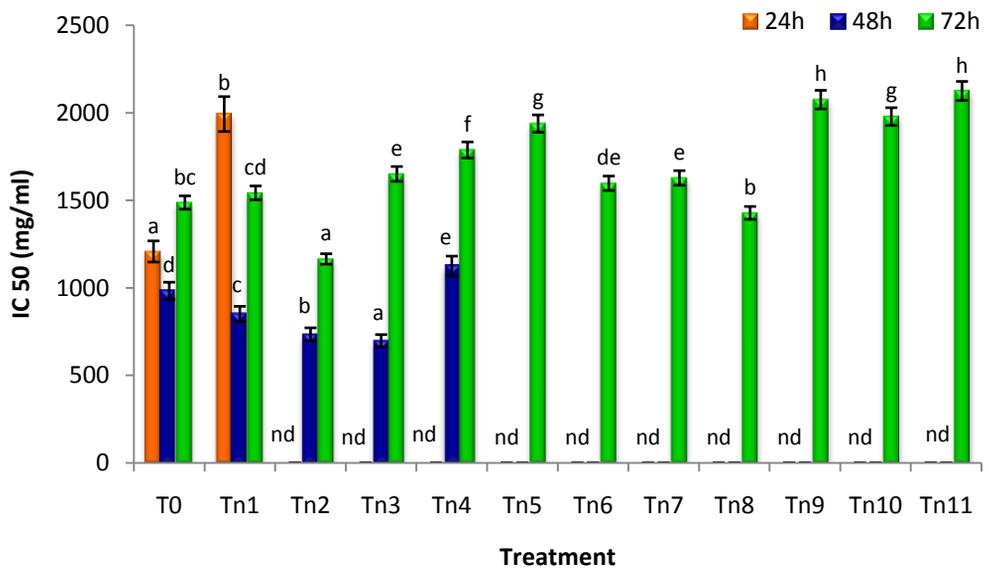
**Figure 3.7:** Metal chelating activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



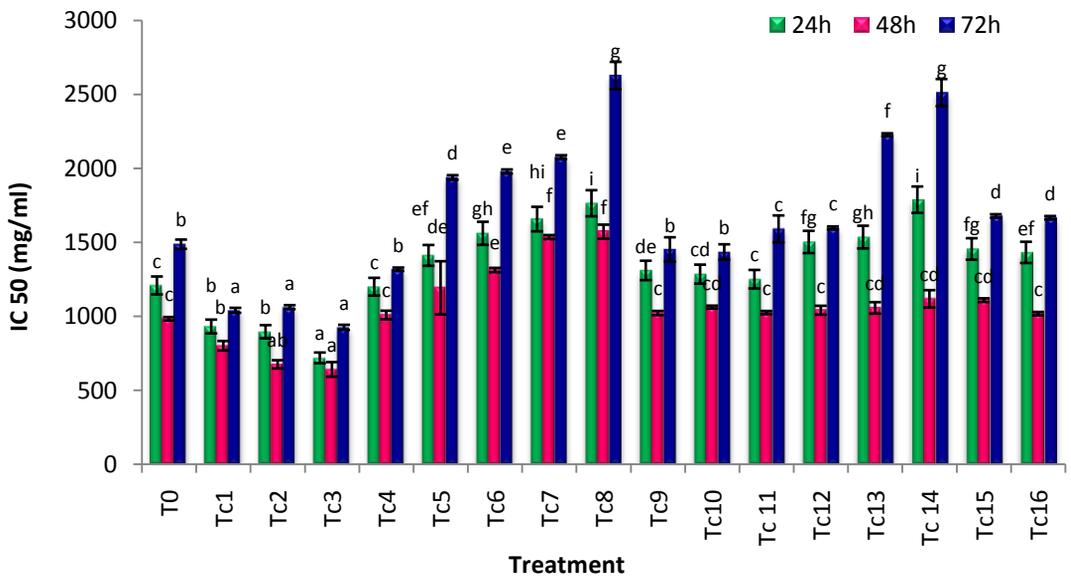
**Figure 3.8:** Metal chelating activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



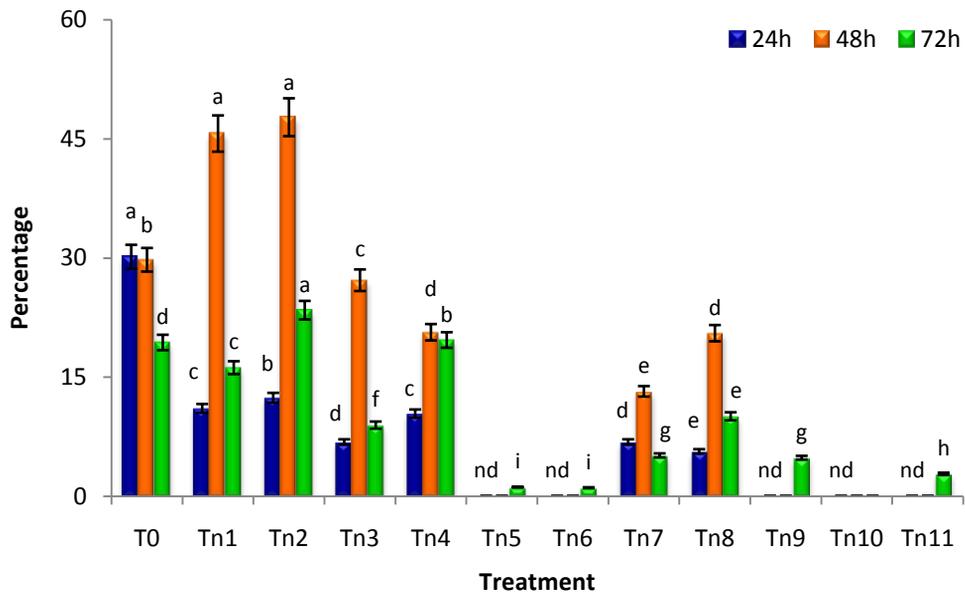
**Figure 3.9:** Superoxide scavenging activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



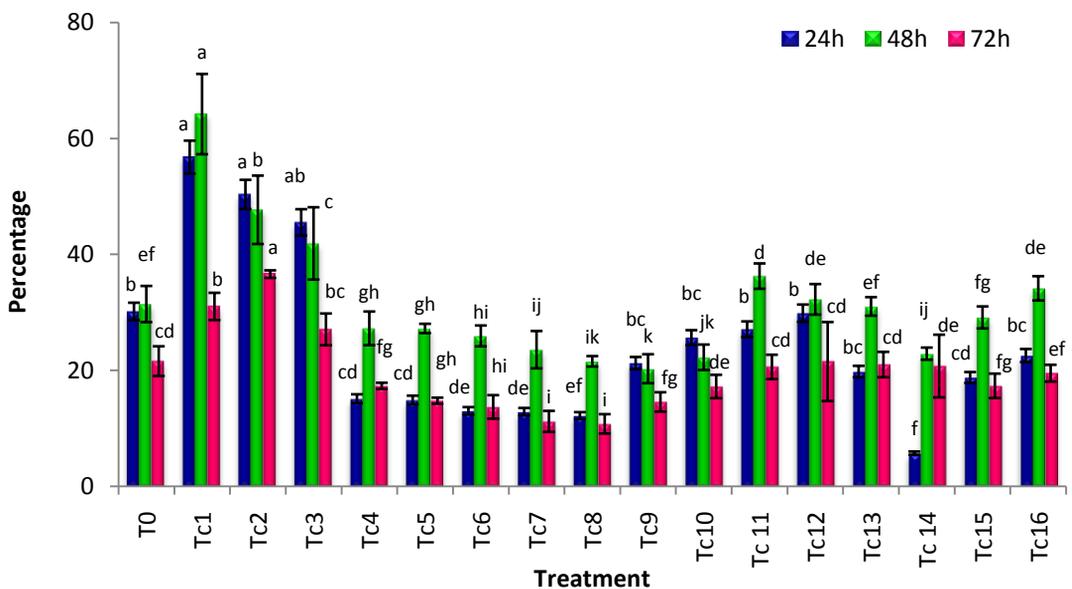
**Figure 3.10:** Superoxide scavenging activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



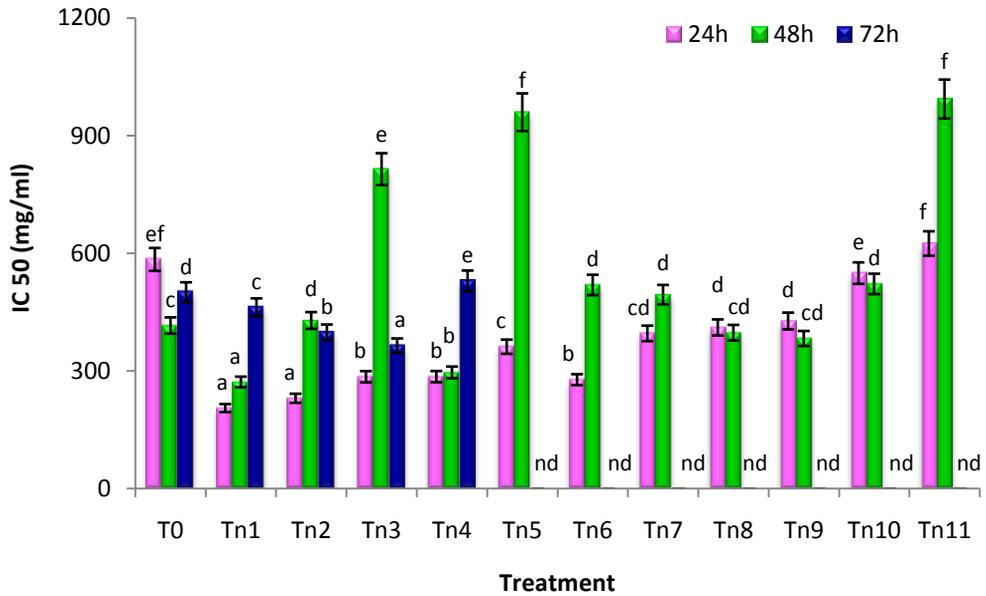
**Figure 3.11:**  $\beta$ -carotene protective activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



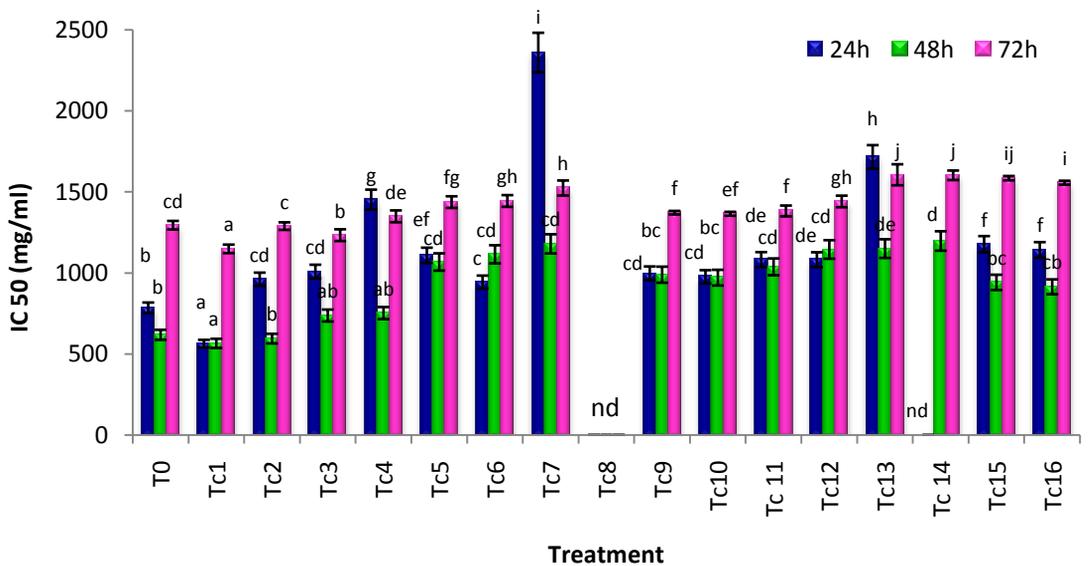
**Figure 3.12:**  $\beta$ -carotene protective activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



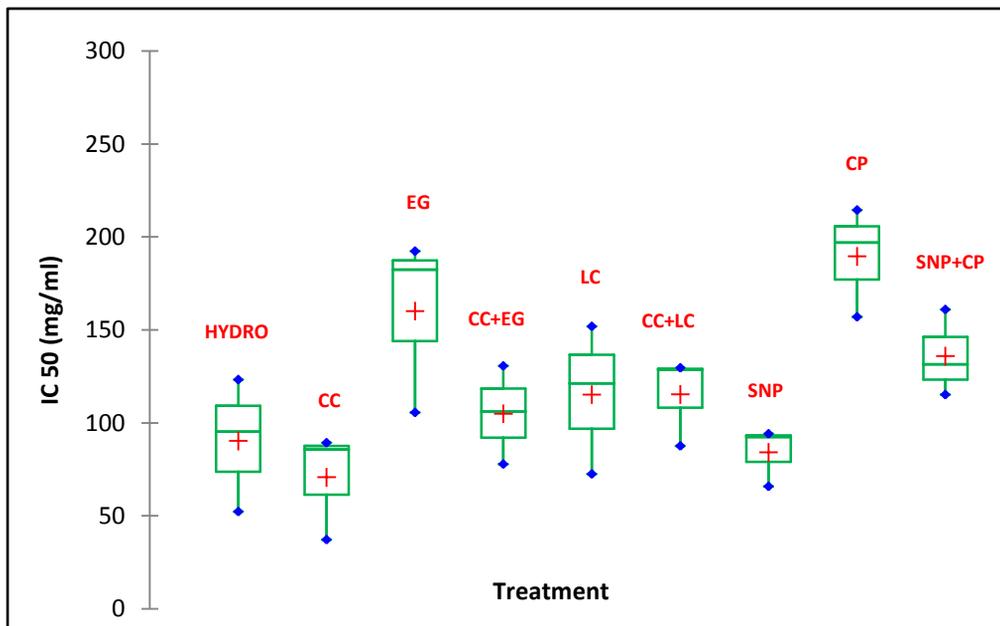
**Figure 3.13:** Anti-Lipid peroxidation activity of fenugreek sprouts primed with elicitors of nitric oxide at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

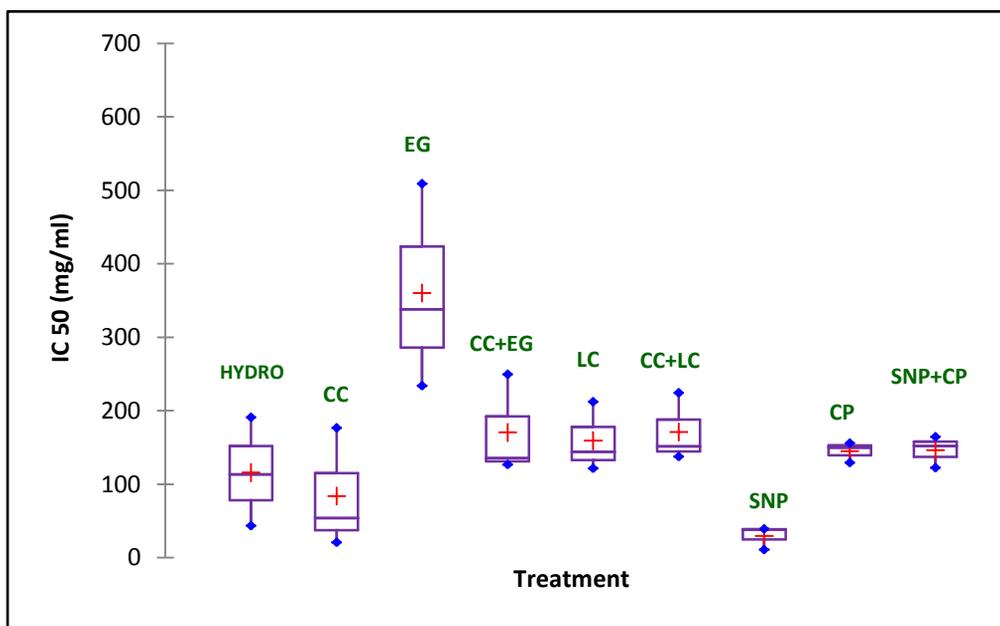


**Figure 3.14:** Anti-Lipid peroxidation activity of fenugreek sprouts primed with elicitors of calcium at 24h, 48h, & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

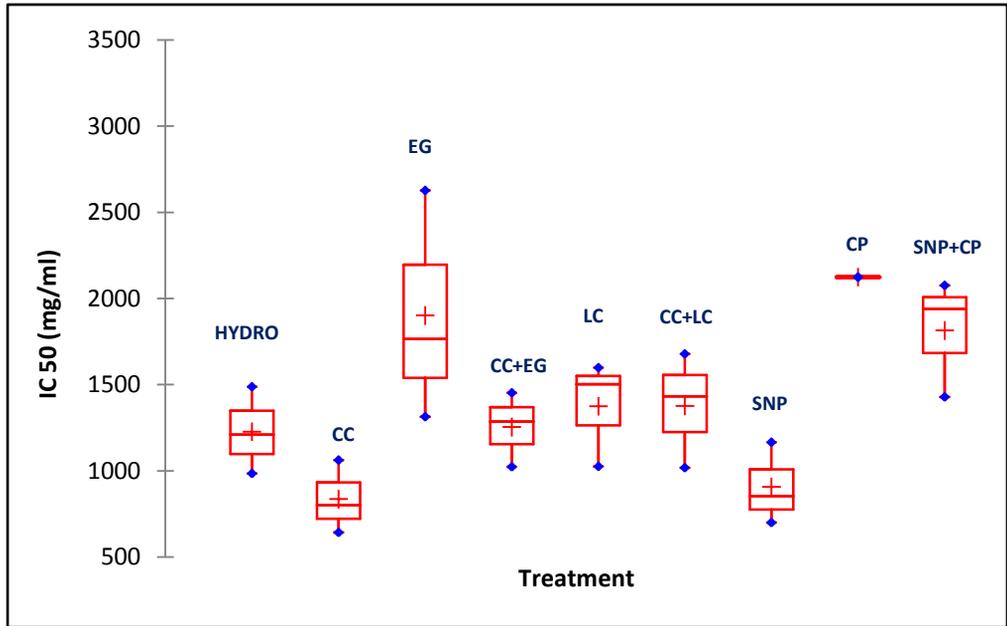


**Figure 3.15:** Effect of different priming agents on DPPH scavenging activity of fenugreek sprouts

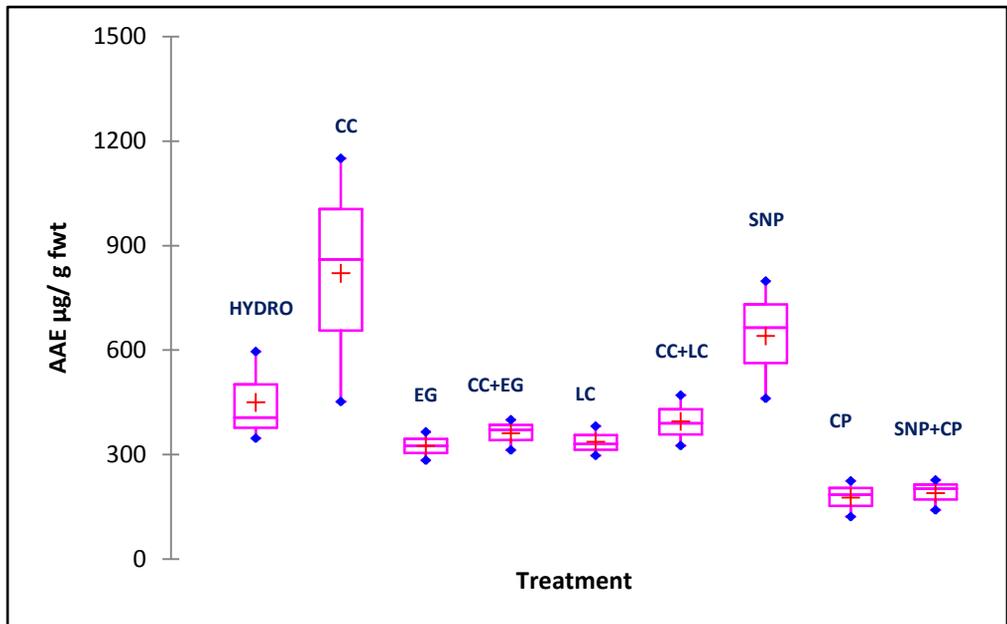


**Figure 3.16:** Effect of different priming agents on ABTS scavenging activity of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

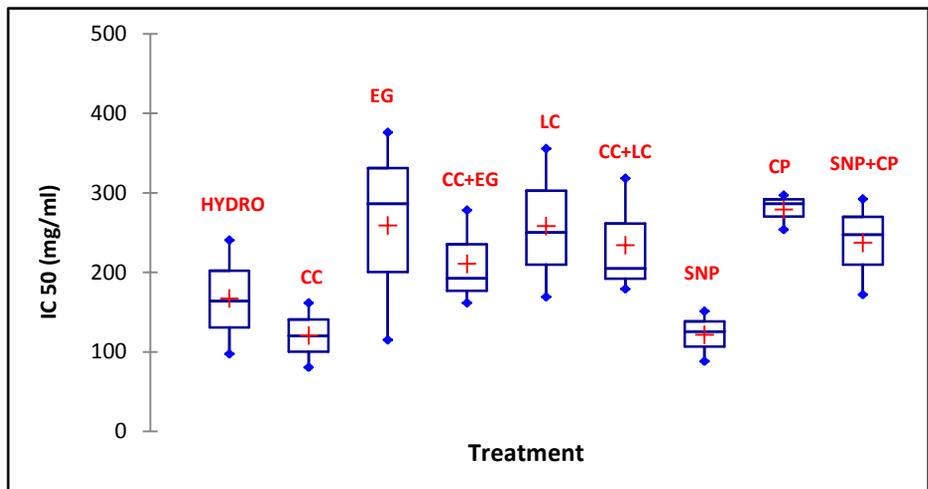


**Figure 3.17:** Effect of different priming agents on Superoxide radical scavenging activity of fenugreek sprouts

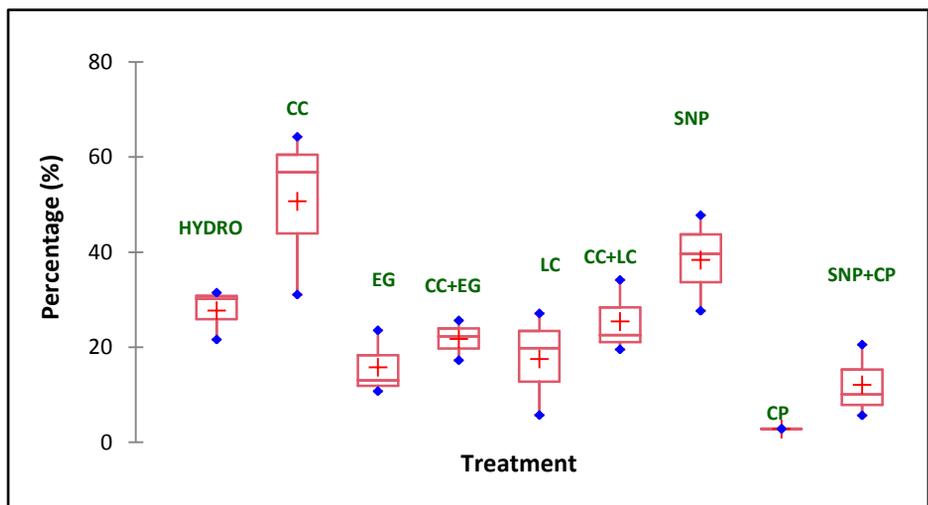


**Figure 3.18:** Effect of different priming agents on Reducing power activity of fenugreek sprouts

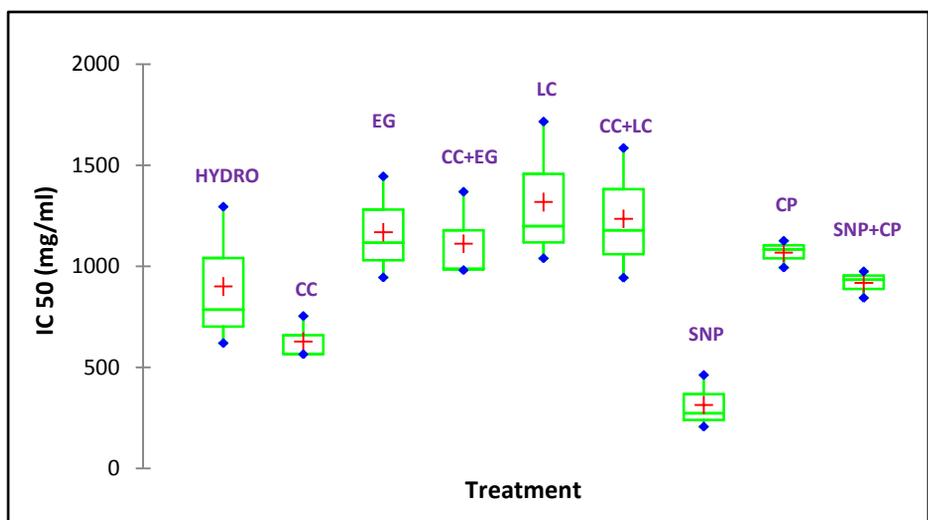
Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 3.19:** Effect of different priming agents on metal chelating activity of fenugreek sprouts

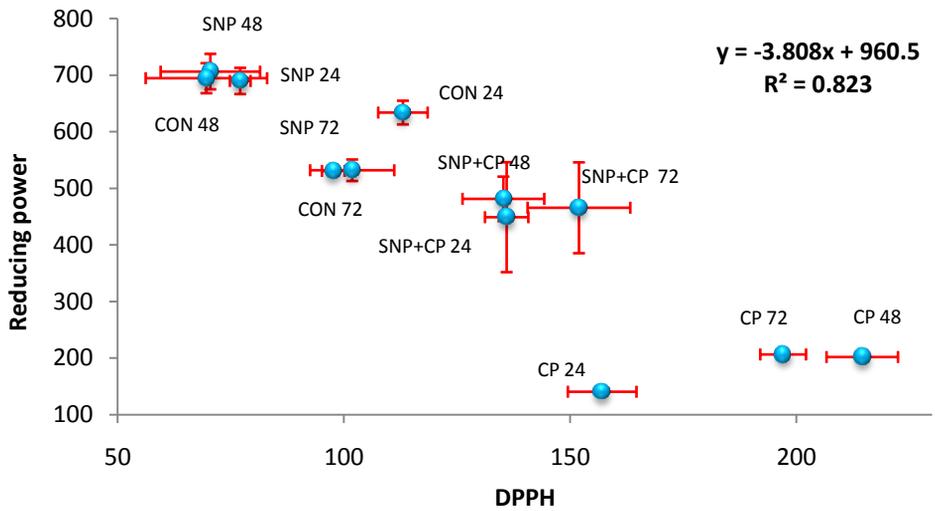


**Figure 3.20:** Effect of different priming agents on  $\beta$ -carotene protective activity of fenugreek sprouts

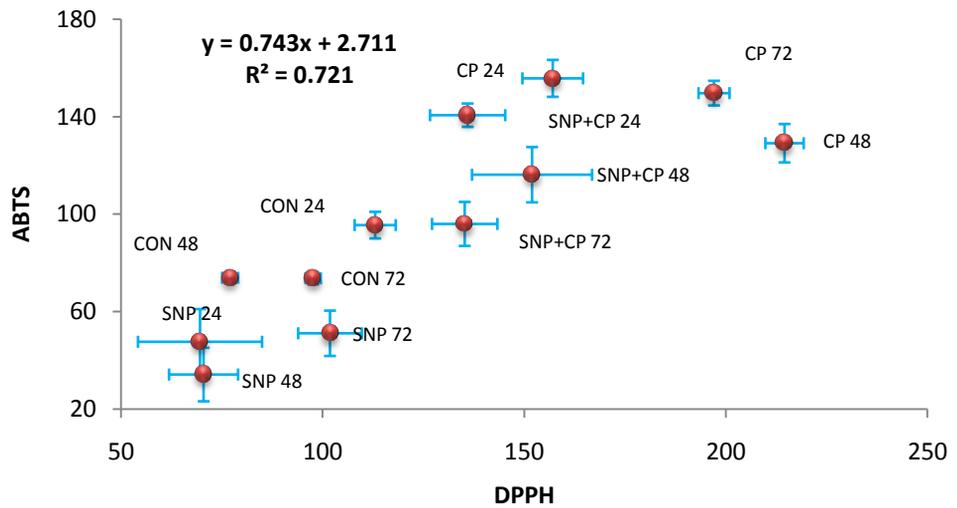


**Figure 3.21:** Effect of different priming agents on Anti-lipid peroxidation activity of fenugreek sprouts

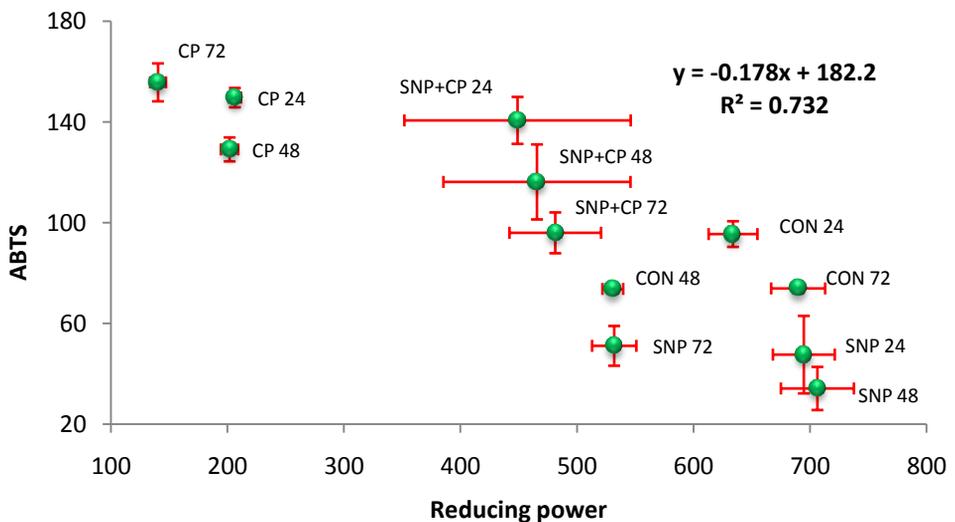
Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 3.22:** Alteration in the DPPH and Reducing power activity of fenugreek sprouts under the influence of nitric oxide signal elicitors

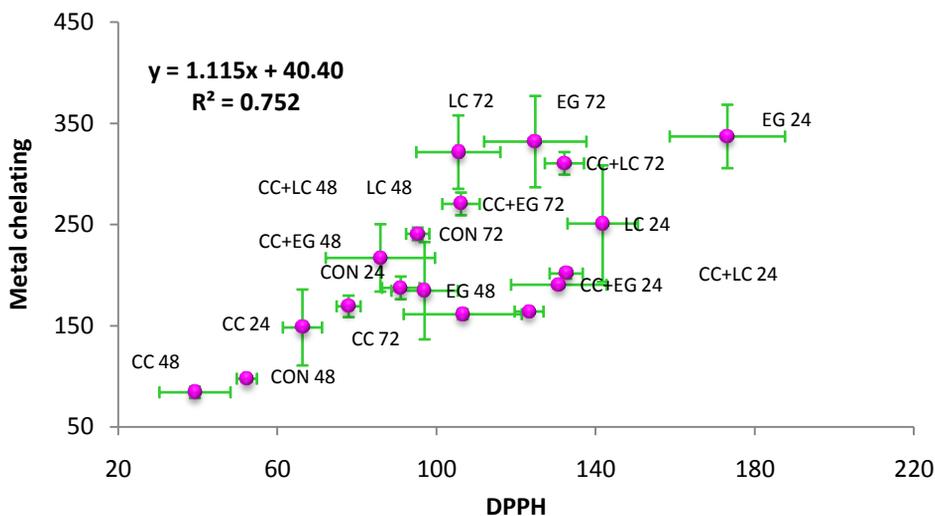


**Figure 3.23:** Alteration in the DPPH and ABTS scavenging activity of fenugreek sprouts under the influence of nitric oxide signal elicitors

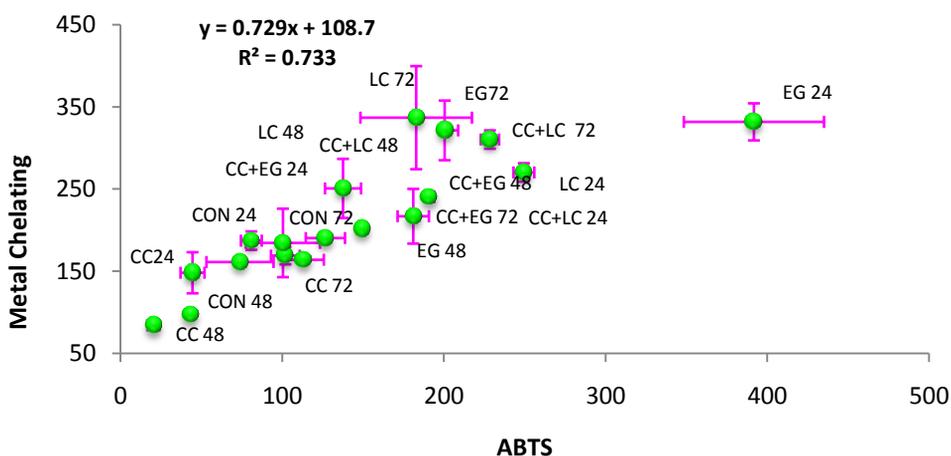


**Figure 3.24:** Alteration in the ABTS and Reducing power activity of fenugreek sprouts under the influence of nitric oxide signal elicitors

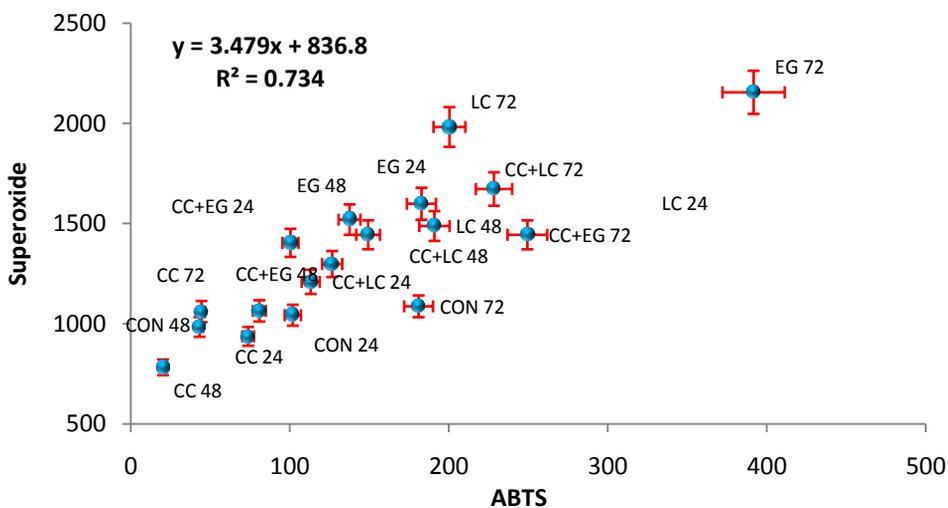
Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 3.25:** Alteration in the DPPH and metal chelating activity of fenugreek sprouts under the influence of calcium signal elicitors

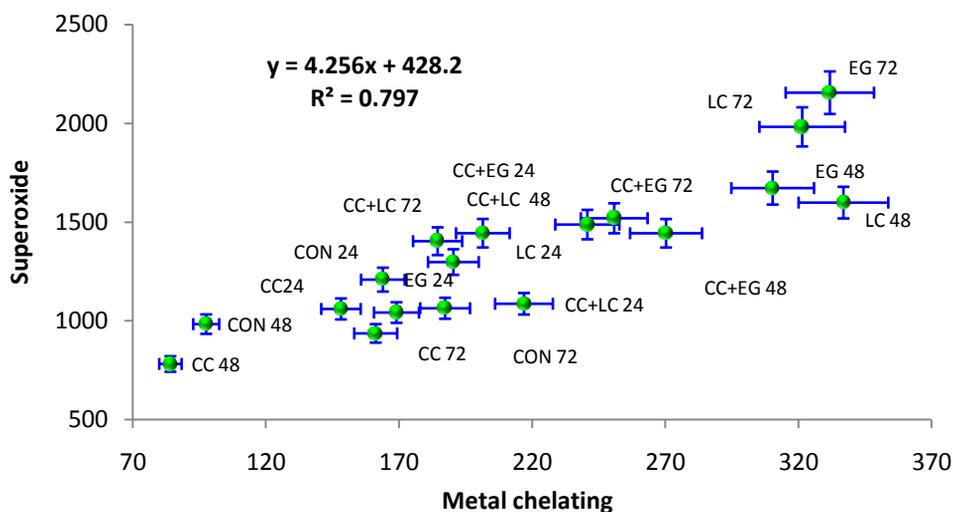


**Figure 3.26:** Alteration in the ABTS and metal chelating activity of fenugreek sprouts under the influence of calcium signal elicitors

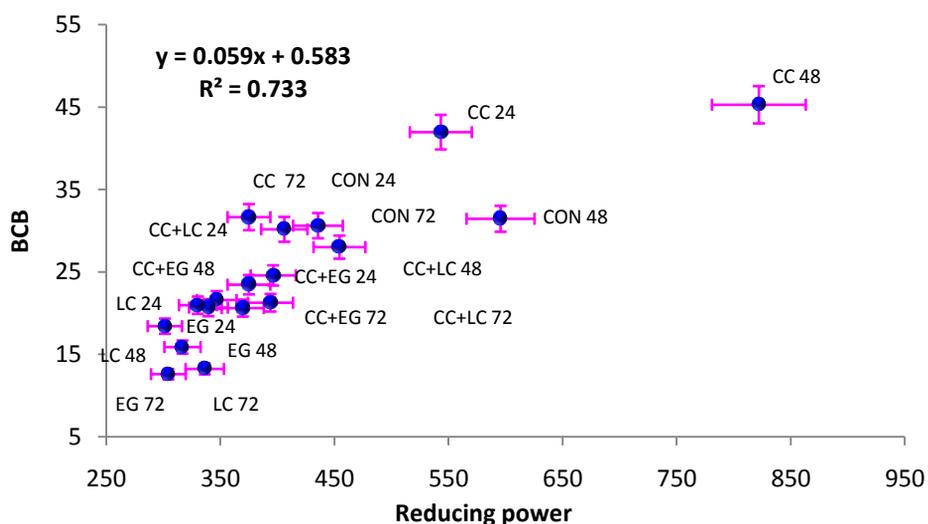


**Figure 3.27:** Alteration in the ABTS and superoxide scavenging activity of fenugreek sprouts under the influence of calcium signal elicitors

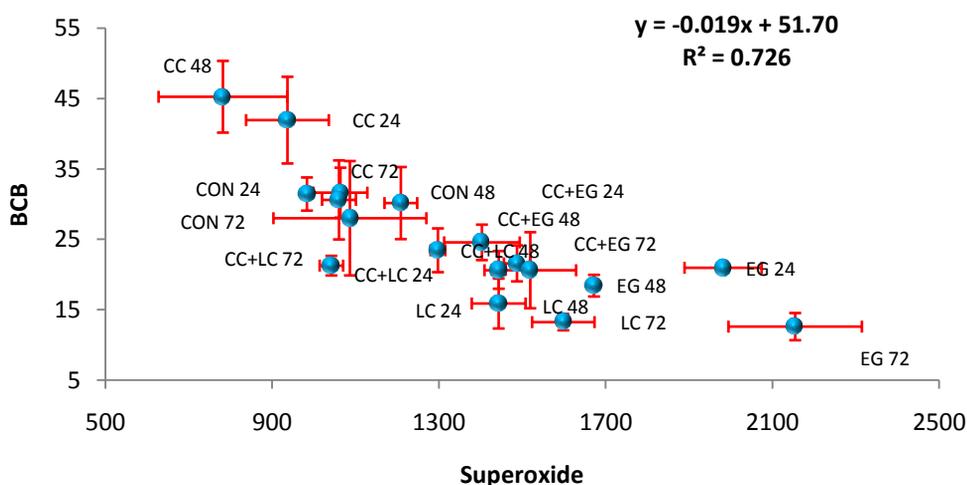
Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 3.28:** Alteration in the superoxide and metal chelating activity of fenugreek sprouts under the influence of calcium signal elicitors



**Figure 3.29:** Alteration in the BCB and reducing power activity of fenugreek sprouts under the influence of calcium signal elicitors



**Figure 3.30:** Alteration in the BCB and superoxide scavenging activity of fenugreek sprouts under the influence of calcium signal elicitors

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 3.2** Correlation matrix of different antioxidant activity of fenugreek sprouts pre-treated with nitric oxide elicitors.

	DPPH	MC	RP	ABTS	SO	ALP
MC	0.363*					
RP	-0.823**	-0.452*				
ABTS	0.776**	0.261	-0.819**			
SO	-0.055	0.224	-0.028	-0.231		
ALP	-0.041	-0.386*	0.049	0.102	-0.549*	
BCB	-0.525*	-0.371*	0.544*	-0.596*	0.180	0.106

\*\*Correlation is significant at the 0.01 level (2-tailed)

\*Correlation is significant at the 0.05 level (2-tailed)

**Table 3.3** Coefficient of determination ( $R^2$ ) values of different antioxidant activity of fenugreek sprouts pre-treated with nitric oxide elicitors.

	DPPH	MC	RP	ABTS	SO	ALP
MC	0.132					
RP	0.678	0.204				
ABTS	0.602	0.068	0.672			
SO	0.003	0.050	0.001	0.054		
ALP	0.002	0.149	0.003	0.010	0.302	
BCB	0.275	0.138	0.296	0.355	0.032	0.011

MC: metal chelating; RP: reducing power; SO: superoxide scavenging assay; ALP: Antilipid peroxidation; BCB:  $\beta$  carotene bleaching

**Table 3.4** Correlation matrix of different antioxidant activity of fenugreek sprouts pre-treated with calcium elicitors.

	<b>DPPH</b>	<b>ABTS</b>	<b>MC</b>	<b>RP</b>	<b>SO</b>	<b>ALP</b>
<b>ABTS</b>	0.556*					
<b>MC</b>	0.738**	0.811**				
<b>RP</b>	-0.646**	-0.596*	-0.697**			
<b>SO</b>	0.627*	0.814**	0.853**	-0.669**		
<b>ALP</b>	0.109	0.160	0.225	-0.288	0.178	
<b>BCB</b>	-0.631**	-0.647**	-0.717**	0.752**	-0.711**	-0.244

\*\*Correlation is significant at the 0.01 level (2-tailed)

\*Correlation is significant at the 0.05 level (2-tailed)

**Table 3.5** Coefficient of determination ( $R^2$ ) values of different antioxidant activity of fenugreek sprouts pre-treated with calcium elicitors.

	<b>DPPH</b>	<b>ABTS</b>	<b>MC</b>	<b>RP</b>	<b>SO</b>	<b>ALP</b>
<b>ABTS</b>	0.309					
<b>MC</b>	0.544	0.657				
<b>RP</b>	0.417	0.355	0.486			
<b>SO</b>	0.393	0.663	0.727	0.449		
<b>ALP</b>	0.012	0.026	0.051	0.083	0.032	
<b>BCB</b>	0.398	0.418	0.515	0.566	0.505	0.059

MC: metal chelating; RP: reducing power; SO: superoxide scavenging assay; ALP: Antilipid peroxidation; BCB:  $\beta$  carotene bleaching

The probable association between DPPH, ABTS and reducing power was also depicted graphically (Figure 3.22, 3.23 & 3.24) The correlation with metal chelation and DPPH scavenging activity was in contradiction to the finding of Wong *et al.*, (2006) and Zhao *et al.*, (2008) who reported a negative correlation between these two variables. On the other hand, ABTS<sup>+</sup> was found to be associated with reducing power and  $\beta$ -carotene protective activity only. The results obtained from the Pearson's correlation of attributes of calcium elicitor primed sprouts were found to exhibit some variation in context of association among the attributes with correspondence to those of nitric oxide elicitors. Here the association between DPPH and ABTS was less having coefficient of correlation (r) value 0.556 (p<0.05) and coefficient of determination (R<sup>2</sup>) value 0.309 in comparison to r = 0.772 (p<0.01) and R<sup>2</sup> = 0.602. Whereas, the association between DPPH and metal chelation was much stronger with r = 0.738 (p<0.01) (Table 3.4) and R<sup>2</sup> = 0.544 (Table 3.5) in comparison to r = 0.363 (p<0.05) (Table 3.2) and R<sup>2</sup> = 0.132 (Table 3.3) thus indicating difference in the degree of elicitation of the factors responsible for these activities by nitric oxide and calcium ion. Additionally, a significant association of DPPH and ABTS was obtained with the superoxide scavenging activity which was not found in case of nitric oxide elicited sprouts; which further suggest better elicitation of components having wide range of activity by calcium ion. The alteration in the various antioxidant attributes in corresponding to other, among them are depicted in Figure 3.25 to Figure 3.30.

The present study has illustrated significant increase of free radical scavenging activity by elicited sprouting in fenugreek with exogenous sources of nitric oxide and calcium ion. When analyzing the time course of germination the stimulatory effect of these signal molecules was found to be most pronounced at the early phases of germination i.e. from 24h to 48h and after that the action was declined during further extension of post-germination phases. The work supports the hypothesis that nitric oxide and calcium offer significant role in enhancement of antioxidant activity during the germination phase of

fenugreek which may be attributed to significant elicitation of bioactive components by these signal molecules. This knowledge can be used to design the sprouting techniques of fenugreek which might have potential application in improving the nutritional and health relevant functional value of legume sprouts with these elicitors.

## **CHAPTER - 4**

### **ALTERATION IN THE PHYTOCHEMICAL CONTENT AND THEIR RELATIONSHIP WITH ANTIOXIDANT ACTIVITY OF FENUGREEK SPROUTS AFTER ELICITATION**

#### 4.1 INTRODUCTION

The plants have been long used as a source of dietary antioxidants for treatment of various oxidative stress mediated disorders (Krishnaiah *et al.*, 2011; Kasote *et al.*, 2015). It is reported that approximately two-third of the entire plant species possess medicinal value, and most of these have potential antioxidant property (Krishnaiah *et al.*, 2011). The reason for such efficient medicinal property of the plants is the presence of wide range of phytochemicals (Gupta *et al.*, 2014). Plants synthesize these phytochemicals for fulfilling their self requirements to either achieve specific function or for defence mechanism against adverse conditions. Interestingly, some of the phytochemicals such as polyphenols, flavonoids, carotenoids, ascorbic acid, and vitamin E have been reported to play important role in human system also (Kasote *et al.*, 2015).

The discovery and isolation of ascorbic acid from plants resulted in increasing interest in the exogenous plant antioxidants (Szent-Giorgyi, 1963). Since then, the phytochemicals with antioxidant activity have drawn tremendous attention of researchers. Plants produce numerous secondary metabolites, out of which polyphenols are considered most important due to their promising antioxidant potential in both *in vivo* and *in vitro* system (Kasote *et al.*, 2015).

It has been claimed that the phytochemicals synthesized by shikimate and phenylpropanoid pathways exhibit pronounced antioxidant activity in biological system (Dewick, 2009). These phytochemicals mainly comprises of phenolics and flavonoids which are said to be strong reducing agents. The antioxidant activity of these phenolic compounds is attributed to their strong redox properties, which accounts for their role as electron donors, reducing agents and metal ion chelators (Tiwary and Rana, 2015). Furthermore, these phenolics are used as suitable substrate by an antioxidant enzyme namely peroxidase for removal of excess H<sub>2</sub>O<sub>2</sub> from the cell system (Sakihama *et al.*, 2002; Michalak, 2006).

Another important compound vitamin C is regarded as one of the most important dietary antioxidants because human beings are not able to synthesize ascorbate due to mutated gulonolactone oxidase gene (Bast and Haenen, 2013). But ascorbate is readily absorbed by human intestinal system and functions as potential electron donor thus, preventing oxidative stress mediated damages (Putchala *et al.*, 2013; Heo *et al.*, 2013). Moreover, interaction of phenolics with ascorbate and tocopherol leading to synergistic amplification of their biological effects has been reported (Croft, 1998).

Fenugreek being rich source of various bioactive phytochemicals has been used for the treatment of wide spectrum of diseases and disorders since ancient times (Puri, 1998). It is considered to be a good source of vitamins, alkaloids, flavonoids, carotene, fibers and other essential mineral nutrients (Srinivasan, 2006; Prajapati *et al.*, 2014). Almost all the plant parts of fenugreek have been reported to provide a potential source of medicinal implications (Randhir *et al.*, 2004; Meghwal and Goswami, 2012).

Considering such diverse implication of fenugreek, the present study was designed aiming the enhancement in the phytochemicals especially associated with the antioxidant and antidiabetic activity as these two pharmacological activities are the major point of concern of our research work. The phytochemicals studied in the present study were phenol, flavonoids, carotenoids and ascorbic acid. Since the phenolics and antioxidant activity recorded in the dry seeds of fenugreek were relatively low (Randhir *et al.*, 2004); therefore, the present work was carried out with objective of the enhancement in the antioxidant activity which we have achieved and discussed in previous chapter along with the above mentioned phytochemicals by the elicitor mediated sprouting of fenugreek sprouts. The elicitors used are mentioned in the previous chapter.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Elicitation process and germination**

Mention in section 3.2.1

### **4.2.2 Preparation of methanolic extract**

Mention in section 3.2.2

### **4.2.3 Total phenolic content**

Total phenolic contents of sprout extracts were determined according to the standard protocol (Chandler and Dodds, 1993). 1 ml of the various methanolic extracts was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 ml of 5% Na<sub>2</sub>CO<sub>3</sub> was added. It was mixed thoroughly and placed in dark for 1h. Finally the absorbance of coloured reaction product was measured at 765nm against the reagent blank. The total phenolic content was expressed as µg of gallic acid equivalent per gram fresh weight.

### **4.2.4 Total flavonoid content**

The flavonoid contents were measured following a standard Spectrophotometric method (Sultana *et al.*, 2009). 1 ml of methanolic extract was diluted with distilled water (4ml) in a 10 ml volumetric flask. Initially, 5% NaNO<sub>2</sub> solution (0.3ml) was added to each volumetric flask; at 5 min, 10% AlCl<sub>3</sub> (0.3ml) was added; and then after 6 min, 1M NaOH (2ml) was added. Next 2.4ml of distill water was added to the reaction flask and mixed well using a vortex. Absorbance of the reaction mixture was read at 510 nm. The total flavonoid content in different extracts was calculated as µg of quercetin equivalent (QE) per gm fresh weight.

#### **4.2.5 Total carotene estimation**

Total carotene contents (TCC) were determined according to the standard protocol (Thimmaiah, 2004). Firstly, the methanolic sample was dried and converted to aqueous extract which was further partitioned thrice with equal volume of peroxide free ether using a separating funnel. The ether layer was collected and evaporated and then after reconstituted in ethanol. To the ethanolic extract 0.1 ml of 60% aqueous (potassium hydroxide) KOH was added and heated for 5 min in water bath. The mixture was kept in dark for overnight. Next day again the mixture was partitioned with ether, the ether layer was evaporated and reconstituted with ethanol and the absorbance of the ethanolic mixture was recorded at 450nm. The carotene content was calculated using a calibration curve prepared against pure  $\beta$ -carotene.

#### **4.2.6 Estimation of Ascorbic acid content**

The content of ascorbic acid was analyzed by the spectrophotometric method described by Mukherjee and Choudhary (1983). 1ml of plant extract was reacted with 0.5ml of 2% Dinitrophenyl hydrazine (DNPH) and 1drop of 10% thiourea (ethanolic) was added to it. The assay mixture was boiled for 15 min in a water bath. After that the mixture was cooled at room temperature in an ice bath and 80% sulphuric acid was added. The absorbance was read at 530nm. Ascorbic acid content was expressed as  $\mu\text{g}$  per gm fresh weight using ascorbate standard curve.

#### **4.2.7 Statistical analysis**

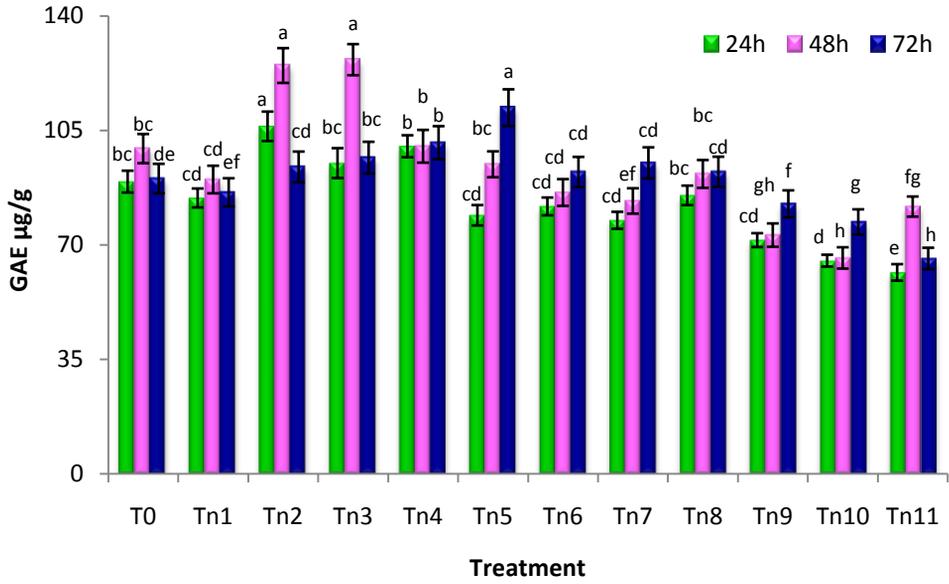
The data were pooled in triplicate and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the phytochemicals of fenugreek seedlings of different experimental set. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Perugia, Italy);  $p < 0.05$  was

considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the values of phytochemicals and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

### **4.3 Results and Discussion**

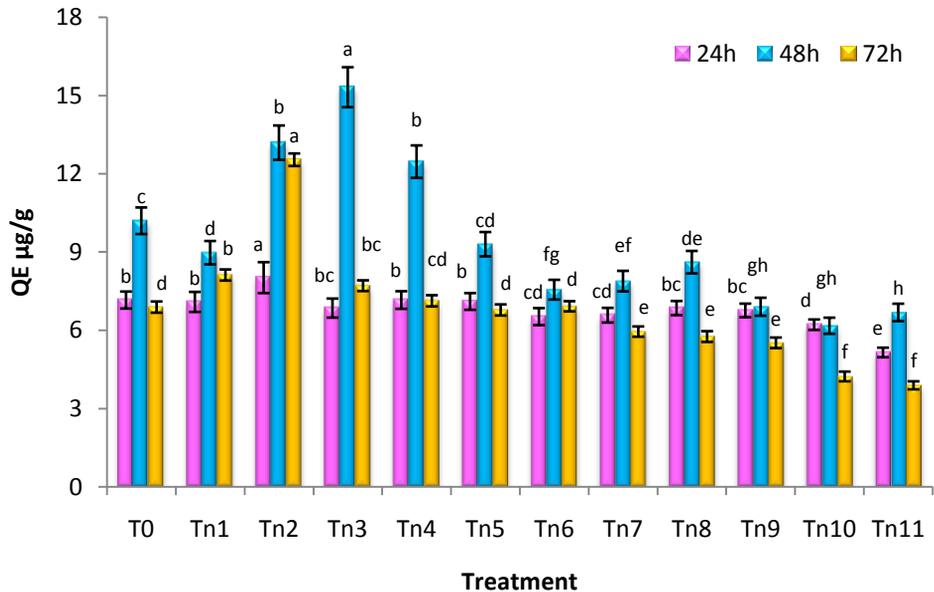
The health benefits of phenolic compounds have been widely investigated considering their potential reducing property. In human beings phenolics are known to exhibit strong antioxidant activity, which is involved in prevention of oxidative damage to biomolecules like DNA, proteins and lipids that may result in chronic diseases, such as cancer and cardiac disorders (Newmark, 1996). The present research aimed at improving the phenolics content and related antioxidant properties of fenugreek through elicited sprouting. The phytochemicals studied were phenol, flavonoids, carotenoids and ascorbic acid. The total phenol content of the fenugreek sprouts were found to be significantly enhanced by the exogenous supply of nitric oxide and calcium ion. The sprouts pre-treated with sodium nitroprusside exhibited maximum of 26% increase in the phenol content (Figure 4.1) whereas those primed with calcium chloride showed about 41% enhancement (Figure 4.5). The priming of fenugreek seeds with the antagonists of nitric oxide and calcium resulted in huge loss of phenol content recording a maximum of 33% by nitric oxide scavenger and 27% by calcium channel blocker, EGTA (Figure 4.9). In agreement to our findings, Joshi *et al*, (2013) have also reported increase in phenolics in the seeds of cucumber after priming with calcium chloride. The other important dietary antioxidant phytochemical known is flavonoid which is also a polyphenolic compound.

As a dietary component, flavonoids are considered to have health-promoting properties due to their potential antioxidant activity both *in vivo* and *in vitro* systems (Cook and Samman, 1996; Kumar and Pandey, 2013). The maximum elicitation of flavonoid content was found in the sprouts primed with sodium nitroprusside and a gradual increase in the enhancement of flavonoid content was observed throughout the germination phases. An increase of about 12%, 50% and 82% was recorded at 24h, 48h and 72h stage of germination by exogenous nitric oxide supply (Figure 4.2). In case of calcium elicitors maximum elicitation of flavonoids was observed to be about 34% in sprouts pre-treated with calcium chloride (5mM) at early stage (Figure 4.6). Likewise, a significant enhancement in the accumulation of flavonoids was reported in *Echinacea purpurea* (Wu *et al.*, 2007) and *Gingko biloba* (El Beltagi *et al.*, 2015) by nitric oxide elicitation. Apparently similar trend of alteration in the carotene content was found in pre-treated fenugreek sprouts (Figure 4.11). Carotenes are another class of phytochemicals known for their antioxidative property and these compounds are involved in the scavenging of specifically singlet molecular oxygen and peroxy radicals (Stahl and Sies, 2003). In the present study considerable enhancement of carotene content was found in the fenugreek sprouts subjected to nitric oxide elicitation (37%) (Figure 4.3) and on the other hand calcium elicitation resulted about 51% (Figure 4.7) increase in the carotene content compared to that of control set. Further, another important component having potential antioxidant property, ascorbic acid was also evaluated in different pre-treated sprouts of fenugreek. The sprouts pre-treated with sodium nitroprusside exhibited maximum of 7% increase in the ascorbate (Figure 4.4) content whereas those primed with calcium chloride showed about 8.5% enhancement (Figure 4.8).



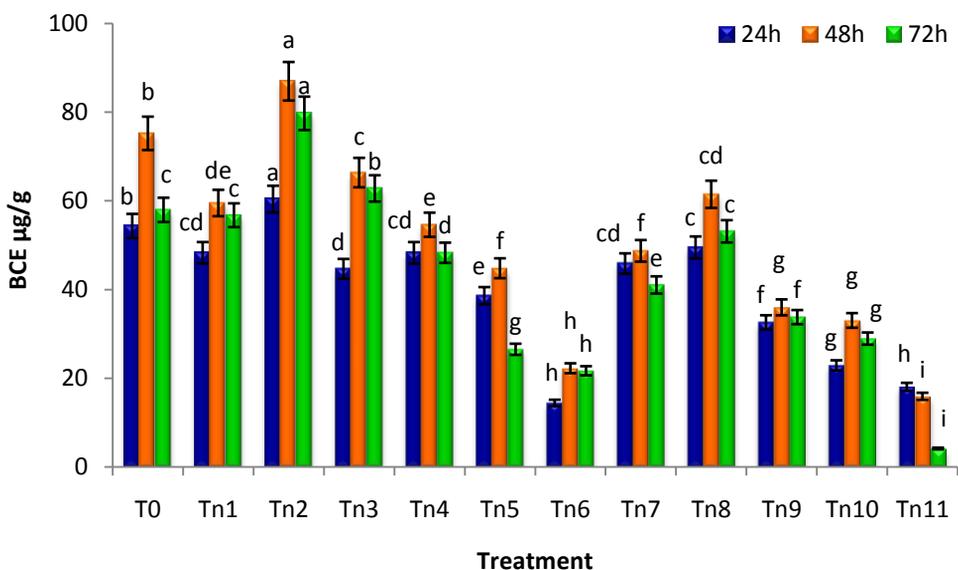
**Figure 4.1:** Total phenol content of fenugreek sprouts primed with different elicitors of nitric oxide at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



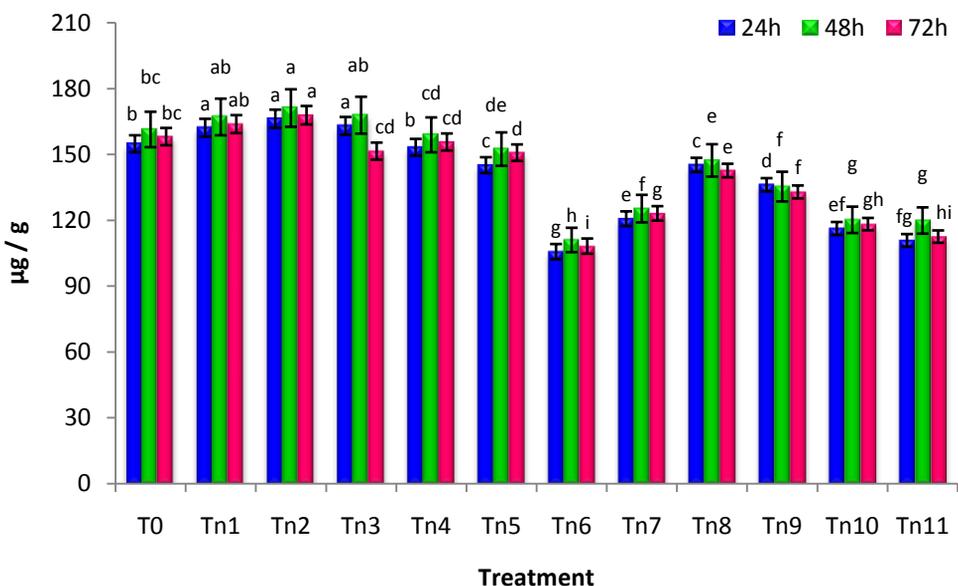
**Figure 4.2:** Total flavonol content of fenugreek sprouts primed with different elicitors of nitric oxide at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



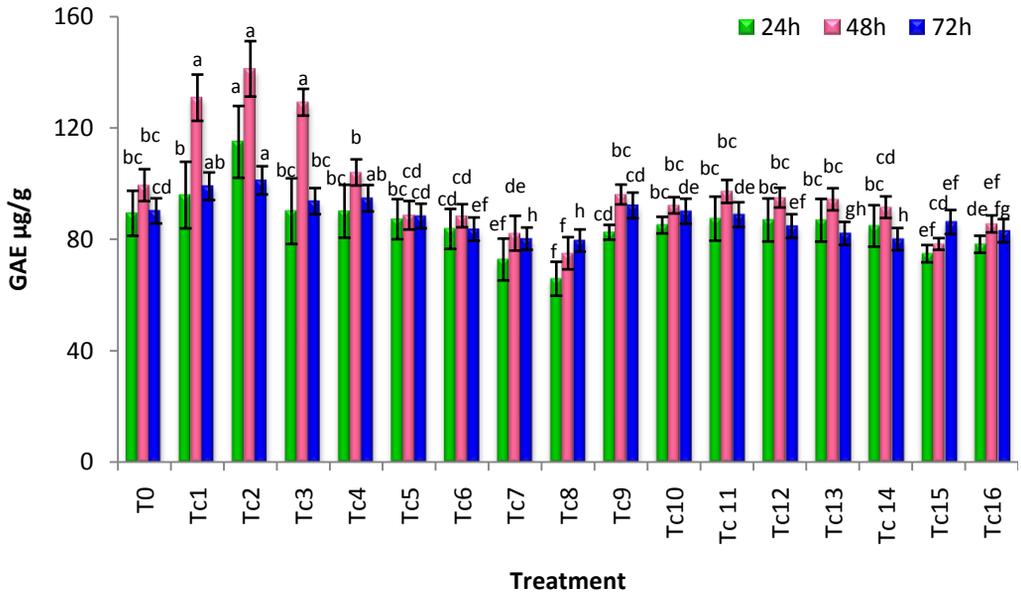
**Figure 4.3:** Total carotene content of fenugreek sprouts primed with different elicitors of nitric oxide at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



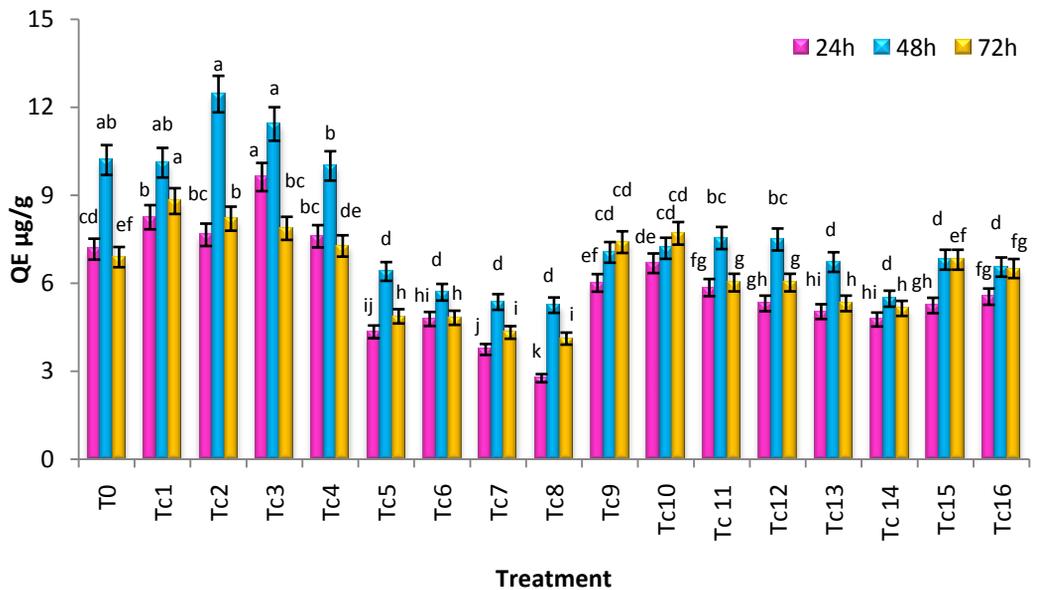
**Figure 4.4:** Total ascorbic acid content of fenugreek sprouts primed with different elicitors of nitric oxide at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



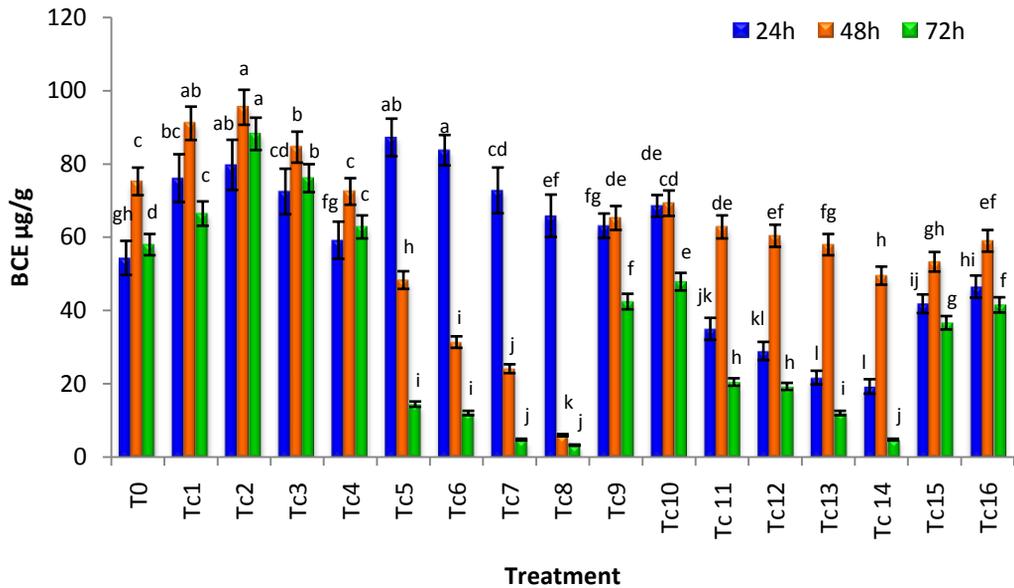
**Figure 4.5:** Total phenol content of fenugreek sprouts primed with different elicitors of calcium at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



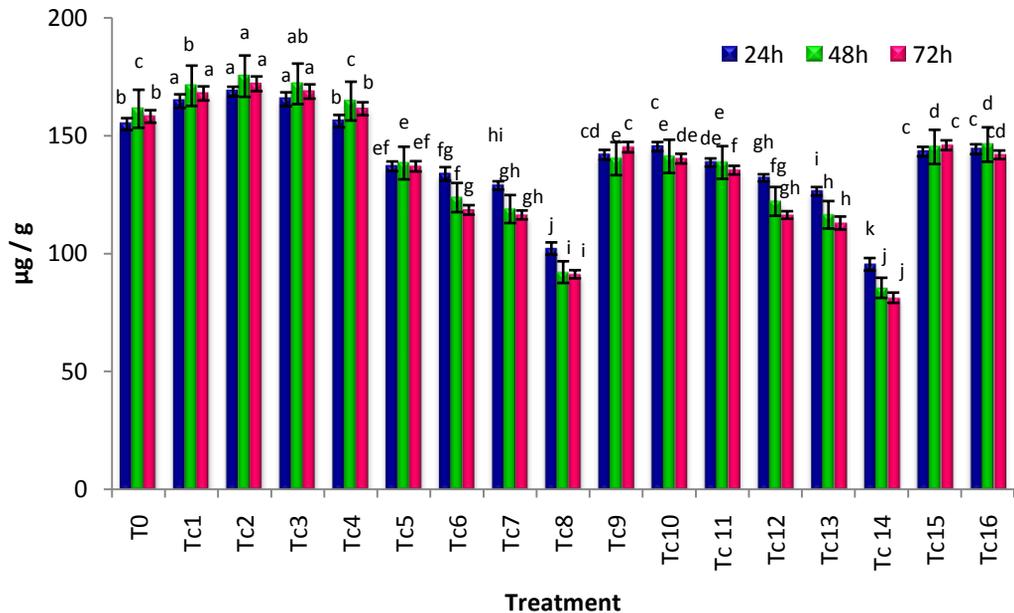
**Figure 4.6:** Total flavonol content of fenugreek sprouts primed with different elicitors of calcium at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



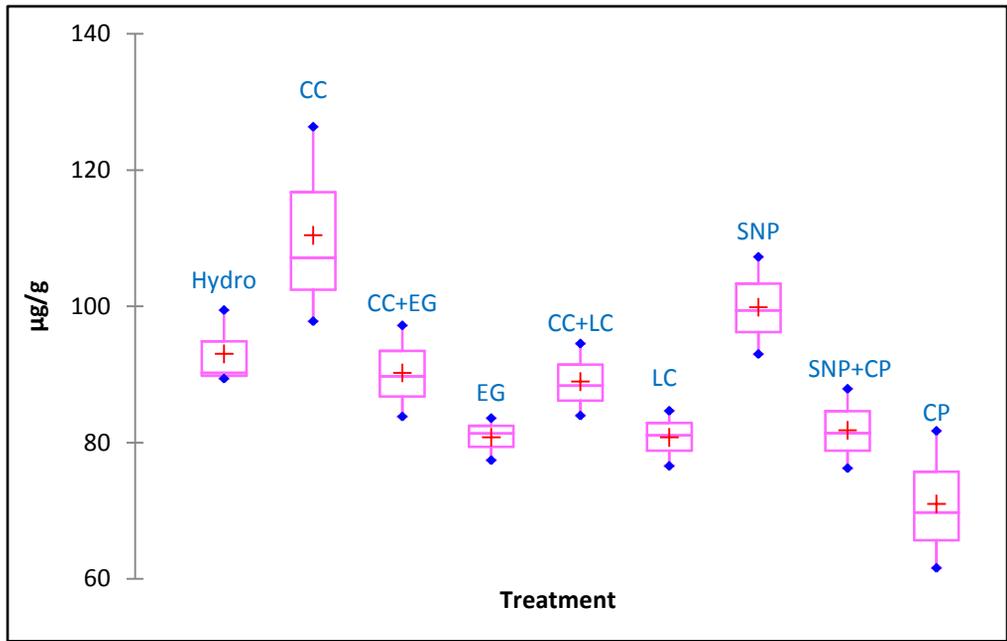
**Figure 4.7:** Total carotene content of fenugreek sprouts primed with different elicitors of calcium at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

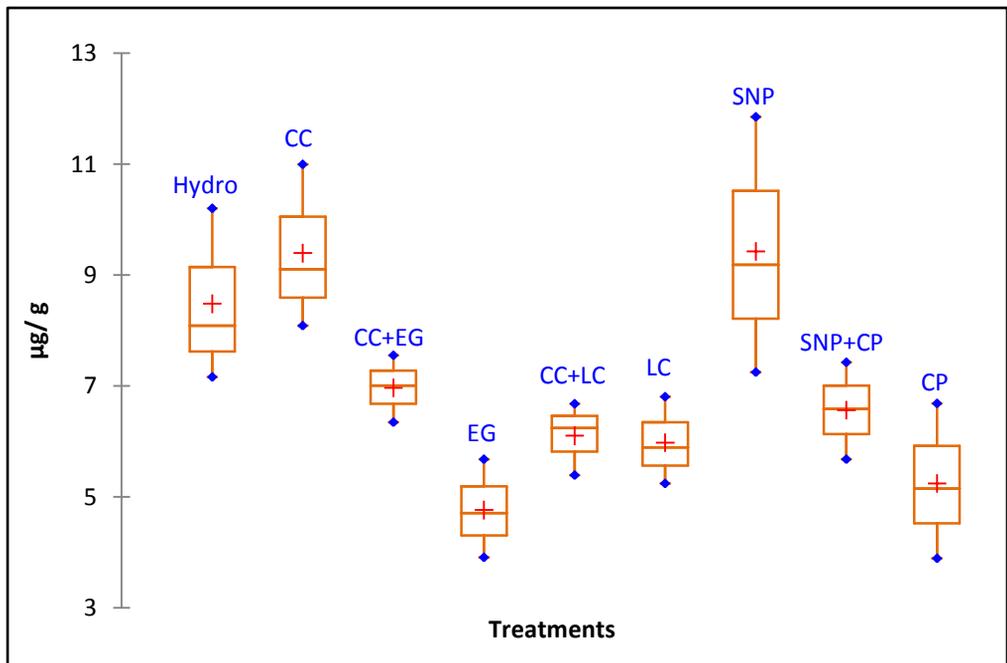


**Figure 4.8:** Total ascorbic acid content of fenugreek sprouts primed with different elicitors of nitric oxide at 24h , 48h & 72h stage of germination

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

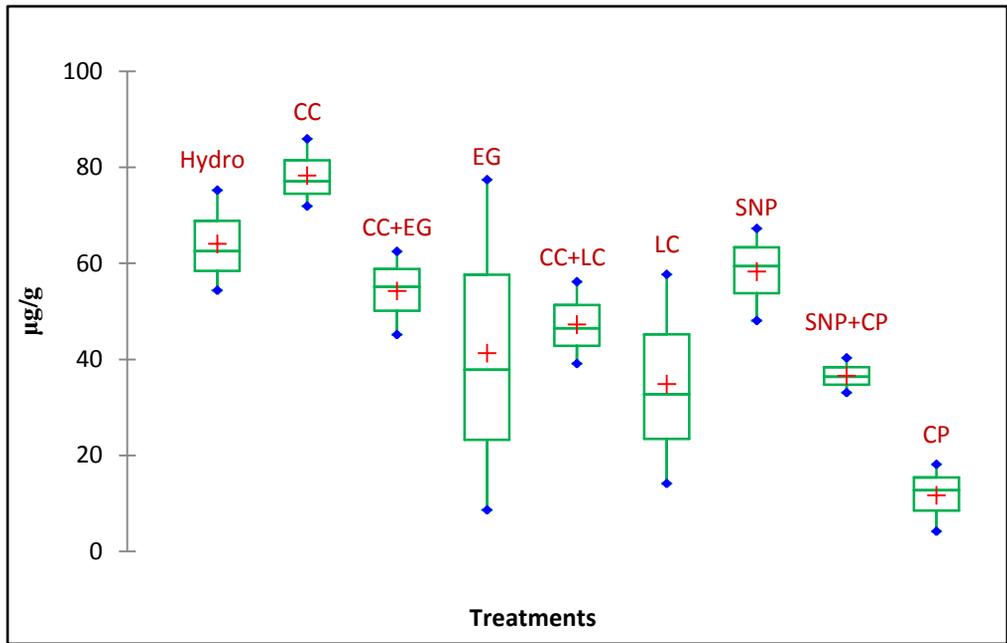


**Figure 4.9:** Effect of different priming agents on the total phenol content of fenugreek sprouts

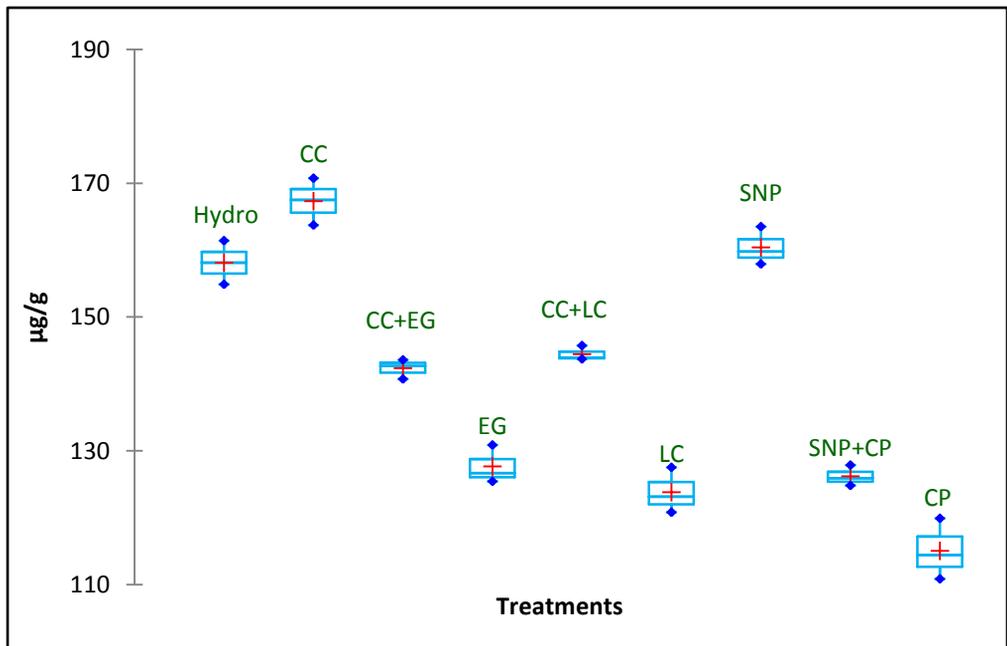


**Figure 4.10:** Effect of different priming agents on the total flavonol content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 4.11:** Effect of different priming agents on the total carotene content of fenugreek sprouts



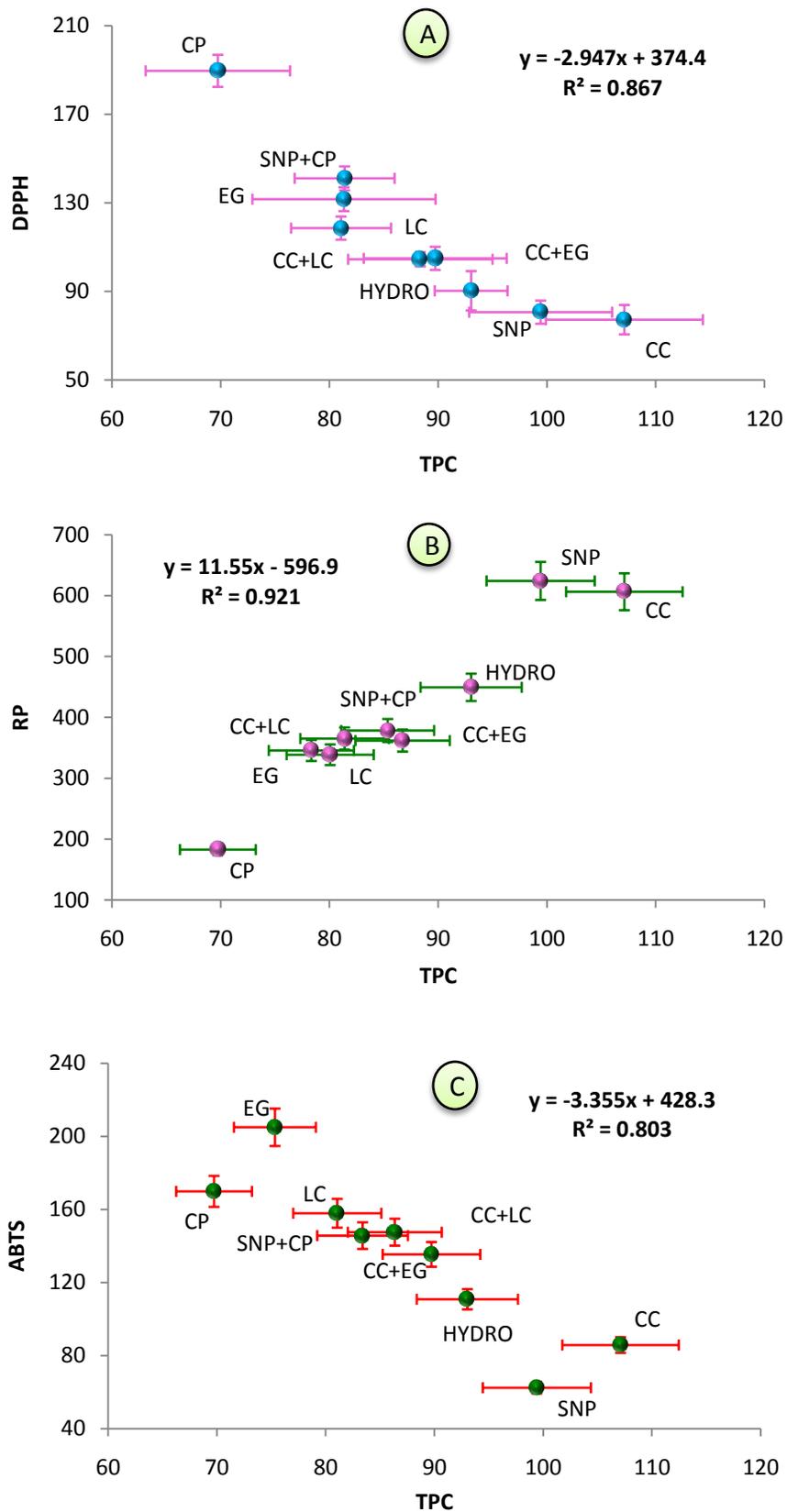
**Figure 4.12:** Effect of different priming agents on the total ascorbic acid content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

The priming of fenugreek seeds with the antagonists of nitric oxide and calcium resulted in loss of ascorbate content recording a maximum of 28% by nitric oxide scavenger; 39% by calcium channel blocker and about 34% by calcium chelator. The effect of the antagonists of nitric oxide and calcium on the phytochemical content of fenugreek sprouts was also investigated and the effect of these elicitors was found to be negative (Figure 4.9 - 4.12). EGTA had the greatest effect on reduction in the antioxidant property of the sprouts (discussed in earlier chapter) with corresponding loss in the synthesis of relative phytochemicals followed by  $\text{LaCl}_3$  and c-PTIO. This further confirms the probable role of the signal molecules, nitric oxide and calcium in the regulation of biosynthesis of antioxidant compounds in fenugreek sprouts during germination.

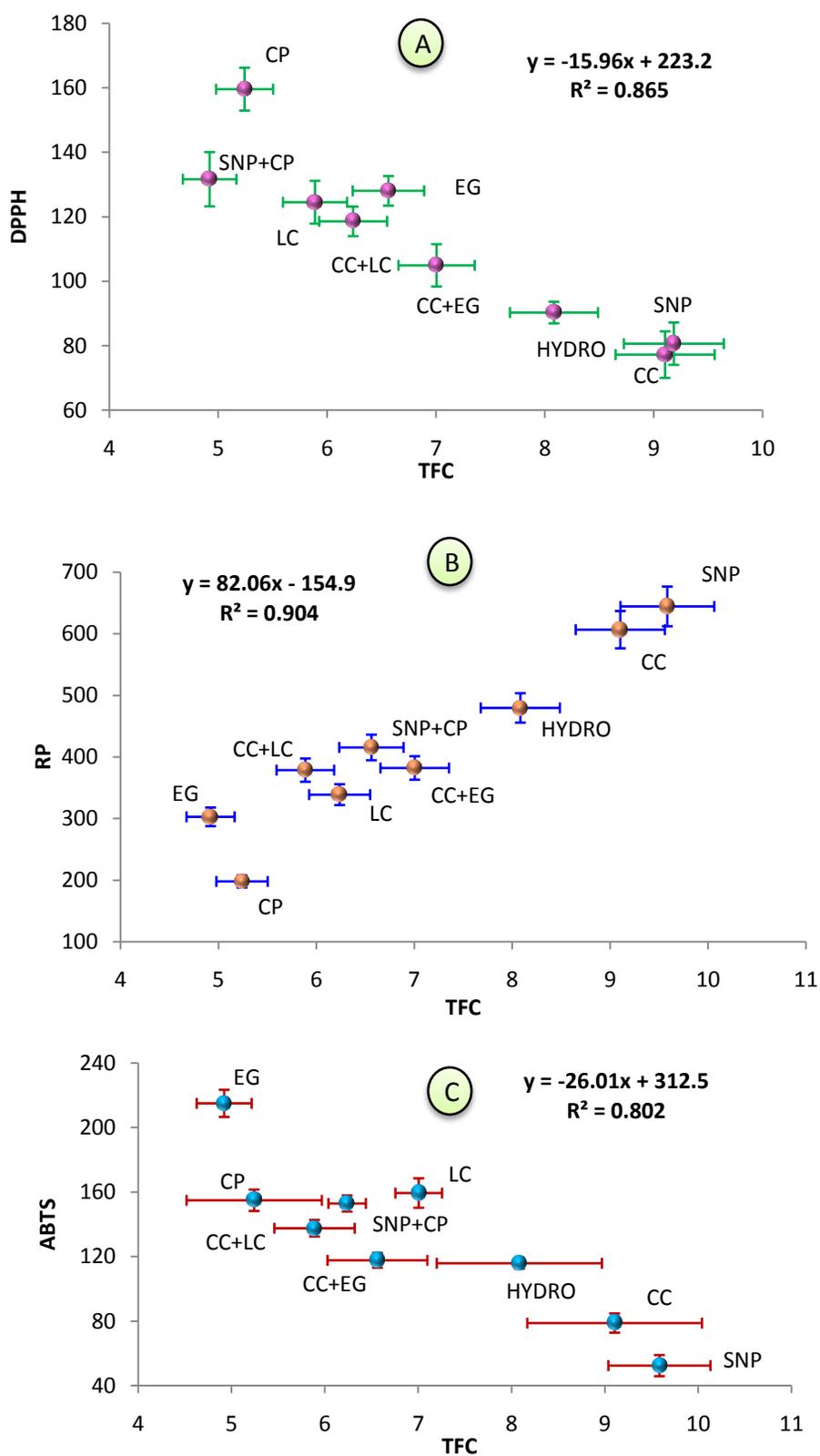
Previously several studies have already reported significant correlation between the antioxidant activity and the phytochemical content (Velioglu *et al.*, 1998; Lai and Lim, 2011). The results obtained from Pearson's correlation revealed that the different antioxidant activity of nitric oxide primed fenugreek sprouts are closely associated with the phytochemical attributes evaluated, thus indicating involvement of these phytochemicals in the removal of free radicals from cellular system. Furthermore, a significant correlation was observed among the entire four phytochemical attributes studied. It has been reported that the efficiency of plant phenolics in protection against oxidative stress depends on their reactivity towards reactive oxygen species (ROS). Reduction of phenoxyl radicals by the intracellular reductants is well-known to recycle phenolic antioxidants, thus enhancing antioxidant property (Kagan and Tyurina, 1998). Accordingly, total phenol content showed significant association with activities such as DPPH, ABTS, reducing power and  $\beta$ -carotene protective activity thus, indicating the potential role of phenol compounds in free radical scavenging activity as a reducing agent (Figure 14 & 15). Further, the impact of alteration in the phenol content of fenugreek sprouts on their antioxidant potential has been represented in Figure 4.13. Flavonoids possess a chemical structure with particular hydroxyl position in

the molecule that is considered to be involved in proton donating and radical scavenging mechanism (Hou *et al.*, 2003). On the other hand, besides DPPH and ABTS the flavonoids were found to be associated with metal chelating activity and also anti-lipid peroxidation. In agreement to our findings the potential role of flavonoids as a chelating agents and their protective effect on peroxidation of lipid bilayers have been suggested by various authors (Korkina and Afanasev, 1997; Durgo *et al.*, 2007). Interestingly, a significant correlation was observed between carotene content and  $\beta$ -carotene protective activity which was similar to the findings of Maisarah *et al.*, (2013) who also claimed a strong correlation between carotene content and  $\beta$ - carotene protective activity in their study. The results obtained from the Pearson's correlation of the antioxidant and phytochemical attributes of calcium elicitors primed sprouts were found to exhibit some variation in context of association among the attributes with correspondence to those of nitric oxide elicitors. Here the association of phenol content was much more strong with DPPH ( $r = -0.733$ ;  $R^2 = 0.537$ ) and reducing power ( $r = 0.894$ ;  $R^2 = 0.799$ ) (Figure 4.16 & Table 4.2) in comparison to that of coefficients value obtained for nitric oxide elicitors with DPPH ( $r = -0.667$ ;  $R^2 = 0.445$ ) and reducing power ( $r = 0.661$ ;  $R^2 = 0.437$ ) (Figure 4.16 & Table 4.1). Similar trend of correlation with different attributes where observed for flavonoids in addition to existence of highest correlation with metal chelating activity with coefficient values ( $r = -0.764$ ;  $R^2 = 0.584$ ) in comparison to that of phenol ( $r = -0.672$ ;  $R^2 = 0.452$ ); ascorbate ( $r = -0.652$ ;  $R^2 = 0.426$ ) and carotene ( $r = -0.596$ ;  $R^2 = 0.356$ ), thus suggesting the most pronounced role of flavonoids as chelating agents.



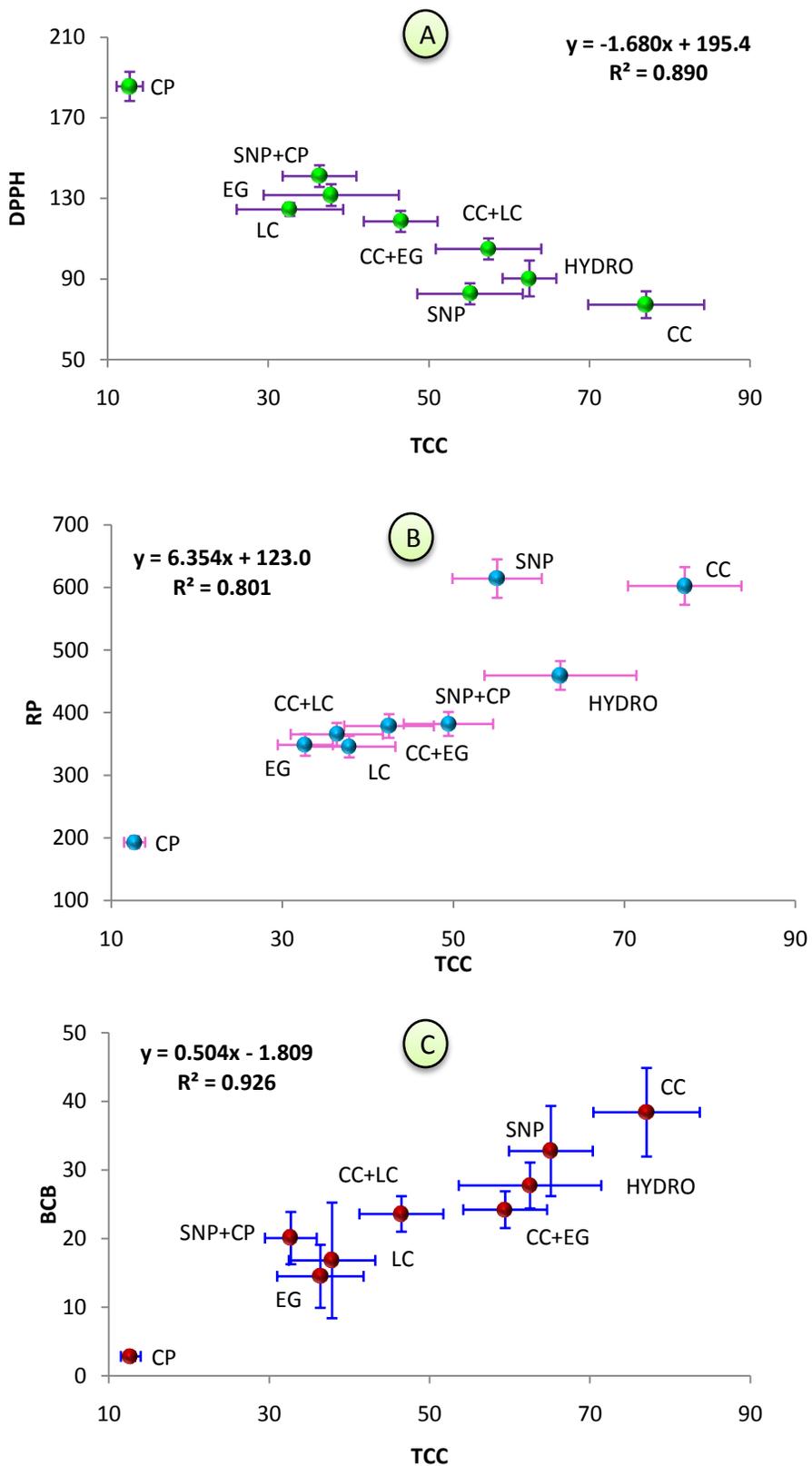
**Figure 4.13:** Effect of different priming agents on the A- DPPH, B-RP and C- ABTS with respect to alteration in the total phenol content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



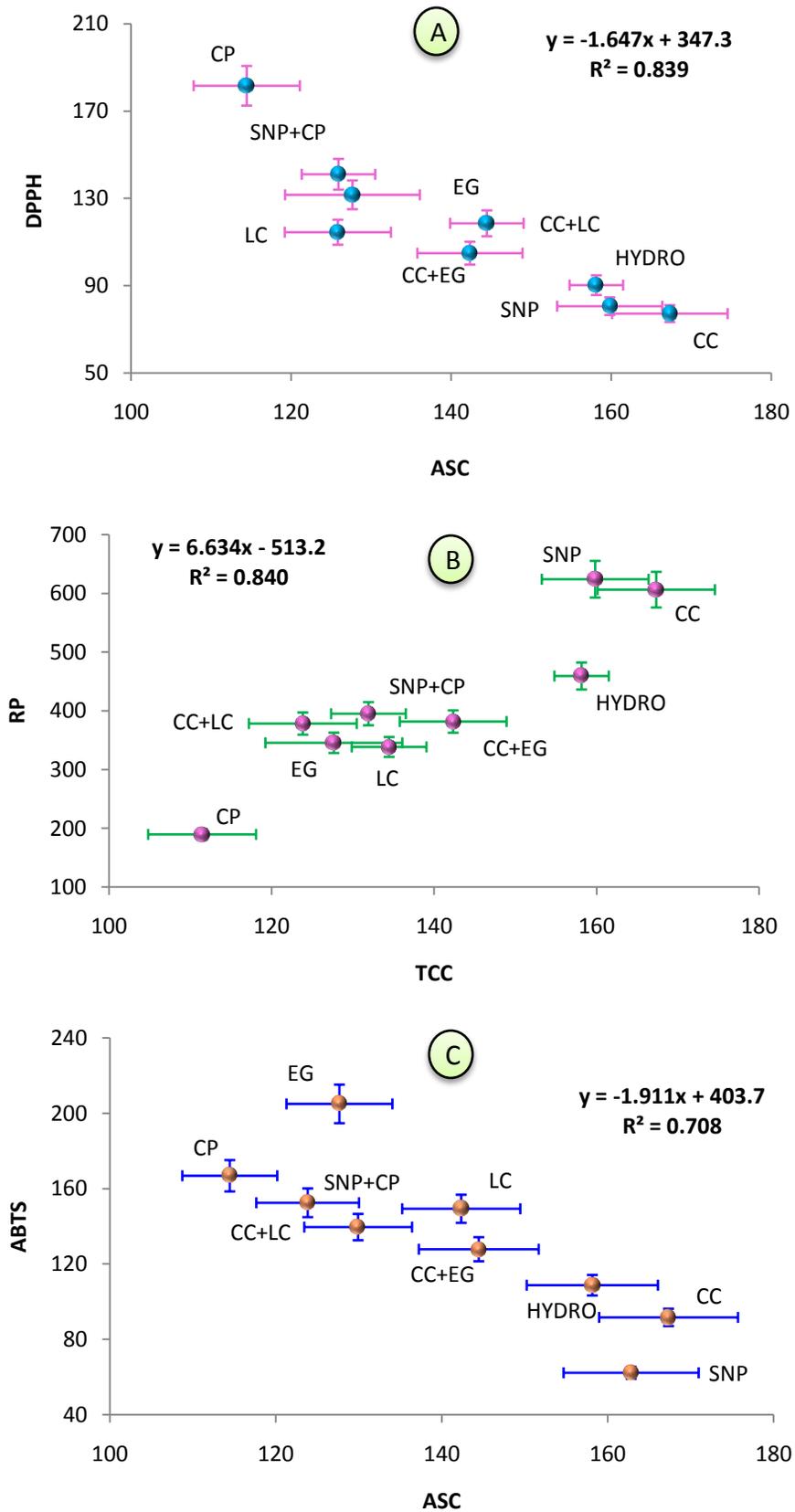
**Figure 4.14:** Effect of different priming agents on the A- DPPH, B-RP and C- ABTS with respect to alteration in the total flavonol content of fenugreek sprouts.

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



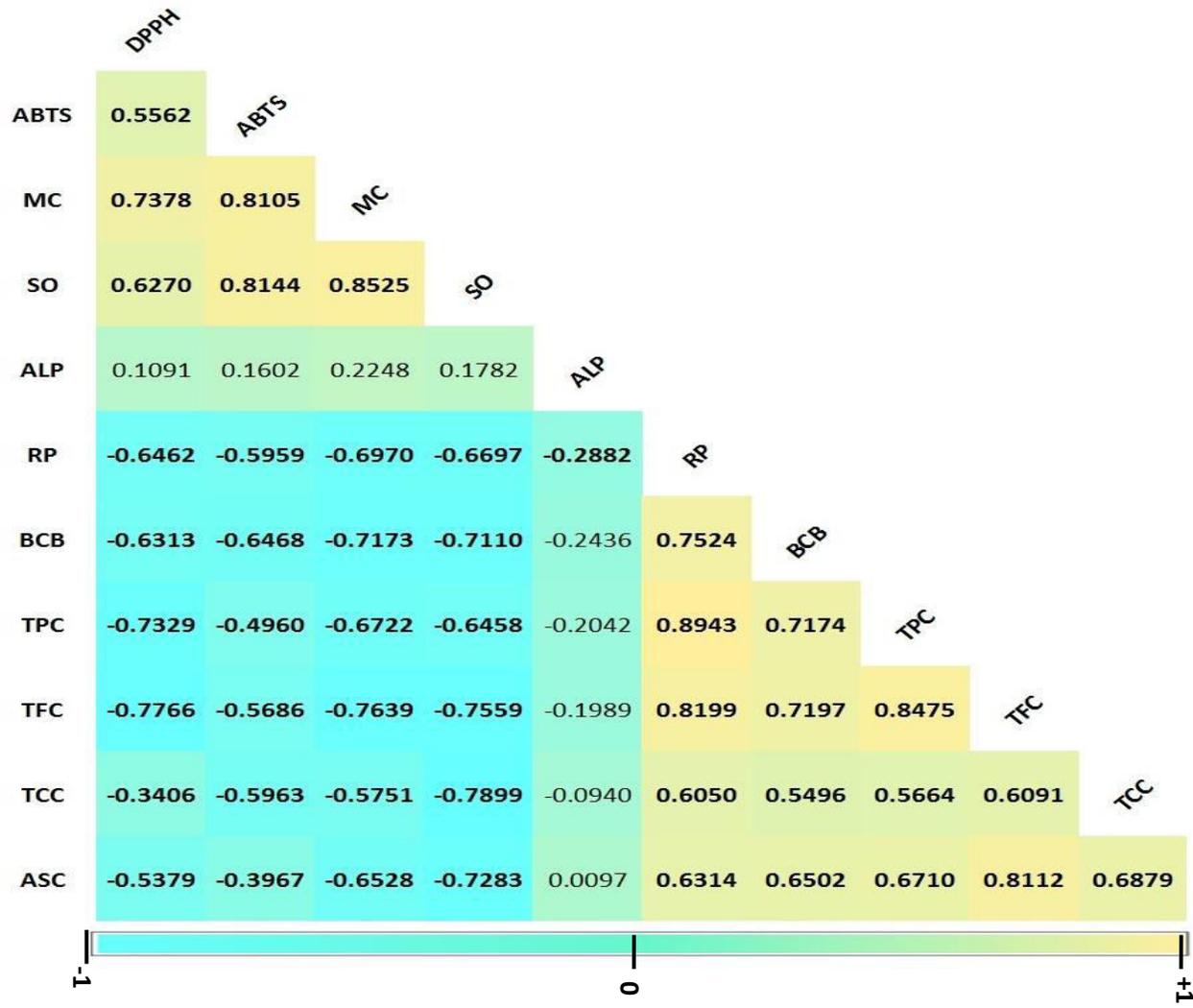
**Figure 4.13:** Effect of different priming agents on the A- DPPH, B-RP and C- BCB with respect to alteration in the total carotene content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 4.14:** Effect of different priming agents on the A- DPPH, B-RP and C- ABTS with respect to alteration in the total ascorbic acid content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



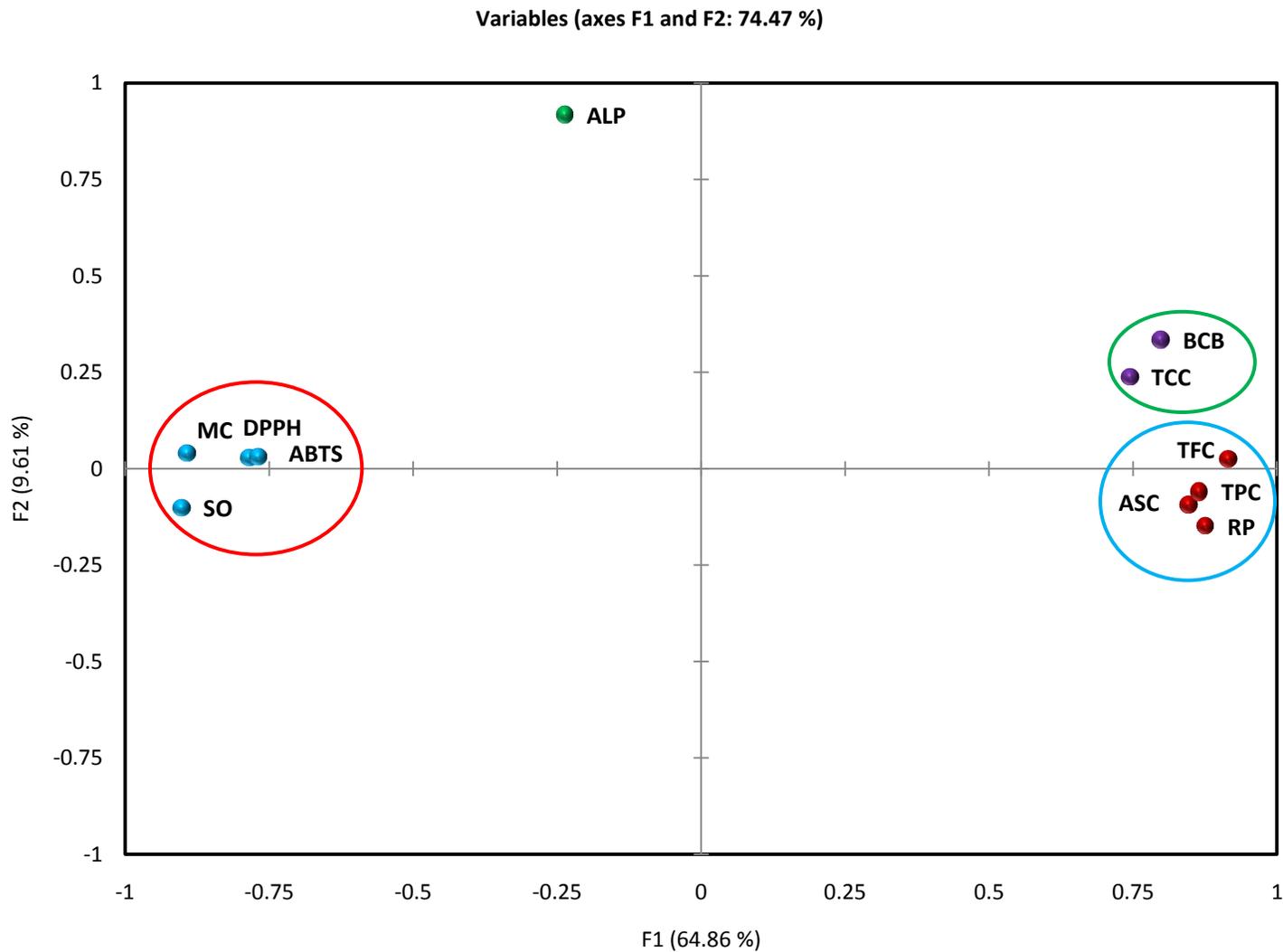
**Figure 4.15:** Correlogram depicting the correlation between different phytochemical and antioxidant attributes elicited by nitric oxide elicitors

Abbr. used: DPPH: DPPH scavenging activity; ABTS: ABTS scavenging activity, C: metal chelating; SO: superoxide scavenging activity; ALP: Antilipid peroxidation; RP: reducing power; BCB: β-carotene bleaching; TPC: total phenol content; TFC: total flavonoid content; TCC: total carotene content; ASC: ascorbic acid content



**Figure 4.16:** Correlogram depicting the correlation between different phytochemical and antioxidant attributes elicited by calcium elicitors

Abbr. used: DPPH: DPPH scavenging activity; ABTS: ABTS scavenging activity, C: metal chelating; SO: superoxide scavenging activity; ALP: Antilipid peroxidation; RP: reducing power; BCB: β-carotene bleaching; TPC: total phenol content; TFC: total flavonoid content; TCC: total carotene content; ASC: ascorbic acid content



**Figure 4.17:** Principal Component analysis of various antioxidant and phytochemical attributes of fenugreek sprouts primed with different priming agents

Abbr. used: DPPH: DPPH scavenging activity; ABTS: ABTS scavenging activity, C: metal chelating; SO: superoxide scavenging activity; ALP: Antilipid peroxidation; RP: reducing power; BCB:  $\beta$ -carotene bleaching; TPC: total phenol content; TFC: total flavonoid content; TCC: total carotene content; ASC: ascorbic acid content

**Table 4.1:** Coefficients of determination ( $R^2$ ) values of different antioxidant and phytochemical attributes of fenugreek sprouts primed with nitric oxide elicitors

	<b>DPPH</b>	<b>MC</b>	<b>RP</b>	<b>ABTS</b>	<b>SO</b>	<b>LP</b>	<b>BCB</b>	<b>TPC</b>	<b>TFC</b>	<b>TCC</b>
<b>TPC</b>	0.446	0.087	0.437	0.566	0.041	0.001	0.350			
<b>TFC</b>	0.444	0.248	0.432	0.346	0.009	0.141	0.450	0.501		
<b>TCC</b>	0.531	0.206	0.467	0.519	0.005	0.017	0.636	0.425	0.521	
<b>ASC</b>	0.666	0.144	0.633	0.703	0.025	0.014	0.494	0.463	0.410	0.702

**Table 4.2:** Coefficients of determination ( $R^2$ ) values of different antioxidant and phytochemical attributes of fenugreek sprouts primed with calcium elicitors

	<b>DPPH</b>	<b>ABTS</b>	<b>MC</b>	<b>RP</b>	<b>SO</b>	<b>ALP</b>	<b>BCB</b>	<b>TPC</b>	<b>TFC</b>	<b>TCC</b>
<b>TPC</b>	0.537	0.246	0.452	0.799	0.417	0.042	0.515			
<b>TFC</b>	0.603	0.323	0.584	0.672	0.571	0.039	0.518	0.718		
<b>TCC</b>	0.116	0.356	0.330	0.366	0.624	0.008	0.302	0.320	0.371	
<b>ASC</b>	0.289	0.157	0.426	0.397	0.530	0.001	0.423	0.450	0.658	0.473

**Table 4.3:** Correlation between different variables and factors obtained from PCA

	<b>F1</b>	<b>F2</b>
<b>DPPH</b>	<b>-0.7835</b>	0.0274
<b>ABTS</b>	<b>-0.7681</b>	0.0302
<b>MC</b>	<b>-0.8920</b>	0.0386
<b>RP</b>	<b>0.8762</b>	-0.1489
<b>SO</b>	<b>-0.9014</b>	-0.1020
<b>ALP</b>	-0.2363	<b>0.9169</b>
<b>TPC</b>	<b>0.8473</b>	-0.0939
<b>TFC</b>	<b>0.8657</b>	-0.0604
<b>ASC</b>	<b>0.9159</b>	0.0247
<b>BCB</b>	<b>0.7443</b>	0.2375
<b>TCC</b>	<b>0.7992</b>	0.3338

**Table 4.4:** Contribution (%) of different variables in factor loading obtained from PCA

	<b>F1</b>	<b>F2</b>
<b>DPPH</b>	<b>8.6042</b>	0.0708
<b>ABTS</b>	<b>8.2694</b>	0.0860
<b>MC</b>	<b>11.1523</b>	1.1412
<b>RP</b>	<b>10.7606</b>	2.0966
<b>SO</b>	<b>11.3892</b>	1.9834
<b>ALP</b>	0.7828	<b>79.5123</b>
<b>TPC</b>	<b>10.0629</b>	1.8347
<b>TFC</b>	<b>10.5041</b>	1.3445
<b>ASC</b>	<b>11.7575</b>	1.0576
<b>BCB</b>	<b>7.7645</b>	5.3355
<b>TCC</b>	<b>8.9525</b>	5.5373

Whereas, the association between carotene and  $\beta$ -carotene protective activity was less with  $r = 0.547$  and  $R^2 = 0.320$  in comparison to  $r = 0.797$  and  $R^2 = 0.636$  thus indicating difference in the degree of elicitation of the antioxidant components responsible for these activities by nitric oxide and calcium ion. Additionally, a significant correlation was observed among phenol, flavonoids, carotene and ascorbate under both the elicitors. Several other findings have suggested such correlation between phenol and flavonoids in various plant samples. Further, similar to our findings such positive correlation between antioxidant and phytochemical such as phenol, flavonoids, carotene and ascorbate have been suggested by several authors earlier (Wang and Linn, 2000; Rekika *et al.*, 2005; Shin, 2012; Salama *et al.*, 2015).

For further determining the precise relationship among different studied phytochemicals and antioxidant activity of fenugreek sprouts and also their contribution in antioxidant activity, principal component analysis (PCA) was performed. PCA on these attributes explained 74.47% of the variability in the data as shown in (Figure 4.17). The first two principal components accounted for 64.86% and 9.61% of the total data variance were chosen on the basis of their eigenvalues ( $>1$ ). The loading of PC1 had a significant correlation with  $IC_{50}$  values of DPPH, ABTS, metal chelation and superoxide scavenging activity; reducing power and  $\beta$ -carotene protective activity (Table 4.3). Additionally, the studied phytochemicals like total phenol, flavonol, carotene and ascorbate content were also found to be loaded heavily on PC1 having negatively correlation with the attributes of free-radical scavenging activity (Table 4.3). The strong association of the above mentioned attributes were evident in Figure 4.17 where they found to form separate clusters exhibiting possible cohesiveness among them. Further it was observed that the clustering of the attributes was found to be based on their contribution percentage on the principal component factor. For instance, DPPH, ABTS, metal chelation and superoxide scavenging activity with a contribution of 8.6%, 8.27%, 11.15% and 11.38% respectively (Table 4.4), were found

forming separate cluster. The reducing power, phenol, flavonoid and ascorbate were found clustered together with their contribution of 10.76%, 10.06%, 10.5% and 11.75% respectively (Table 4.4), thus further highlighting the role of these phytochemicals as reducing agents. The formation of another cluster by carotene content and  $\beta$ -carotene protective activity with their contribution of 7.76% and 8.9% in loading of F1 indicates the potential role of carotene content in the  $\beta$ -carotene protective activity of the fenugreek extract. Lipid peroxidation attribute was found to be exclusively separated from all other components in correspondence to its sole contribution of 79.51% in the loading of second principal component or F2 with correlation coefficient of 0.916. Overall, it was observed that though a significant correlation was established between the phytochemicals and antioxidant activities; on the other hand, insignificant correlation as well as difference in the significance level of correlation was also found between various attributes. This might be due to the variation in the stoichiometry of the reactions between the antiradical components in the sprout extracts and the various radicals, which may be considered as a probable reason for the difference in their scavenging potential of these free radicals (Khan *et al.*, 2012).

In conclusion, the present study illustrated significant increase of antioxidant activity along with phenolics and ascorbate content by elicited sprouting in fenugreek with exogenous nitric oxide and calcium. When analyzing the time course of germination the stimulatory effect of the elicitors was most pronounced at the early phases of germination i.e. from 24h to 48h and after that the action was declined confirming that the molecules responsible for antioxidant activity were elicited appropriately at this stage. Another reason could be that, during initial germination stages there is a higher oxygen demand and therefore phenolics might be involved in protecting the cells from possible oxidation-induced damages (Randhir *et al.*, 2004). Higher antioxidant activity was observed in pre-treated sprouts compared to control during late stages of germination indicates that most phenolics under natural conditions are diverted towards lignification with growth which was

also observed during elicitation of corn (Randhir and Shetty, 2005). Also, perhaps the change in the physiological condition due to elicitation might reduce the amount of phenolics partitioned for lignification because some are employed for antioxidant function against the ROS generated during the process. The work supports the hypothesis that nitric oxide and calcium offer significant role in enhancement of phenolics and antioxidant activity during the germination phase of fenugreek. This knowledge may be used to design the sprouting techniques of fenugreek for improving the nutritional and health relevant functional value of legume sprouts with these elicitors.

## **CHAPTER- 5**

### ***IN VITRO* ANTIDIABETIC ACTIVITY OF GERMINATING FENUGREEK SPROUTS UNDER THE INFLUENCE OF CALCIUM AND NITRIC OXIDE ELICITORS**

## 5.1 INTRODUCTION

Plants are the basic source of knowledge of modern medicine. Almost all the parts of a plant, namely leaves, flowers, fruits, bark, roots, stem and seeds are known to have various medicinal properties (Mori and Prance, 1978). The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of potential bioactive components (Ramma *et al.*, 2002; Senthilkumar *et al.*, 2014).

India with highest number of people suffering from diabetic disorders has been considered as the diabetic capital of the world by the 'International Journal of Diabetes in Developing Countries'. There is an alarming rise in diabetes patients in India; approximately 3.4 million deaths occur due to complication related to high blood sugar (Kumar *et al.*, 2012).

Diabetes mellitus is a serious metabolic disorder that leads to hyperglycemic condition due to decreased insulin production or inefficient insulin utilization. It is usually characterized by hyperglycemia, lipoprotein abnormalities, high basal metabolic rate, impairment in the activity of important enzymes and oxidative stress which damages the pancreatic beta cells. It is the most common endocrine disorder which disrupts glucose homeostasis causing severe diabetic associated complications in major organs like eye, blood vessels and brain (Sharma *et al.*, 2010; Jaiswal *et al.*, 2014). Multiple risk factors responsible for the disease to occur include persistent stress and depression, obesity, environmental pollutants and sedentary life style (Ghamarian *et al.*, 2012).

Some of the synthetic antidiabetic components such as metformin, acarbose, biguanides and voglibose are found to be used clinically in combination with other diet to control diabetes but moreover they exhibit adverse side effects after long term use (Asano 2009; Standl and Schnell, 2012; Patel *et al.*, 2012). To prevent or overcome the side effects

of these synthetic drugs and also to create other safer alternative drug choices, it has become essential to seek other inhibitors for further drug development. Thus, in recent years, several efforts have been made for increasing the availability of glucosidase inhibitors from natural sources (Jung *et al.*, 2006; Jin *et al.*, 2013).

Likewise, fenugreek besides having several pharmacological properties it is also reported to possess potent antidiabetic property both *in vitro* and *in vivo* system. Fenugreek is known to be a rich source of various bioactive components having different therapeutical property such as saponins, fenugreekine, nicotinic acid, phytic acid, and trigonelline (Puri, 1998; Randhir *et al.*, 2004). Trigonelline is a pyridine alkaloid known to be mostly found in Fabaceae members and is reported to be metabolically active as hypocholesterolemic agent along with potential hypoglycemic effect (Taguchi, 1986; Olthof *et al.*, 2011; Mathur and Kamal, 2012). Fenugreek has been successfully implemented as antidiabetic remedy for both type I and II diabetes (Zia *et al.*, 2001). Moreover, fenugreek has been reported to be enriched with wide spectrum of pharmacological and folkloric significance (Meghwal and Goswami, 2012).

The present study deals with the isolation and identification of trigonelline by 1-D and 2-D NMR from fenugreek sprouts. Further the elicitors of calcium and nitric oxide were applied for the enhancement in the alkaloid content of fenugreek sprouts along with the *in vitro* anti-diabetic property. The present study may provide an insight in the role of these signaling molecules in modulating the biosynthesis of alkaloids, a potential hypoglycemic agent; additionally the utility of 2-D NMR spectroscopy in identification of bioactive compounds.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Elicitation process and germination

Mentioned in section 3.2.1

**Table 5.1: Different priming agents applied**

Calcium elicitors		Nitric oxide elicitors	
T0	H <sub>2</sub> O	T0	H <sub>2</sub> O
Tc1	CC-1mM	Tn1	SNP-10mM
Tc2	CC-2mM	Tn2	SNP-20mM
Tc3	CC-5mM	Tn3	SNP-40mM
Tc4	CC-1mM+EG500µM	Tn4	SNP-80mM
Tc5	CC-2mM+EG500µM	Tn5	SNP-120mM
Tc6	CC-5mM+EG500µM	Tn6	SNP10mM+CP125µM
Tc7	EG-2mM	Tn7	SNP20mM+CP125µM
Tc8	CC-1mM+LC500µM	Tn8	SNP40mM+CP125µM
Tc9	CC-2mM+LC500µM	Tn9	SNP80mM+CP125µM
Tc10	CC-5mM+LC500µM	Tn10	SNP120mM+CP125µM
Tc11	LC-2mM	Tn11	CP125 µM

### 5.2.2 Determination of antidiabetic property by *in vitro* method

#### 5.2.2.1 Preparation of extract

The fenugreek sprouts of 3 different stages: 24h, 48h & 72h were crushed in mortar-pestle and individually processed through soxhlet extraction apparatus with methanol for 8h duration. The refluxed samples were separated from the residues by filtering through

Whatman No. 1 filter paper and the extract was concentrated to a uniform concentration of 1 g/ml using a vacuum rotary evaporator at 50°C. The obtained methanolic extracts were further used for experimental analysis for determination of antidiabetic activity and total alkaloid estimation.

#### **5.2.2.2 Estimation of $\alpha$ -amylase enzyme inhibitory activity**

The alpha-amylase (AA) inhibition potential of the extract was estimated by standard spectrophotometric method (Paul and Banerjee, 2013). 0.5 ml of aqueous extract was reacted with 0.5 ml of  $\alpha$ -amylase solution and incubated at 37°C for 5 min. After incubation, 0.5 ml starch solution (1%) was added and was further incubated for 10 min. To the above reaction mixture, 1 ml of DNSA reagent was added to terminate the reaction and was heated for 10 min in a hot water bath till the colour of reaction mixture colour changed to orange-red. After change in colour, the reaction mixture was cooled and diluted up to 5 ml with distilled water. The OD value was measured at 540 nm. The  $\alpha$ -amylase inhibitory activity was estimated by measuring the concentration of inhibitor required to inhibit 50% of the enzyme activity.

$$\% \text{ Inhibition} = (A_{540 \text{ control}} - A_{540 \text{ sample}}) / (A_{540 \text{ control}}) \times 100$$

Where  $A_{540 \text{ control}}$  = Absorbance of control at 540 nm and  $A_{540 \text{ sample}}$  = Absorbance of sample at 540 nm.

#### **5.2.2.3 Estimation of $\alpha$ -glucosidase enzyme inhibitory activity**

The  $\alpha$ -Glucosidase (AG) inhibitory property of the sample extract was assayed according to Dong *et al.*, (2012) with slight modifications. The reaction was initiated with 0.05 ml each of the samples at different concentrations in 0.2 mM phosphate buffer (pH 6.8), followed by incubation at 37°C for 15 min, after which 0.1 ml of enzyme solution was immediately added to the mixture before mixing and incubation at 37°C. Then, 3 mM *p*-nitrophenyl

glucopyranoside (pNPG) (0.25 ml) was added, after which the reaction was terminated by the addition of 4 ml of 0.1 M Na<sub>2</sub>CO<sub>3</sub>. Alpha-Glucosidase inhibitory activity was determined by measuring the release of pNPG at 405 nm. The control contained all reagents without the tested sample. The reactions were conducted in triplicate. The  $\alpha$ -glucosidase inhibitory activity was calculated as follows:

$$\text{Inhibitory \%} = [1 - (A_s - A_b) / A_c] \times 100$$

where A<sub>c</sub>, A<sub>s</sub>, and A<sub>b</sub> represent the absorbance levels of the control, sample, and blank, respectively. The concentration of  $\alpha$ -glucosidase inhibitor required to inhibit 50% of  $\alpha$ -glucosidase activity under the assay conditions is defined as the IC<sub>50</sub> value.

### **5.2.3 Total alkaloid estimation**

The extract was gently heated and methanol was evaporated on a rotary evaporator under vacuum at a temperature of 45°C to dryness. The residue was dissolved in 2 N HCl and then filtered. One ml of this solution was transferred to a separation funnel and washed with 10 ml chloroform (3 times). The pH of the solution was adjusted to neutral with 0.1N Sodium hydroxide. Then 5 ml of Bromocresol Green solution and 5 ml of phosphate buffer were added to the reaction mixture. The mixture was shaken and the complex formed was extracted with 5ml chloroform by vigorous shaking. The extracts were collected in a 10-ml volumetric flask and diluted to same volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank (Shamsa *et al.*, 2008). Similarly trigonelline content was also estimated from the purified extract.

### **5.2.4 Isolation of Trigonelline**

The extraction procedure was followed as suggested by Ahmed *et al.*, (2011) with some modifications. The fenugreek sprouts of about 100 g were homogenized in 10 M HCl solution and gently boiled for 2h in an Erlenmeyer flask. Next the mixture was filtered and zinc dust was added to the mixture and kept on magnetic stirrer for overnight. In next

morning, the mixture was filtered again and extracted with equal volume of dichloromethane ( $\text{CH}_2\text{Cl}_2$ ). The  $\text{CH}_2\text{Cl}_2$  layer containing the alkaloid was separated and later evaporated. The obtained extract after  $\text{CH}_2\text{Cl}_2$  evaporation was re-constituted in methanol for further analysis.

#### **5.2.5 Thin Layer Chromatography (TLC)**

The extract dissolved in methanol was applied manually on activated TLC plates, along with standard trigonelline. After loading the samples the TLC plates were developed in pre-saturated chromatography solvent chamber containing solvent system composed of ethylacetate:methanol: $\text{H}_2\text{O}$  (100:13.5:10). After running the solvent through the TLC plates, the plates were sprayed with Dragendorff's reagent to develop spot corresponding to standard trigonelline simultaneously.

After confirming the presence of trigonelline, in next TLC experiment after running the solvent through the TLC plate, the portion of plate containing pure trigonelline was cut and sprayed with Dragendorff's reagent and then the area of TLC plate containing unknown extract corresponding to band developed on the plate with standard trigonelline was scrapped and collected, dried and later dissolved in methanol for further quantitative and spectral analysis.

#### **5.2.6 Infra-red (IR) spectral analysis**

IR spectra of the crude, purified sample and standard trigonelline were recorded on FTIR spectrophotometer (model: 8300 Shimadzu) by nujol mulling for liquid sample and KBr pellets for solid sample.

#### **5.2.7 NMR analysis**

The confirmation of the structure of the extracted compound was done by NMR- spectra in 300 MHz Bruker-Avance spectrometer using 5mm BBO probe and Methanol-d<sub>4</sub> was used as solvent.

### **5.2.8 Statistical analysis**

Each experiment was performed in triplicate. The software package, MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antidiabetic activity and alkaloid content of the fenugreek sprouts. The different group means were compared by Duncan's Multiple Range Test through DSAASTAT software (version 1.002; DSAASTAT, Perugia, Italy);  $p < 0.05$  was considered significant in all cases. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC<sub>50</sub> values of antidiabetic activity and their standard error of estimates.

## **5.3 RESULTS AND DISCUSSION**

The key enzymes in the digestive system,  $\alpha$ -amylase and  $\alpha$ -glucosidase are actively involved in the release of glucose in the blood via breakdown of starch. Hence, the inhibition of these enzymes would minimize the breakdown of starch, which may lead to a reduction in the post-prandial hyperglycemic level (Tarling *et al.*, 2008). Therefore, determination of the capability of an extract to inhibit the activity of these enzymes  $\alpha$ -amylase and  $\alpha$ -glucosidase is essential for investigating the antidiabetic potentiality of the extract. Since fenugreek has been well known for its antidiabetic activity, the present study aimed at enhancing the antidiabetic activity of fenugreek sprouts through elicited sprouting. The inhibition capacity of the sprouts was expressed in terms of IC<sub>50</sub> values. The results revealed that the fenugreek sprouts exhibited potential antidiabetic property, and interestingly, it was also observed that the exogenous supply of Ca<sup>2+</sup> and nitric oxide resulted in the enhancement of this activity in fenugreek sprouts. Both the  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition capacity of fenugreek sprouts was enhanced by CC (Table 5.2) and SNP (Table 5.3).

In many plant species the alkaloids are reported to get accumulated in seeds in relatively higher amount as a chemical defense. During germination these alkaloids are metabolized and their nitrogen is further used for seedling's metabolism (Wink and Witte, 1985). The antidiabetic property of most legumes including fenugreek is usually attributed to their trigonelline content, an alkaloid with scientifically well characterized hypoglycemic effect (Randhir *et al.*, 2004; Yoshinari *et al.*, 2013). Trigonelline is reported to act by regulating cell regeneration, insulin secretion, enzymes associated with glucose metabolism. It is further known to mitigate oxidative stress during the diabetic complications (Yoshinari *et al.*, 2009; Amaro *et al.*, 2014). Considering such potential role of alkaloids in controlling the diabetic disorders, the alkaloid content of the fenugreek seedlings subjected to various priming agents were determined. Interestingly, it was found that the sprouts pre-treated with CC and SNP exhibited significantly higher alkaloid content with respect to control sprouts Figure 5.1. Similar trend in the effect of different priming agent was observed in the trigonelline content of the fenugreek sprouts (Figure 5.2). The potential hypoglycaemic property of trigonelline was further supported by our results which clearly depicted the significant influence of trigonelline content on the AG (Figure 5.3) and AA (Figure 5.4) inhibitory capacity of fenugreek sprouts pre-treated with different priming agents. On the other hand, the antagonists, EG, LC and CP showed inhibitory effect on the alkaloid accumulation in the sprouts. In agreement to the earlier cited statement, it was noted that the sprouts with higher trigonelline content exhibited stronger  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition activity thus suggesting the probable role of alkaloids especially trigonelline as hypoglycemic compounds in the management of diabetic disorders.

Furthermore, various authors have proved that the enhancement in the hypoglycemic activity of fenugreek is considerably associated with increase in the level of bioactive components such as trigonelline and 4-hydroxy leucine (Ahmed *et al.*, 2011; El-Soud *et al.*, 2007; Narender *et al.*, 2006).

**Table 5.2:** *In vitro*  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition activity of fenugreek sprouts under the influence of different nitric oxide elicitors.

Treatment	24h		48h		72h	
	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)
<b>T0</b>	1010±47.76e	1186.75±38.72de	715.20±31.24c	840.36±42.36d	1122.81±29.98d	1319.30±24.21c
<b>Tn1</b>	533.33±22.16b	981.33±11.05c	420.01±9.22ba	772.8±8.72c	765.14±21.94b	1407.86±18.44d
<b>Tn2</b>	377.86±12.78a	581.90±12.62a	350.91±8.56a	540.41±7.16a	630.38±17.04a	970.78±8.54a
<b>Tn3</b>	635.81±8.15c	915.57±9.29b	451±11.05b	649.44±7.95b	839.75±18.69c	1209.25±12.05b
<b>Tn4</b>	822.96±12.33d	1431.95±12.67f	836.32±12.06d	1455.21±18.52g	1215.47±27.24e	2114.92±22.48i
<b>Tn5</b>	1313.16±11.22g	1497.0±26.38g	1464.12±19.14i	1669.09±18.32h	1962.27±39.97i	2236.99±19.86j
<b>Tn6</b>	1341.30±12.56gh	1166.93±9.08d	1354.88±14.17h	1178.75±10.22f	2375.56±23.15j	2066.73±21.62hi
<b>Tn7</b>	1160.0±9.42df	1032.40±14.12bc	1197.09±12.32f	945.71±8.25e	1992.78±13.73i	1574.29±18.24e
<b>Tn8</b>	1071.33±11.24f	1229.90±8.54e	1146.82±13.16e	860.11±8.66d	1406.67±15.67f	1448.86±16.92d
<b>Tn9</b>	1376.40±13.05h	1575.97±14.16h	1294.28±12.11g	1481.95±18.45g	1625.24±17.14g	1860.90±24.68f
<b>Tn10</b>	1704.09±19.19j	1917.0±17.32j	1820.76±22.04k	2048.36±24.35j	1819.86±12.05h	2047.33±18.78h
<b>Tn11</b>	1559.38±14.22i	1748.06±21.41i	1608.13±14.28j	1802.71±22.08i	1768.12±11.36h	1982.06±21.86g

Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) in columns are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test

**Table 5.3:** *In vitro*  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition activity of fenugreek sprouts under the influence of different calcium elicitors

Treatment	24h		48h		72h	
	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)
<b>T0</b>	1010±47.76b	1186.75±38.72c	715.20±31.24c	840.36±42.36d	1122.81±29.98d	1319.30±24.21c
<b>Tc1</b>	336.52±28.05a	619.20±15.02b	330.0±22.37a	607.20±12.08b	717.50±21.94b	1320.20±19.12c
<b>Tc2</b>	339.42±22.32a	522.71±10.33a	301.12±13.77a	463.71±20.48a	599.80±17.04a	923.69±14.34a
<b>Tc3</b>	348.94±16.69a	502.48±12.05a	464.68±23.13b	669.15±23.95c	795.38±18.69c	1145.35±12.72b
<b>Tc4</b>	1055.23±22.42b	1202.97±32.15c	773.20±16.08d	1345.36±27.99i	1176.25±27.24d	1576.17±18.79e
<b>Tc5</b>	1593.33±12.54d	1386.20±36.44e	1289.22±18.33g	1121.43±11.86f	1869.88±39.97h	1626.79±16.76ef
<b>Tc6</b>	1634.5±15.69d	1291.97±9.56d	1610.71±25.36h	1272.46±13.40h	1906.36±23.15h	1506.02±23.17d
<b>Tc7</b>	2276.66±29.58e	1707.50±24.45f	1732.11±17.87i	1299.08±13.25hi	2284.50±13.73i	1713.37±13.90g
<b>Tc8</b>	1047.41±10.56b	1199.29±13.22c	832.85±12.42e	953.63±10.55e	1326.92±15.67e	1519.32±17.09d
<b>Tc9</b>	1141.54±12.36c	1284.23±12.42d	1075.21±19.11f	1209.62±19.23g	1598.89±17.14f	1798.75±20.33h
<b>Tc10</b>	1148.09±9.44c	1287.02±8.56d	1258.18±12.45g	1410.42±17.19j	1738.04±12.05g	1885.77±18.90i
<b>Tc11</b>	2545±34.22f	1679.70±22.72f	1294.58±14.62g	1513.34±12.14k	1700.80±11.36g	1632.76±18.24df

Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) in columns are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test

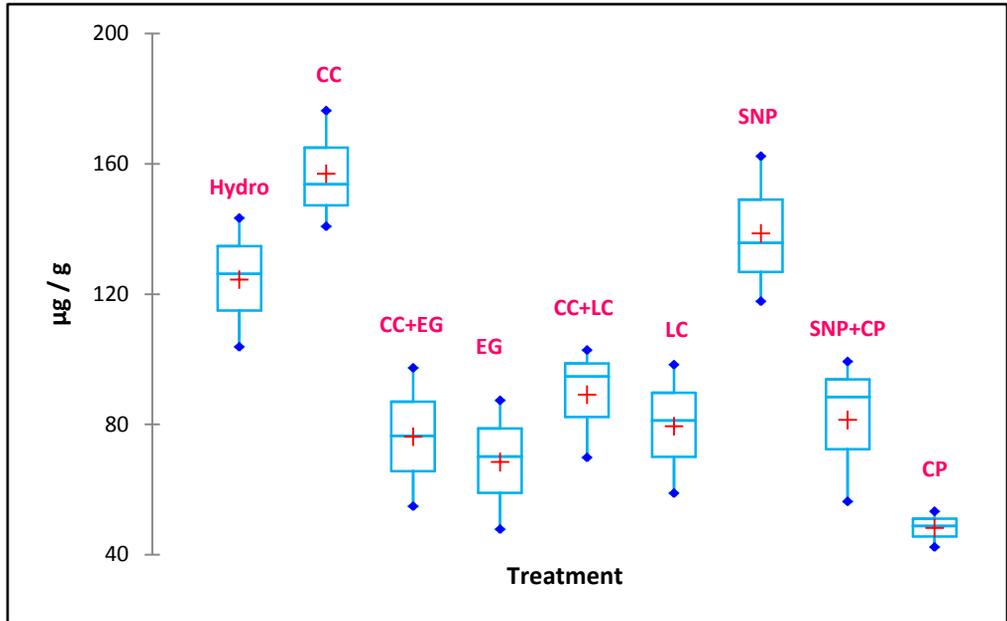
**Table 5.4:** Two way ANOVA analysis of antidiabetic and trigonelline content of differently primed fenugreek sprouts at various days of germination stage

Source of Variation	df	<i>F crit</i>	AG		AA		TC	
			<i>F</i>	<i>P-value*</i>	<i>F</i>	<i>P-value*</i>	<i>F</i>	<i>P-value*</i>
<b>Treatment</b>	23	1.604555	91163.68	1.1E-287	70049.33	1.8E-279	1314.238	2.6E-155
<b>Days of germination</b>	2	3.058928	131427.4	1.5E-235	178824.7	3.5E-245	1838.919	3E-103
<b>Interaction</b>	46	1.453191	4813.891	5.2E-209	7558.876	4.1E-223	69.94316	1.9E-78
<b>Within</b>	144							
<b>Total</b>	215							

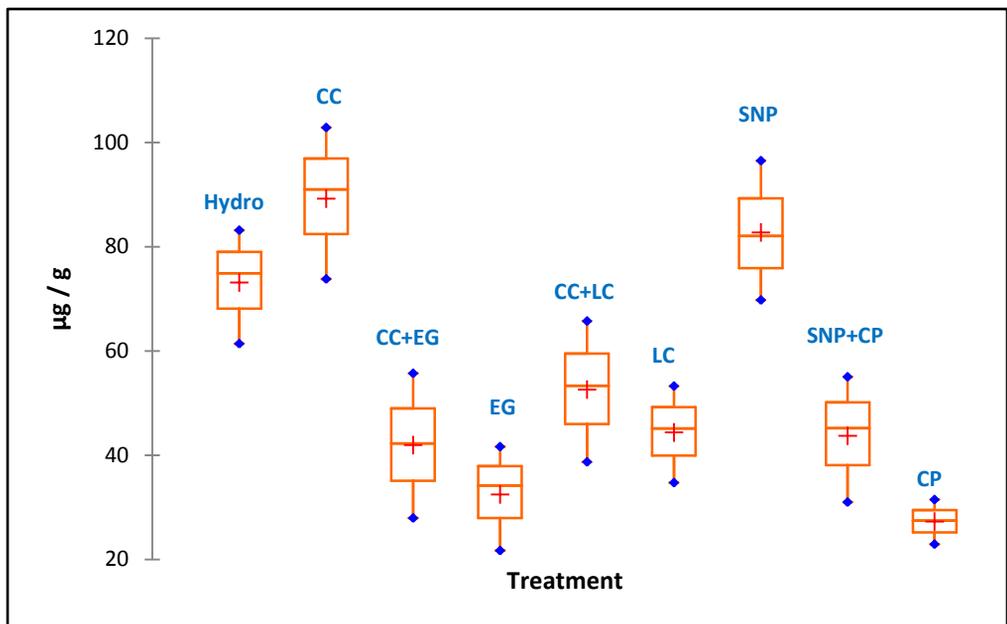
\*Significant at  $P < 0.01$

Further, two-way ANOVA analysis with replication was performed to determine the impact of different priming agents and various germination stages on antidiabetic and trigonelline content of fenugreek sprouts. As a result, a significant impact of both the variance i.e. priming agents and germination stages was found on the AA and AG inhibitory activity along with the trigonelline content (Table 5.4). Interactions between both were also significant at  $p < 0.05$  level.

The crude extract and the purified extract of fenugreek sprout were subjected to IR spectral analysis. The finger print region of both the crude and purified extract matched exactly with the that of standard trigonelline (Figure 5.6), this indicates all the samples are of same nature, and further confirms the predominance of trigonelline in both the extract (crude and purified). In the frequency range 3000 to 3500  $\text{cm}^{-1}$  broad strong absorption indicates presence of -OH groups, both intra and intermolecular H-bonded. At 1715  $\text{cm}^{-1}$  the absorption corresponds the presence of carboxylic acid group.

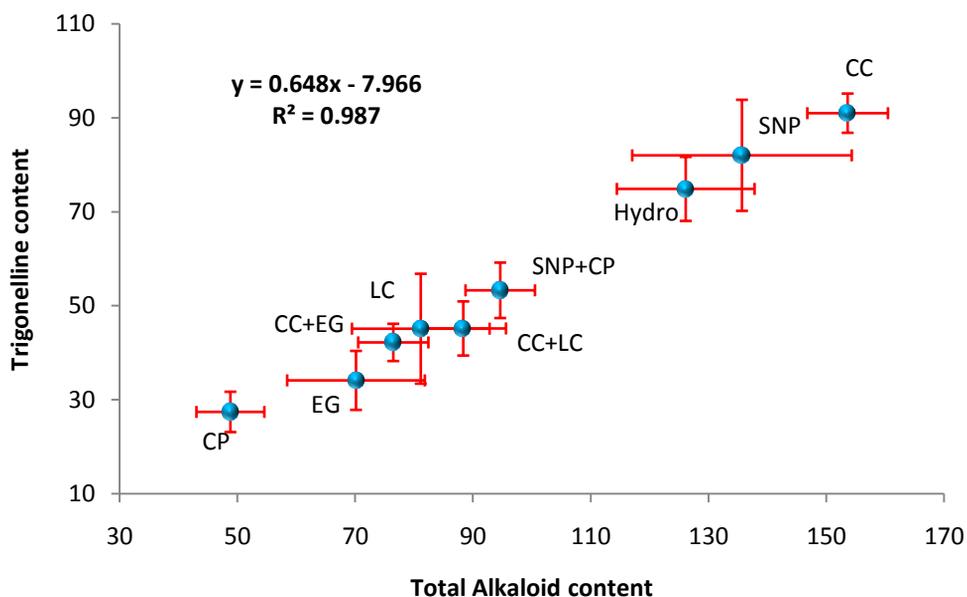


**Figure 5.1:** Effect of different priming agents on total alkaloid content of fenugreek sprouts

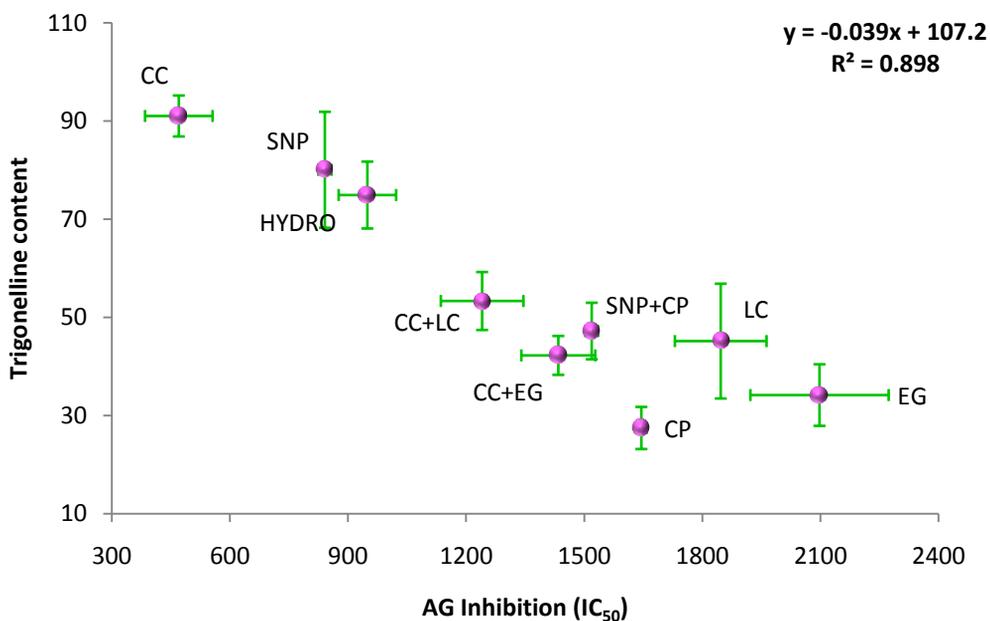


**Figure 5.2:** Effect of different priming agents on trigonelline content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

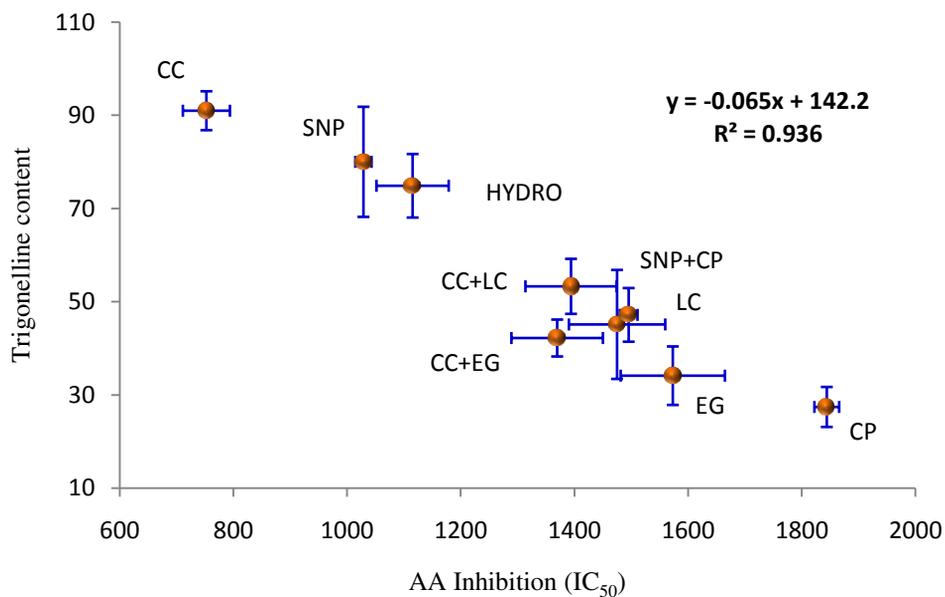


**Figure 5.3:** Effect of different priming agents on the total alkaloid and trigonelline contents of fenugreek sprouts

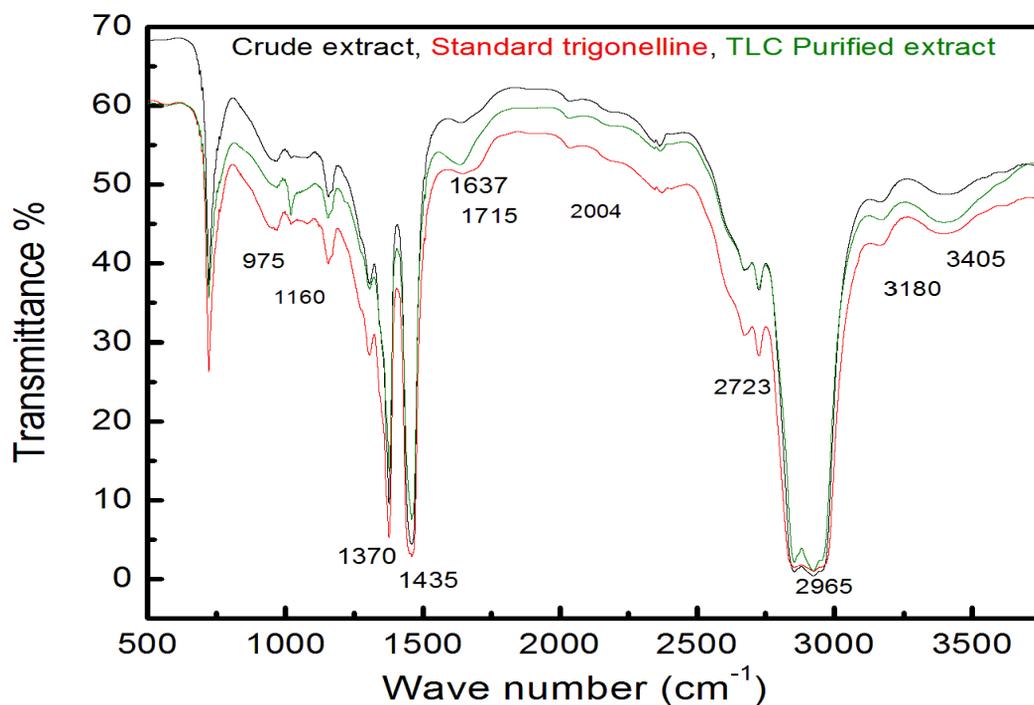


**Figure 5.4:** Alteration in the  $\alpha$ -glucosidase inhibition activity of fenugreek sprouts with respect to the trigonelline contents of fenugreek sprouts resulted due to different priming agents

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



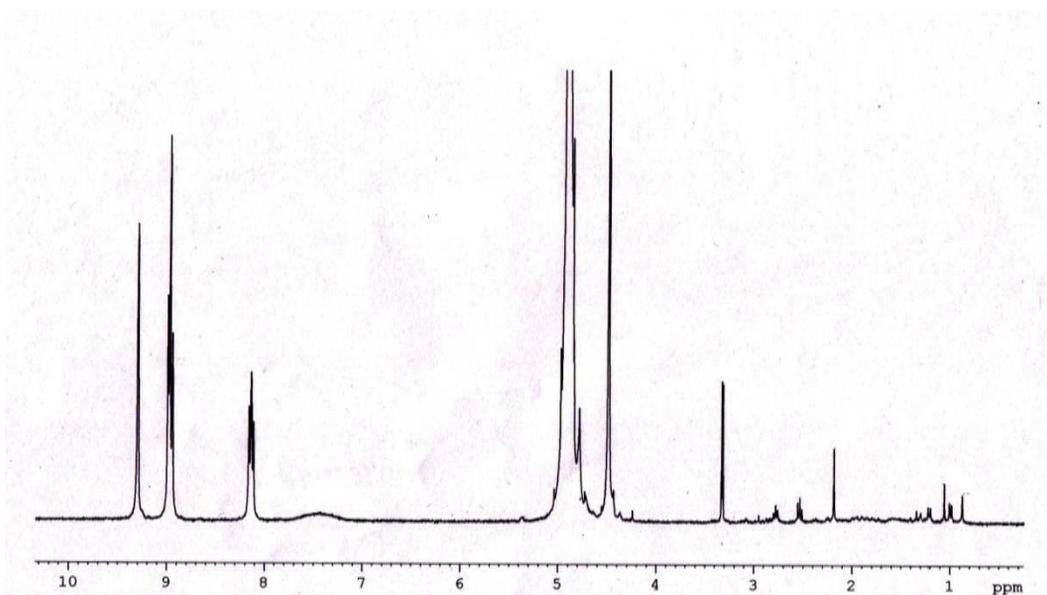
**Figure 5.5:** Alteration in the  $\alpha$ -glucosidase inhibition activity of fenugreek sprouts with respect to the trigonelline contents of fenugreek sprouts resulted due to different priming agents



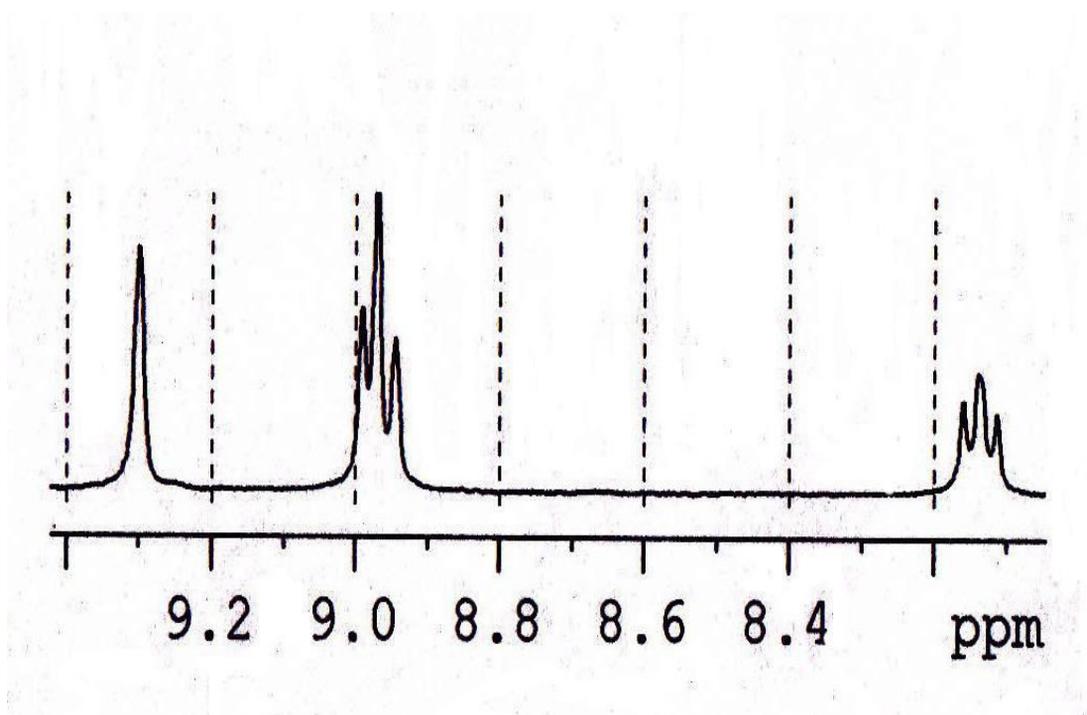
**Figure 5.6:** IR spectra of crude extract and TLC purified extract compared with standard trigonelline

The presence of some sharp medium absorption peaks at around  $2725\text{ cm}^{-1}$  indicates presence of C=C-H aromatic bonds supported by the presence of aromatic ring unsaturation vibrational bands around  $1600\text{cm}^{-1}$ , the weak absorption for the ring bending of benzene derivatives in the range of  $900\text{ cm}^{-1}$  was also observed. The aliphatic C-H stretching modes at around  $2900\text{cm}^{-1}$  are masked with that of the nujol. After confirming the presence of trigonelline in purified extract by IR analysis the extract was further analysed by NMR spectroscopy.

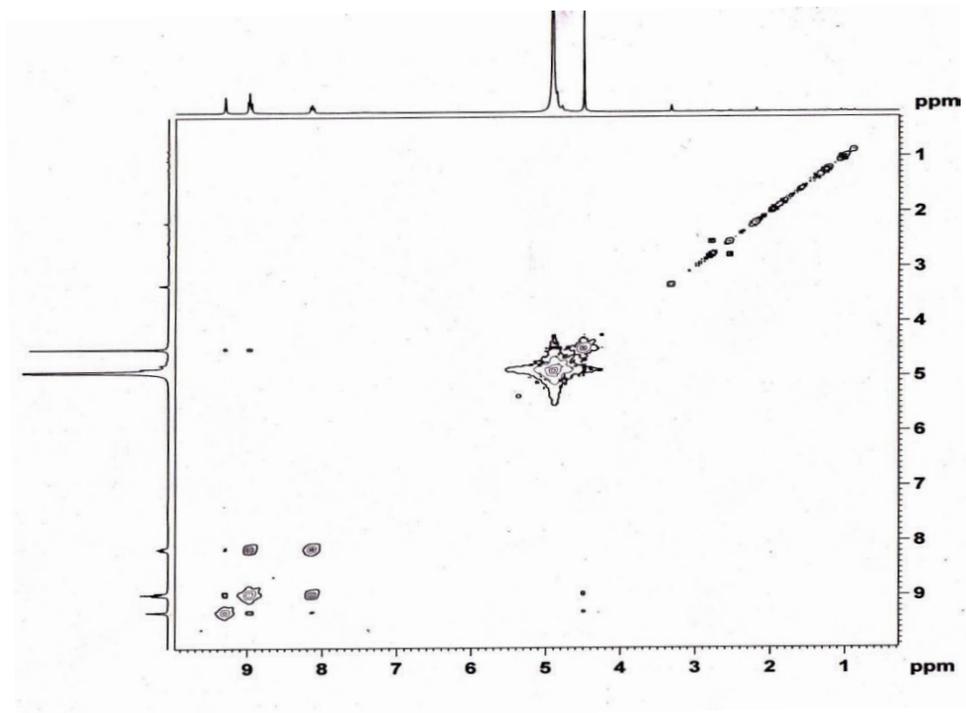
The implementation of NMR spectroscopy for metabolite fingerprinting has been considered highly reliable by the researchers. One dimensional NMR (1D-NMR) spectra has been used successfully for analyzing compounds in a crude extract, further two dimensional NMR (2D-NMR) spectra have been reported to be more efficient in identification of bioactive compounds in plant extracts (Kim *et al.*, 2010; Farag *et al.*, 2015). Likewise, the purified extract was subjected to 2D-NMR for the identification of compound. The  $^1\text{H}$  NMR spectra of purified fenugreek extract obtained by NMR spectroscopy is depicted in (Figure 5.7 & 5.8). The proton ( $^1\text{H}$ ) NMR spectra derived from the purified extract of fenugreek was characterized by  $\text{N}^+\text{-CH}_3$  (3H) peak at 4.5 ppm ( $\delta$ ); aromatic protons are observed at 8.1 ppm ( $\delta$ ) (1H), at 9 ppm ( $\delta$ ) (2H) and at 9.3 ppm ( $\delta$ ) (1H). In proton correlated spectroscopy (COSY) (Figure 5.9) and total correlation spectroscopy (TOCSY) spectra the correlation of the protons are confirmed and in the heteronuclear correlation (HETCOR) (Figure 5.10) spectra correlation of the spectra with that of carbon shifts are ascertained. This spectral analysis unequivocally suggests that the purified extract contains 1-Methylpyridinium-3-carboxylate (trigonelline). Overall, it was observed that among all treatments, CC and SNP priming exhibited enhancement in the alkaloid and antidiabetic potential of fenugreek, among which CC was found to exhibit the best result.



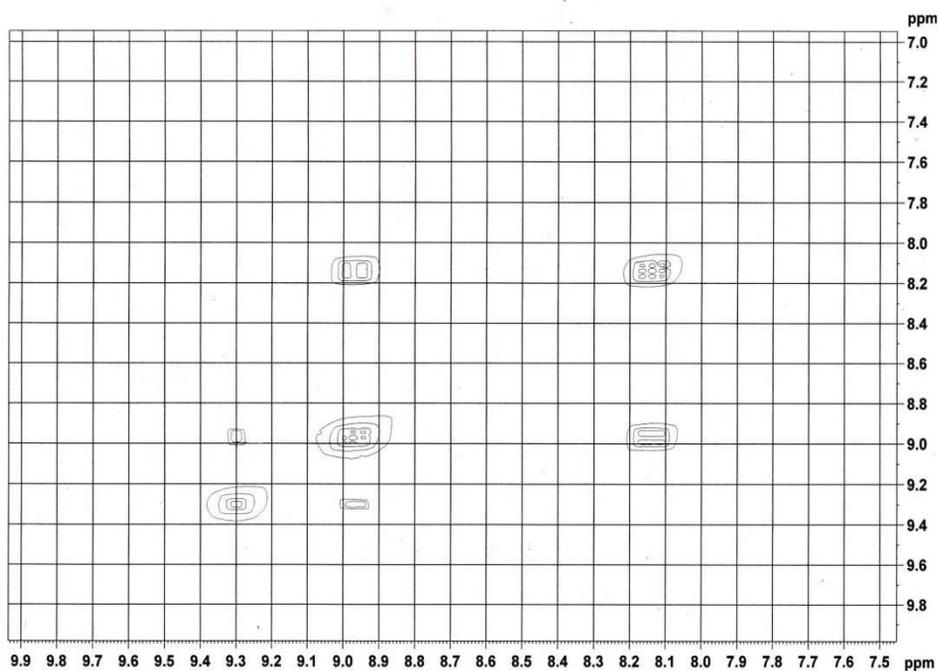
**Figure 5.7:**  $^1\text{H}$  NMR spectra of TLC purified extract of fenugreek sprout



**Figure 5.8:** Expanded regions of  $^1\text{H}$  NMR spectra of TLC purified extract of fenugreek sprout



**Figure 5.9:** COSY spectra of TLC purified extract of fenugreek sprout



**Figure 5.10:** HETCOR spectra of TLC purified extract of fenugreek sprout

Such enhancement in the antidiabetic activity and the level of trigonelline in fenugreek has reported by various authors through biotic and abiotic elicitors (Ahmed, 2011; Qaderi, 2016). The therapeutic property of trigonelline is highly attributed to its hypoglycaemic effect, thus the enhancement in the biosynthesis of alkaloids further boosts the antidiabetic potential of plant. The observation of best antidiabetic activity of sprouts during 48h stage indicates that the components including trigonelline responsible for the therapeutic potential were elicited appropriately at this stage. The impact of nitric oxide scavenger (CP), calcium chelator (EG) and calcium channel blocker was also assessed on the antidiabetic as well as alkaloid content of the fenugreek sprouts. The effect of these elicitors was found to be deteriorative on both the aspects of the sprouts, i.e. therapeutic (antidiabetic) as well phytochemical (alkaloid) content.

It can be suggested that the increase in the alkaloid content was found to be responsible for the enhancement in antidiabetic property of fenugreek sprouts. The negative effects of the antagonists of calcium and nitric oxide indicate that the flux of these signalling molecules within the cellular system is essential during germination phases to regulate the mechanisms responsible for the biosynthesis of antidiabetic compounds. The presence of trigonelline as a major alkaloid component was further confirmed by IR and NMR analysis. Thus, this knowledge can be used to design the priming based sprouting techniques which might have potential application in improving the nutraceutical quality of legume sprouts.

## **CHAPTER - 6**

### **ALLEVIATION OF SALINITY STRESS IN FENUGREEK SEEDLINGS BY NITRIC OXIDE AND CALCIUM DURING GERMINATION**

## 6.1 INTRODUCTION

The environmental factors are known to have significant impact on the morphological, biochemical attributes along with the growth and development of plants. When any of these factors exceeds the tolerance level, a stress is imposed on the plant which influences its development and structural, physiological and biochemical processes (Jaleel *et al.*, 2007). The increase in the salt content above optimum level, which creates salinity stress is considered one among these environmental factors which are responsible for threatening the crop productivity worldwide (Manivannan *et al.*, 2007).

The deleterious effect of salinity which affect the normal growth and development of the plant is attributed to a reduced osmotic potential, specific ion toxicity and nutrient deficiency of the substratum (Luo *et al.*, 2005; Bhattacharjee, 2008). The reduced osmotic potential affects water availability due to the prevention of water uptake by the plants, leading to a condition known as physiological drought (Kim *et al.*, 2009; Ozdener and Kutbay, 2008). In addition, salinity is reported to result in the generation of reactive oxygen species which further leads to membrane disruption and metabolic toxicity in plant system (Mittler, 2002).

To protect themselves from the oxidative stress mediated damages, plants are found to develop scavenging mechanisms of these destructive free radicals. This involves detoxification processes regulated by an integrated system of non-enzymatic antioxidants such as ascorbic acid and glutathione (Sharma *et al.*, 2004), and the enzymatic system which comprises of efficient antioxidants such as catalase (CAT) ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) (Reshmi and Rajalakshmi, 2012).

*Trigonella foenum-graecum* also known as fenugreek, the plant parts has been reported to possess wide pharmacological and folkloric significance. The leaves have been found to exhibit potential antioxidant property, antibacterial and anti-diabetic activity. In

addition, its seeds are found to possess potential hypocholesterolemic effect, antioxidant property and also have been effective in the treatment and/or prevention of diabetic disorders (Meghwal and Goswami, 2012).

In plant system, Calcium ( $\text{Ca}^{2+}$ ) is considered as a key second messenger as well as signal transducer, which is involved in coupling a wide spectrum of extracellular stimuli to intracellular responses (Sneeden and Formm, 2001), and play vital role in plant growth and development (Arshi *et al.*, 2006). It has been considered to be involved in linking stress perception as well as regulation of adaptive cellular responses (Torrecilla *et al.*, 2000). In the cited literature  $\text{Ca}^{2+}$  is found to enhance tolerance against various environmental stresses, including salinity by mitigating oxidative stress and regulating membrane stabilization (Larkindale and Knight, 2002; Kader and Lindberg, 2010). Likewise, nitric oxide (NO) a bioactive gaseous signalling molecule, exhibits central role in mediating several physiological processes and responses towards biotic and abiotic stresses including salinity (Wendehenne *et al.*, 2005).

Seed priming is a pre-sowing technique in which seeds are subjected to low external water potential that limits hydration which does not allow the protrusion of radicle through the seed coat. This technique is known to enhance the primary development of seeds under unfavourable environment (Rozbeh *et al.*, 2011; Nasri *et al.*, 2011). The priming of seeds with various substances such as water, inorganic salts, osmolytes and hormones has been successfully reported as a cost-effective strategy to enhance tolerance under saline conditions (Joshi *et al.*, 2013).

However, literature suggests that reports on the role of nitric oxide and calcium ion and their effect on growth and metabolism of fenugreek during the developmental phases under salinity stress are not studied till date. Considering this fact, the present study was undertaken to investigate the role of these signalling molecules on saline mediated

alterations in the morphological, biochemical and antioxidant defense system of fenugreek during post-germination phases.

## **6.2 MATERIALS AND METHOD**

### **6.2.1 Elicitation process and germination**

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of 5mM sodium nitroprusside (SNP) as an exogenous source of nitric oxide; 100  $\mu$ M of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CP), a nitric oxide scavenger; 5mM calcium chloride (CC) as an exogenous source of calcium ion; a calcium chelator: 1mM of Ethylene glycol-bis(2-aminoethylether)-N,N,N',N, tetra acetic acid (EG); and 1mM of Lanthanum chloride (LC): a calcium channel blocker. For control set, seeds were primed with normal water and placed in a rotary shaker along with the treated seeds. After priming for 24h, the seeds were washed thrice with sterile water and kept in the seed germinator for germination. To provide saline conditions the NaCl at the level of (0ds  $m^{-1}$ , 4ds  $m^{-1}$  and 8ds  $m^{-1}$ ) was applied to the seeds for 7days.

### **6.2.2 Measurement of growth parameters**

The growth performance of the seedlings was assessed by calculating various morphological parameters. The average length of the roots and shoots of the seedlings of each experimental set up was recorded along with their fresh weight and dry weight.

### **6.2.3 Relative water content**

The fenugreek seedlings of different experimental set up were kept in double distilled water for duration of 24h after taking initial weight (fresh weight). The seedlings were then taken out and excess water on the surface of the seedlings was blotted with

blotting paper and the seedlings were weighed to measure the turgid weight. After recording the turgid weight, the seedlings were dried for 24h at 80°C and finally the dried seedlings were weighed to obtain their dry weight.

The relative water content (RWC) of the seedlings was calculated using the following equation (Gonzalez and Gonzalez-Vilar, 2001):

$$\text{RWC (\%)} = (\text{Fresh weight} - \text{Dry weight}) / (\text{Turgid weight} - \text{Dry weight}) \times 100.$$

#### **6.2.4 Stress tolerance index**

The variation in the tolerance level of the fenugreek seedlings against salinity was measured as the tolerance index. The stress tolerance index (STI) of the seedlings was calculated using the following equation Seydi (2003):

$$\text{STI} = [(\text{DWc} - \text{DWs}) / \text{DWc}] \times 100$$

where DWc: Dry weight of control seedlings and DWs: Dry weight of seedlings under stress

#### **6.2.5 Histochemical detection**

##### **6.2.5.1 Detection of H<sub>2</sub>O<sub>2</sub> localization**

For detection of hydrogen peroxide localization in the roots, the seedlings were kept submerged for about 45min in potassium iodide (0.1M) /starch (4% w/v) reagent as suggested by Olson and Varner, (1993). All the stained roots were photographed using Nikon SLR camera (Model: D3200).

##### **6.2.5.2 Detection of Superoxide radical localization**

For detection of superoxide anion radical the seedlings were submerged in 50mM phosphate buffer (pH 7.5) containing (0.05% w/v) nitroblue tetrazolium (NBT) for 20 min in the presence of light (Doke, 1983) and then the roots were submerged in ethanol for

terminating the reaction. All the stained roots were photographed using Nikon SLR camera (Model: D3200).

#### **6.2.5.3 Detection of lipid peroxidation**

The detection of lipid peroxidation was conducted according to Pompella *et al.* (1987). The fenugreek roots were placed in Schiff's reagent until the coloured stain developed on the roots. The extra stain imparted by the reagent was removed using potassium sulphite solution (0.5% w/v) in 0.05 M HCl. All the stained roots were photographed using Nikon SLR camera (Model: D3200).

#### **6.2.5.4 Detection of plasma membrane integrity**

The integrity of plasma membrane was detected by the method suggested by Yamamoto *et al.* (2001). Firstly, the roots were stained using Evans blue solution (0.025% w/v in 100 mM CaCl<sub>2</sub>) for about half an hour and then the stained roots were rinsed thrice with distilled water for removal of extra stain. All the stained roots were photographed using Nikon SLR camera (Model: D3200).

#### **6.2.6 Quantitative estimation of H<sub>2</sub>O<sub>2</sub> content**

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content in the fenugreek seedlings was estimated as per Singh *et al.* (2006). 1gm of fenugreek seedlings of different experimental set up were extracted with 10ml of 0.05M phosphate buffer (pH 7.0) and the extraction mixture was centrifuged at 3000 rpm for 15 min and the obtained supernatant was used for further analysis. H<sub>2</sub>O<sub>2</sub> concentration was determined spectrophotometrically at 390 nm in a reaction mixture containing 50 mM phosphate buffer (pH 7), potassium iodide (1M) along with sample extract. H<sub>2</sub>O<sub>2</sub> content was expressed as  $\mu\text{mol g}^{-1}$  fwt.

### **6.2.7 Estimation of lipid peroxidation**

The extent of lipid peroxidation was determined by using 2-thiobarbituric acid (TBA) reagent for quantifying malondialdehyde (MDA) (Fu and Huang, 2001). 1g of fenugreek seedlings were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid solution followed by centrifugation at 10, 000 rpm for 10 min. 500 µl of the plant extract was reacted with thiobarbituric acid (TBA) reagent (2 ml of 20% trichloroacetic acid solution containing 0.5% (w/v) TBA); the reaction was stopped by cooling the reaction tubes in an ice water bath, after an incubation of 30 min at 95°C. The absorbance of the end product MDA was monitored at 532, 600 and 440 nm using UV-Visible Spectrophotometer (Model 2202, Systronics).

### **6.2.8 Determination of glutathione content**

Total glutathione content in fenugreek seedlings was determined according to Griffith, (1980) with some modification. The fenugreek seedlings were homogenized by 5% (w/v) sulfosalicylic acid under cold conditions and centrifuged for 10 min at 1000 g and the obtained supernatant was neutralized by addition of 0.6 ml of 10% sodium citrate. 0.5 ml of sample extract was reacted with a reaction mixture containing 100 mM phosphate buffer (pH 7.0), 0.3 mM NADPH and 6mM of 5-5'- dithiobis-2-nitrobenzoic acid (DTNB) and the whole reaction mixture was incubated for 3 min at room temperature. After incubation, 10 µl of glutathione reductase enzyme was added and the absorbance was recorded at 412 nm in a UV-visible spectrophotometer. The resulted glutathione content was expressed as µmol g<sup>-1</sup> fw using glutathione standard curve.

### **6.2.9 Determination of ascorbic acid content**

The content of ascorbic acid was analyzed by the spectrophotometric method described by Mukherjee and Choudhary (1983). The fenugreek seedlings of different experimental set up were homogenized in 5% TCA under ice-cold condition and the homogenate was centrifuged at 3000rpm for 15min and the obtained supernatant was used for further analysis. One ml of plant extract was reacted with 0.5 ml of 2% Dinitrophenyl hydrazine (DNPH) and 1 drop of 10% thiourea was added to it. The assay mixture was boiled for 15 min in a water bath. After that the mixture was cooled at room temperature in an ice bath and 80% sulphuric acid was added. The absorbance was read at 530nm. Ascorbic acid content was expressed as  $\mu\text{g g}^{-1}$  fwt using ascorbate standard curve.

### **6.2.10 Extraction and estimation of total soluble sugar and reducing sugar content**

#### **6.2.10.1 Extraction**

The total and reducing sugars were extracted following Harborne (1973) method with some modifications. First, seedlings were extracted with 95% ethanol at 1:10 ratio of tissue and solvent and the alcoholic fraction of the mixture was evaporated on a boiling water bath at about 80°C. Secondly, the tissues were re-extracted with 80% ethanolic (10ml) solution and the mixture was filtered and the ethanolic fraction was collected and evaporated once again. The dried fraction was reconstituted in 5ml of distilled water and then centrifuged at 5000 rpm for 15min. The supernatant was taken and stored for further analysis.

#### **6.2.10.2 Estimation of total soluble sugar**

Total soluble sugar content was estimated following the anthrone method (Thimmaiah, 2004). The assay mixture containing 1 ml extract and 4 ml anthrone reagent was incubated in a water bath at 100° C for 10 min. After appearance of blue-green colour,

the assay mixture was cooled to room temperature and its absorbance was monitored at 620 nm. The total soluble sugar in the extract was calculated using a standard curve prepared from sucrose.

### **6.2.10.3 Estimation of reducing sugar content**

Reducing sugars were measured by following the standard DNS method (Sadasivam and Manickam, 1996). Here, 1 ml extract was reacted with 1 ml of DNS reagent and incubated in a boiling water bath for 5 min. After appearance of colour, 1 ml 40 % Rochelle salt solution was added and vortexed. Absorbance was recorded at 510 nm against reagent blank.

### **6.2.11 Extraction and estimation of proline content**

#### **6.2.11.1 Extraction**

Free proline was extracted and estimated from fenugreek seedlings using 3% sulfosalicylic acid solution following the method of Bates *et al.* (1973). 2g of fenugreek seedlings were homogenized in 10ml of 3% sulfosalicylic acid solution and the extraction mixture was filtered through Whatman No. 1 filter paper. The final volume of extract was maintained up to 5ml by adding 3% sulfosalicylic acid solution wherever required. The extracts were stored for further experimental analysis.

#### **6.2.11.2 Estimation**

For the estimation of free proline, 1mL of extract was reacted with mixture containing, 3ml distilled water and 1ml of (4% w/v) ninhydrin solution (1g ninhydrin was dissolved in 10ml acetone and further 15ml distilled water was added). The whole reaction mixture was kept on boiling water-bath for 30min and cooled under tap water. After cooling the mixture was extracted with equal volume of toluene in a separating funnel, the coloured

toluene layer was separated and the remaining layer was discarded. The OD values of the toluene fraction were recorded at 520 nm in a spectrophotometer against a reaction blank. The total proline content of each sample was calculated from a standard curve of proline.

## **6.2.12 Extraction and estimation of protein content**

### **6.2.12.1 Extraction of proteins**

The fresh seedlings (1g) of fenugreek were homogenized in chilled 5ml of 0.05M potassium phosphate buffer (pH 7.0) containing 1% PVP (polyvinyl pyrrolidone) using pre-chilled mortar and pestle. The homogenates were subjected to centrifugation at 10,000g for 30min at 4°C and then the supernatants were stored at -20°C until further analysis.

### **6.2.12.2 Quantitative estimation of soluble proteins**

Quantitative estimation of protein was performed by the method of Lowry *et al* (1951). 0.2ml of the protein extract was diluted with 0.8ml of distilled water and was reacted with 4.5ml of freshly prepared reagent I (Reagent I comprises of 2% sodium carbonate dissolved in 100 ml of 0.1N NaOH to which further 1ml each of 0.5% CuSO<sub>4</sub> and 1% Na<sup>+</sup>-K<sup>+</sup> tartarate was added) and vortexed after which it was incubated at room temperature for 10mins. After the incubation 0.5ml of reagent II (50% Folin-ciocalteau) was added to the reaction mixture and further incubated for 30 min at room temperature. The absorbance value was recorded in a spectrophotometer at 660 nm against a reaction blank. The total soluble protein content of each sample was calculated from a standard curve of BSA (Bovine serum albumin).

### **6.2.13 Extraction of antioxidant enzymes**

#### **6.2.13.1 Catalase**

For the extraction of catalase enzyme from the sample, 1g of fenugreek seedlings of each experimental set up were homogenized in 5 ml of ice-cold 0.05M sodium phosphate buffer, pH 7.0, containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1% triton using liquid nitrogen in a pre-chilled mortar-pestle. The homogenate was then subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

#### **6.2.13.2 Guaiacol Peroxidase**

The peroxidase enzyme of the fenugreek seedlings were extracted by homogenizing 1g of seedlings in 5ml of ice-cold 0.05M sodium phosphate buffer, pH 6.6, containing 1% (w/v) PVP and 0.2mM EDTA using liquid nitrogen in a pre-chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

#### **6.2.13.3 Superoxide dismutase**

The superoxide dismutase enzyme of the fenugreek seedlings of different experimental set up were extracted by homogenizing 1g of seedlings in 5ml of ice-cold 0.1M tris buffer (pH 7.5) containing 1% (w/v) PVP and 0.2mM EDTA along with liquid nitrogen in a chilled mortar and pestle. The homogenate was then subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

#### **6.2.13.4 Ascorbate peroxidase**

For the extraction of ascorbate peroxidase enzyme 1g of fenugreek seedlings were homogenized in 5ml of ice-cold 0.05M of potassium phosphate buffer (pH 7.2) containing 1% (w/v) PVP and 5mM ascorbate along with liquid nitrogen in a pre-chilled mortar and

pestle. The homogenate was then subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

#### **6.2.13.5 Glutathione-S-transferase**

The Glutathione-S-transferase enzyme of the fenugreek seedlings were extracted by homogenizing 1g of seedlings in 5ml of ice-cold 0.1M potassium phosphate buffer (pH 6.5) containing 1% (w/v) PVP and 0.2mM EDTA using liquid nitrogen in a pre-chilled mortar-pestle. The homogenate was then subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

#### **6.2.13.6 NADPH oxidase**

For extraction of NADPH oxidase enzyme the fenugreek seedlings were homogenized in ice-cold 0.05M phosphate buffer (pH 6.8) with 1% PVP and 0.05% Triton X-100 in a chilled mortar and pestle. The enzyme extract was then centrifuged at 10,000rpm for 15min at 4 °C and the supernatant was collected for further analysis.

#### **6.2.13.7 Glutathione reductase**

The glutathione reductase and peroxidase enzyme of the fenugreek seedlings were extracted by homogenizing 1g of seedlings in 5ml of ice-cold 0.05M potassium phosphate buffer (pH 7.6) containing 1% (w/v) PVP and 0.2mM EDTA using liquid nitrogen in a pre-chilled mortar-pestle. The homogenate was then subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was separated and used for enzyme assays.

## **6.2.14 Assay of enzyme activities**

### **6.2.14.1 Catalase**

The method of Aebi (1983) was followed for the estimation of the Catalase (CAT) (EC 1.11.1.6) activity in which the breakdown of hydrogen peroxide was measured at 240 nm. The reaction mixture containing 3ml of 50 mM sodium phosphate buffer (pH 7.0) and 20 mM H<sub>2</sub>O<sub>2</sub> was reacted with 0.1ml of enzyme aliquot. The decrease in absorbance of the reaction mixture was recorded at 240nm in a UV-VIS spectrophotometer at time interval of 60sec for 3min. The blank was prepared by adding equal volume of buffer instead of the enzyme extract. The enzyme activity was calculated using the extinction coefficient  $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . Each test was done in three replicates.

### **6.2.14.2 Guaiacol Peroxidase**

The Guaiacol peroxidase (GPX) (EC 1.11.1.7) activity was measured by following the method involving oxidation of guaiacol (Hemeda and Klein 1990). This method is based on the scavenging of H<sub>2</sub>O<sub>2</sub> by GPX using guaiacol as hydrogen donor. 3ml of assay mixture composed of 50mM phosphate buffer (pH 6.6), 1% guaiacol (v/v) and 0.3% H<sub>2</sub>O<sub>2</sub> (v/v) was reacted with 0.1ml aliquot of enzyme extract. The increase in absorbance of tetra-guaiacol was monitored at 470 nm in a UV-VIS spectrophotometer. The enzyme activity was calculated using extinction coefficient  $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$  and was expressed as  $\mu\text{mol}$  of guaiacol oxidized  $\text{min}^{-1} \text{ mg}^{-1}$  protein. The blank was prepared by adding equal volume of extraction buffer to the reaction mixture in place of enzyme extract, each test was done in triplicates.

### **6.2.14.3 Superoxide dismutase**

The superoxide dismutase (SOD) (EC 1.15.1.1) enzyme activity was performed by the method based on the inhibition of photochemical reduction of nitro blue tetrazolium by

enzyme extract (Giannopolitis & Ries 1977) with some modification. The assay mixture contained 0.2ml of 63  $\mu\text{M}$  NBT (nitroblue tetrazolium chloride), 0.1ml of 3mM EDTA, 0.2ml of 13 mm methionine, 0.1ml of 0.5M sodium carbonate, 0.1mL of 1.3  $\mu\text{M}$  riboflavin, 1.5ml of 0.1M potassium phosphate buffer (pH 7.6), and 0.1ml of enzyme extract was added and the final volume was made up to 3ml by adding distilled water. The whole reaction mixtures were illuminated in glass test tubes of uniform thickness and colour. After illumination, the absorbance of the samples was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme responsible for 50% inhibition of the NBT reduction as monitored at 560 nm. The blank was prepared by adding equal volume of extraction buffer to the reaction mixture in place of enzyme extract, each test was done in triplicates.

#### **6.2.14.4 Glutathione reductase**

Glutathione reductase (EC 1.6.4.2) activity was determined by monitoring the change in absorbance at 340 nm due to GSSG-dependent oxidation of NADPH (Schaedle & Bassham 1977). The reaction mixture consist of 0.75ml of 0.2M potassium phosphate buffer (pH 7.6) containing 2mM EDTA (Ethylenediaminetetraacetic acid), 75 $\mu\text{l}$  of freshly prepared 2mM NADPH (Nicotinamide adenine dinucleotide phosphate reduced tetrazolium salt), 75 $\mu\text{l}$  of freshly prepared 20mM oxidized glutathione and 0.1ml of enzyme extract. The resulted change in OD value was monitored at 340nm an interval of 60sec for 2min in a UV-VIS spectrophotometer. The enzyme activity was calculated using extinction coefficient  $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  and was expressed as  $\mu\text{mole of NADPH oxidized min}^{-1} \text{ mg}^{-1} \text{ protein}$ . The blank was prepared by adding equal volume of extraction buffer to the reaction mixture in place of enzyme extract, each test was done in triplicates.

#### **6.2.14.5 Glutathione S-transferase**

The Glutathione S-transferase (GST) (EC 2.5.1.18) activity was estimated by the method described by Habig and Jacoby (1981) with some modifications. The potassium phosphate buffer (0.1M, pH 6.5) containing 1mM reduced glutathione (GSH) and 1mM of 1-Chloro-2,4-dinitrobenzene (CDNB) was subjected to reaction with the enzyme extract; the volume of reaction mixture was made up to 3ml by adding required amount of distilled water. The change in the absorbance of the reaction mixture was monitored at 340nm at an interval of 30sec for 3min against distilled water. The reaction mixture except the enzyme extract served as control to monitor the nonspecific binding of the substrates. The enzyme activity was calculated using extinction coefficient  $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$  and was expressed as  $\mu\text{mole CDNB conjugate min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

#### **6.2.14.6 Ascorbate peroxidase**

The activity of ascorbate peroxidase (EC 1.11.1.11) was measured by monitoring the rate of oxidation of ascorbate (Nakano and Asada, 1981) with some modification. 3ml of reaction mixture containing 0.05 M sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM  $\text{H}_2\text{O}_2$  was reacted with 0.1ml aliquot of enzyme extract. The change in the absorbance of the reaction mixture was recorded at 290 nm at an interval of 60 sec for 3 min. The enzyme activity was calculated using extinction coefficient  $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  and was expressed as  $\mu\text{mole ascorbate min}^{-1} \text{ mg}^{-1} \text{ protein}$ . The blank was prepared by adding equal volume of extraction buffer to the reaction mixture in place of enzyme extract, each test was done in triplicates.

#### **6.2.14.7 NADPH oxidase**

The NADPH oxidase was determined by following the reduction of sodium, 3'-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid

hydrate (XTT) by superoxide (Sagi and Fluhr 2001) with some modifications. The assay mixture of 3 ml containing 0.05 mM Tris-HCl buffer (pH 7.5) and 0.5 mM XTT was reacted with 100  $\mu$ l of protein extract after which the reaction was initiated by adding 100  $\mu$ M NADPH. The reduction of XTT was monitored at 470 nm in a UV-VIS spectrophotometer. The enzyme activity was calculated using extinction coefficient  $\epsilon = 21.6 \text{ mM}^{-1} \text{ cm}^{-1}$ . The blank was prepared by adding equal volume of extraction buffer to the reaction mixture in place of enzyme extract, each test was done in triplicates.

## **6.2.15 Analysis of Isozyme patterns by polyacrylamide gel electrophoresis**

### **6.2.15.1 Preparation of reagents and stock solutions**

#### **6.2.15.1.1 Stock solution for gel preparation**

For the isozyme analysis the method as introduced by Ornstein and Davis (1970) was followed with minor modifications. A stock solution for resolving gel was prepared by dissolving 29.2% (w/v) acrylamide and 0.8% (w/v) N'N'-methylene bis-acrylamide (29.2 g of acrylamide and 0.8g of N'N'-methylene bis-acrylamide) in 100 ml double distilled water. The mixture solution was filtered through Whatman No. 1 filter paper and stored in brown bottle at 4°C for further to be used within one month from the date of preparation.

#### **6.2.15.1.2 Tris buffer for resolving gel pH 8.8**

1.5M tris buffer for resolving gel was prepared by dissolving 9.08g of tris base in 50ml of distilled water and the pH of the solution was adjusted to 8.8 using 6N HCl. The final volume of the buffer solution was made up to 75ml by adding required amount of distilled water and stored at -4°C.

#### **6.2.15.1.3 Tris buffer for stacking gel pH 6.8**

0.5M tris buffer required for stacking gel preparation was made by dissolving 3.02g of tris base in 30ml of distilled water and the pH of the buffer was adjusted to 6.8 using 6N HCl. The final volume of the solution was made up to 50mL by adding required amount of distilled water and was stored at -4°C.

#### **6.2.15.1.4 Sample loading buffer**

Sample buffer or loading buffer was prepared by making a mixture of 5.55ml of deionized water, 1.25ml of 0.5M Tris-HCl (pH 6.8), 3ml of glycerol and 0.2ml of 0.5%(w/v) Bromophenol blue solution stored.

#### **6.2.15.1.5 Electrophoresis running buffer**

For the preparation of electrophoresis buffer or electrode running buffer 3.02g of tris base and 18.8g of glycine was dissolved in 200ml of distilled water and later the final volume was made up to 1litre by adding required amount of distilled water and stored at -4°C.

#### **6.2.15.1.6 Ammonium per sulphate (APS)**

10% (w/v) APS solution was freshly prepared each time when required.

#### **6.2.16 Preparation of sample**

The enzymes from fenugreek seedlings of different experimental set were extracted following the method of Zhou *et al.*, 2009 with some modifications. 1g of fenugreek seedlings were homogenized in 5ml of ice cold 50mM phosphate buffer (pH 7.0) containing 0.2mM EDTA and 1% (w/v) polyvinyl pyrrolidone (PVP). The resulted homogenate was subjected to centrifugation at 10,000rpm at 4°C for 20 min. The obtained supernatant was stored at -20°C for further electrophoretic analysis.

For electrophoresis, the sample for loading in the gel was prepared by mixing 50µl of sample protein with 20µl sample loading buffer and immediately loaded into the wells of stacking gel using a micropipette.

#### **6.2.17 Preparation of gel and electrophoresis set up**

The resolving gel (10%; 10ml) was prepared by mixing the following mentioned solution. So, 10ml of 10% resolving gel contained 4.2ml of distilled water, 3.3ml stock solution of acrylamide and bisacrylamide and 2.5ml stock solution of 1.5M Tris (pH 8.8). To the above prepared monomer solution 50µl of freshly prepared 10% APS solution and 5µl of TEMED (N,N,N',N'-Tetramethylethylenediamine) was added and mixed gently.

The resolving gel (8%; 10ml for catalase isozyme) was prepared by mixing the following mentioned solution. 8% resolving gel contained 4.8ml of distilled water, 2.7ml stock solution of acrylamide and bisacrylamide and 2.5ml stock solution of 1.5M Tris (pH 8.8). To the above prepared monomer solution 50µl of freshly prepared 10% APS solution and 5µl of TEMED (N,N,N',N'-Tetramethylethylenediamine) was added and mixed gently. Immediately after preparation, the resolving mixture was loaded inside the gel plates leaving sufficient space for the stacking gel.

A thin layer of distilled water was over layered with the help of micropipette on the resolving gel and then the gel was left undisturbed for 1hr to get polymerised. After the polymerisation of resolving gel the water layer was removed carefully using blotting paper. Now the stacking gel (5%; 10ml) was prepared by mixing the following mentioned solution. 5ml of 5% stacking gel comprised of 5.8ml of distilled water, 1.7ml stock solution of acrylamide and bisacrylamide and 2.5ml of stock solution of 0.5 M Tris (pH 6.8). To the above prepared monomer solution 50µl of freshly prepared 10% APS solution and 10µl of TEMED was added and mixed gently.

For catalase isozyme the stacking gel (4%; 10ml) was prepared by mixing the following mentioned solution. 10ml of 4% Stacking gel comprised of 6.2ml of distilled water, 1.3ml stock solution of acrylamide and bisacrylamide and 2.5ml of stock solution of 0.5 M Tris (pH 6.8). To the above prepared monomer solution 50 $\mu$ l of freshly prepared 10% APS solution and 10 $\mu$ l of TEMED was added and mixed gently. Immediately after loading the stacking gel on the resolving gel a comb was inserted for make wells on the stacking gel leaving a space between the stacking gel and the resolving gel. It was also left undisturbed and allowed to polymerise for 1hr.

After the polymerisation of stacking gel, the comb was removed and the wells were cleaned using capillary tube. The gel set up was then properly inserted inside the buffer tank of gel electrophoresis system and was connected with electrodes. 700ml of electrophoresis running buffer was poured in the gel electrophoresis buffer tank and then was allowed to run for about 60mins before loading the protein sample. 30 $\mu$ l of protein samples in each lane was loaded on the gel.

The isozymes of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7) and NADPH oxidase (NOX; EC 1.6.3.1) were separated on discontinuous polyacrylamide gels (composed of 5% stacking gel and 10% resolving gel) and for catalase (CAT; EC 1.11.1.6) (4% stacking gel and 8% resolving gel) under non-denaturing conditions. Proteins were electrophoresed at 4°C and 80V in the stacking gel followed by 120V in the resolving gel.

After the separation of protein samples in the gel electrophoresis apparatus, the gel plate was taken from the electrophoresis system and released from the glass plate with the help of gel releaser after the stacking layer was cut off from the resolving gel. The resolving gel where the isozymes were separated was subjected to respective gel staining procedure mentioned in the following section.

## **6.2.18 Staining procedures**

### **6.2.18.1 Catalase**

The staining of catalase isozyme in the resolving gel was performed by following the method of Verma and Dubey, (2003) with minor modifications. After the completion of electrophoretic separation of isozymes the resolving gel was separated from the stacking gel and washed thrice with chilled distilled water. The resolving gel was soaked in 50 mM sodium-phosphate buffer (pH 7.0) containing 5 mM H<sub>2</sub>O<sub>2</sub> for 15min. The gel was then rinsed in chilled distilled water and further washed thrice. It was then stained in freshly prepared mixture solution composed of equal volume of 1% ferric chloride and 1% potassium ferricyanide for about 20min in dark at room temperature.

After the completion of staining process, the gel was scanned under GS800 densitometer and the obtained image was used for analysis of protein bands by image lab software.

### **6.2.18.2 Superoxide dismutase**

The isozyme pattern of superoxide dismutase activity was detected on gel as described by Pereira *et al.*, 2002. After electrophoretic separation of isozyme the gels were rinsed in distilled water thrice. The gel was incubated in an assay mixture containing 0.05M potassium phosphate buffer (pH 7.8), EDTA (1 mM), riboflavin (0.05 mM), nitroblue tetrazolium (0.1 mM) and 0.3% N,N,N',N'-tetramethyl-ethylenediamine (TEMED) in the dark condition for 30 min at room temperature. After incubation the gels were again rinsed with distilled water and then illuminated on a light box until the colourless bands of SOD appeared against a purple-stained gel background.

After the completion of staining process, the gel was scanned under GS800 densitometer and the obtained image was used for analysis of protein bands by image lab software.

### **6.2.18.3 Peroxidase**

The staining of peroxidase isozyme in the resolving gel was performed by following the method of Janda *et al.*, (1999) in ice-cold condition. A staining solution was freshly prepared by dissolving 5mM benzidine in 0.2M acetate buffer and filtered through Whatman no. 1 filter paper. After filtration, 5mM H<sub>2</sub>O<sub>2</sub> was added to the previous solution and immediately poured on top of the resolving gel in a gel staining box. The gel was stained till coloured bands were observed on the gel.

After the completion of staining process, the gel was scanned under GS800 densitometer and the obtained image was used for analysis of protein bands by image lab software.

### **6.2.18.4 NADPH oxidase**

The staining of NADPH oxidase (NOX) isozyme in the resolving gel was performed by following the method of Frahy and Schopfer, (2001) in ice-cold condition. After electrophoretic separation of isozyme the gel was rinsed in distilled water and washed thrice. The gels were soaked in a staining solution composed of nitroblue tetrazolium (0.5 mg/ml) and 134 µM NADPH dissolved in 10 mM Tris buffer, (pH 7.4). The gel was soaked in the staining solution until the bands were visible on the gel.

After the completion of staining process, the gel was scanned under GS800 densitometer and the obtained image was used for analysis of protein bands by image lab software.

### **6.2.19 Statistical analysis**

The data were pooled in triplicate and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing various attributes of different elicited fenugreek sprouts. Different group means were compared by Duncan's Multiple Range Test (DMRT) through

DSAASTAT software (version 1.002; DSAASTAT, Perugia, Italy);  $p < 0.05$  was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the values of different parameters and their standard error of estimates (SEE). In order to examine and visualize relationships between different biochemical, morphological and antioxidative attributes, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

### **6.3 Result and discussion**

Since germination being the initial stage of plant development defines the quality of yield and development of plant; therefore, the plants must be provided best condition during initial stages for better germination. It has been well known that saline environment constrains the growth and development of plant by virtue of their adverse effect on the various physiological and biochemical processes which includes osmolytes accumulation and metabolism along with the antioxidant enzyme system (Li *et al.*, 2014). The basic criteria for a stress tolerant plant is said to be survival of stress; maintenance of biomass production; growth performance, especially elongation of root and accumulation of biochemical markers such as proline, soluble sugars, polyamines, amino acids and also reduced level of lipid peroxidation (Ashraf and Harris, 2004; Juan *et al.*, 2005). As a result, it was observed that the growth performance of fenugreek seedlings was extensively affected by the saline condition as it was evident by the reduction in the fresh weight and dry weight of the seedlings. In comparison to control seedlings, the fresh weight of the unprimed seedlings was found to be reduced by 35% and 42% (Figure 6.1 & 6.5) and dry weight by 12% and 47% (Figure 6.2 & 6.6) at 4ds  $m^{-1}$  and 8ds  $m^{-1}$  salinity respectively with respect to control (0ds  $m^{-1}$ ) seedlings. A decrease in the reduction of the growth performance was

observed in the seedlings subjected to calcium chloride priming. The increase in the fresh weight was found to be 23% and 18% (Figure 6.1 & 6.5) and dry weight by 7% and 23% (Figure 6.2 & 6.6) when compared to unprimed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively. Also the fresh weight of SNP primed seedlings was found to be increased by 11% and 13% and dry weight by 6% and 29% approximately. On the other hand, the decrease in fresh weight was further enhanced in the seedlings pre-treated with the antagonists of these signalling molecules which has been recorded to be 61% and 67% for EG; 66% and 71% for LC and 52% and 64% for CP (Figure 6.1) and the decrease in dry weight was recorded to be 36% and 58% for EG; 44% and 67% for LC and 42% and 55% for CP at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively with respect to control (0ds m<sup>-1</sup>) seedlings (Figure 6.2). The average length of the roots was measured to study the effect on the root elongation; the root length of the unprimed seedlings was 1.67±0.25cm and 0.97±0.21cm, whereas those pre-treated with calcium chloride showed comparatively elongated roots of 2.7±0.14cm and 1.63±0.19cm and the roots of sodium nitroprusside primed seedlings were 2.07±0.12cm and 1.5±0.09cm long at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Table 6.1). Similar effect of different priming agents were observed on the length of shoot in fenugreek seedlings; the shoot length of the unprimed seedlings was 2.03±0.41cm and 1.57±0.29cm, whereas those pre-treated with calcium chloride showed comparatively elongated shoots of 3.50±0.11cm and 2.30±0.25cm and the shoots of sodium nitroprusside primed seedlings were 2.57±0.21cm and 2.03±0.12cm long at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Table 6.1). Meanwhile the length of the seedling were tremendously affected by the antagonist molecules the seedling length of EG primed seedlings was 1.74±0.27cm and 1.40±0.22cm; for LC was 1.24±0.18cm and 0.93±0.14cm and for CP seedling length was 1.66±0.16cm and 1.17±0.18cm when compared to that of unprimed seedlings having length of 3.70±0.69cm and 2.54±0.48cm at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Table 6.1). Another important parameter, relative water content was

calculated and it was observed to be 90.06% for control, 80.56% and 70.32% for unprimed seedlings, 86.91% and 80.96% for calcium pre-treated, 62.78% and 45.03% for EGTA primed and 55.46% and 49.44% for lanthanum chloride, 87.26% and 75.13% for SNP primed and 65.89% and 51.78% for those primed with CP at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Figure 6.3 & 6.7). The parameter such as relative water content has been considered as one of the vital factors for the assessment of the extent of salinity induced effects and the degree of tolerance in plants towards stress environment. Our findings suggesting the positive effect of exogenous calcium and nitric oxide priming on the RWC of fenugreek seedlings under salinity stress was in agreement to previous studies on several plants including mustard (Zeng *et al.*, 2011), rice (Habib and Ashraf, 2014) chick pea (Ahmad *et al.*, 2016) and linseed (Khan *et al.*, 2010). Further earlier studies have claimed that the stress tolerant plants exhibit higher RWC than those susceptible ones which are found to lose significant amount of water content from their body parts. Though it is yet to be explored the mechanism of the involvement these signalling molecules in maintenance of RWC in stressed plants; however, Ke *et al.*, (2013) had proposed that NO could decrease solute potential thus enhancing the water potential in plant system under osmotic stress. The stress tolerance index of each set of seedlings subjected to salinity was also calculated, the STI of unprimed was 88.01% and 75.67%; for calcium chloride primed 94.44% and 82.42%; for EGTA primed 74.56% and 50.51%; for lanthanum chloride 65.78% and 40.23%; for SNP 92.98% and 80.42% and for CP primed seedlings the STI was found to be 67.54% and 55.69% at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Figure 6.4 & 6.8).

The morphological parameters such as fresh weight, dry weight, relative water content (RWC), root length, shoot length and stress tolerance index of the seedlings which were unprimed and primed with antagonists of calcium and nitric oxide were found to be extensively affected by the salinity stress in a dose-dependent manner. On the other hand, the seedlings pretreated with exogenous sources of calcium and nitric oxide were found to

develop pronounced tolerance towards salinity. The seedlings subjected to calcium chloride and nitric oxide priming exhibited minimal reduction in root elongation during saline stress, which is considered to be one the major physiological parameter for salinity tolerance (Tari *et al.*, 2015).

The seedlings subjected to calcium chloride and nitric oxide priming exhibited minimal reduction in root elongation during saline stress, which is considered to be one the major physiological parameter for salinity tolerance (Tari *et al.*, 2015). The growth performance of the seedlings under the influence of calcium was found to exhibit considerable improvement followed by nitric oxide in comparison to other treated seeds which were found to be susceptible as their growth performance was drastically dropped (Figure 6.5-6.8). In agreement with our study, various other workers have reported such positive improvement in the growth performance of the plants subjected to calcium and nitric oxide influence during salinity stress (Murillo-Amador *et al.*, 2006; Garcia-Legaz *et al.*, 2008; Khan *et al.*, 2010; Tian *et al.*, 2015). The growth performance of the plants under stress conditions also depends upon the physiological status of various biochemical factors and antioxidant enzymes which are discussed later.

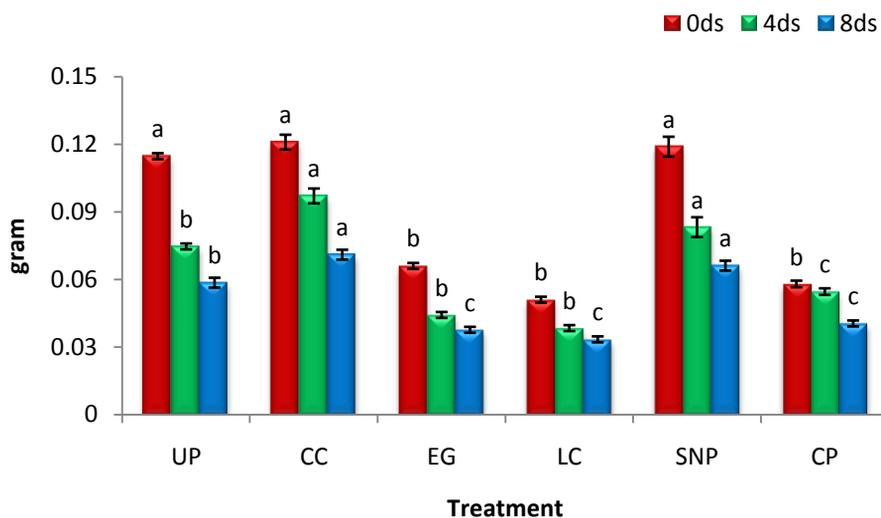
The major reactive forms of oxygen also termed as reactive oxygen species such as hydrogen peroxide, and superoxide radicals are known to be the molecules with high toxic potentials to plant tissues (Asada 2006). After detailed analysis of morphological parameters; histochemical detection was performed for the study of specific localization of free radicals such as  $H_2O_2$  and superoxide anion and their pattern of accumulation in the tissue. A major enhancement in the ROS generation both  $H_2O_2$  and  $O^{2-}$  was observed, wherein the seedlings exposed to saline condition as well as those primed with the antagonists of both the signalling molecules considerably higher rate of accumulation was noted with respect to those primed with exogenous calcium and nitric oxide.

**Table 6.1:** Shoot length, Root length and Seedling length of fenugreek seedlings pre-treated with different elicitors of calcium and nitric oxide on 7<sup>th</sup> day under salinity stress.

<b>Treatment</b>	<b>Shoot length (cm)</b>	<b>Root length (cm)</b>	<b>Seedling length (cm)</b>
<b>UP (0ds m<sup>-1</sup>)</b>	3.8±0.26	3.23±0.34	7.03±0.83
<b>UP (4ds m<sup>-1</sup>)</b>	2.03±0.41	1.67±0.25	3.70±0.69
<b>UP (8ds m<sup>-1</sup>)</b>	1.57±0.29	0.97±0.21	2.54±0.48
<b>CC (0ds m<sup>-1</sup>)</b>	4.21±0.31	3.64±0.19	7.85±0.42
<b>CC (4ds m<sup>-1</sup>)</b>	3.50±0.11	2.7±0.14	6.20±0.52
<b>CC (8ds m<sup>-1</sup>)</b>	2.30±0.25	1.63±0.19	3.93±0.45
<b>EG (4ds m<sup>-1</sup>)</b>	1.85±0.09	0.96±0.07	2.85±0.14
<b>EG (4ds m<sup>-1</sup>)</b>	1.27±0.16	0.47±0.10	1.74±0.27
<b>EG (8ds m<sup>-1</sup>)</b>	1.07±0.15	0.33±0.08	1.40±0.22
<b>LC (4ds m<sup>-1</sup>)</b>	1.52±0.14	0.68±0.11	2.10±0.36
<b>LC (4ds m<sup>-1</sup>)</b>	0.87±0.09	0.37±0.05	1.24±0.18
<b>LC (8ds m<sup>-1</sup>)</b>	0.63±0.07	0.30±0.06	0.93±0.14
<b>SNP (0ds m<sup>-1</sup>)</b>	3.98±0.54	3.32±0.32	7.30±0.92
<b>SNP (4ds m<sup>-1</sup>)</b>	2.57±0.21	2.07±0.12	4.64±0.35
<b>SNP (8ds m<sup>-1</sup>)</b>	2.03±0.12	1.5±0.09	3.53±0.24
<b>CP (0ds m<sup>-1</sup>)</b>	1.78±0.14	0.71±0.11	2.49±0.32
<b>CP (4ds m<sup>-1</sup>)</b>	1.23±0.06	0.43±0.05	1.66±0.16
<b>CP (8ds m<sup>-1</sup>)</b>	0.87±0.07	0.30±0.04	1.17±0.18

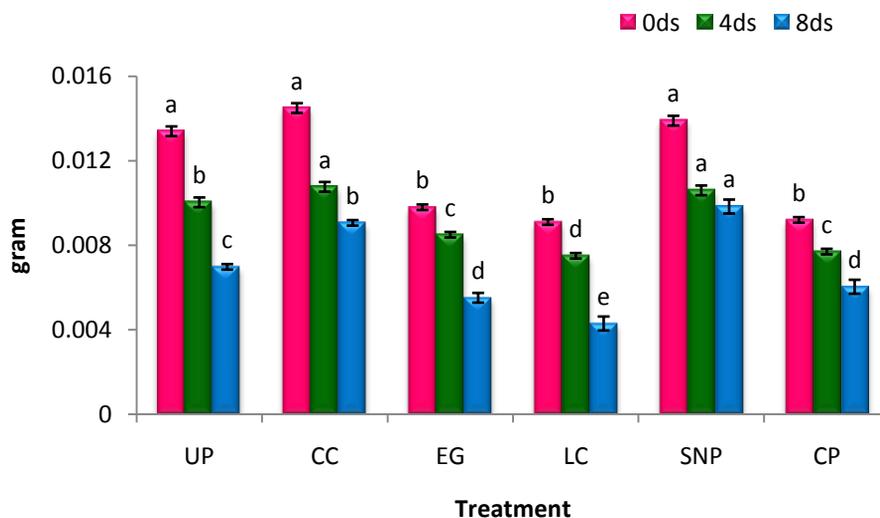
Results are expressed as a mean of 10 seedlings each

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.1:** Fresh weight of fenugreek seedlings primed with different elicitors under saline condition

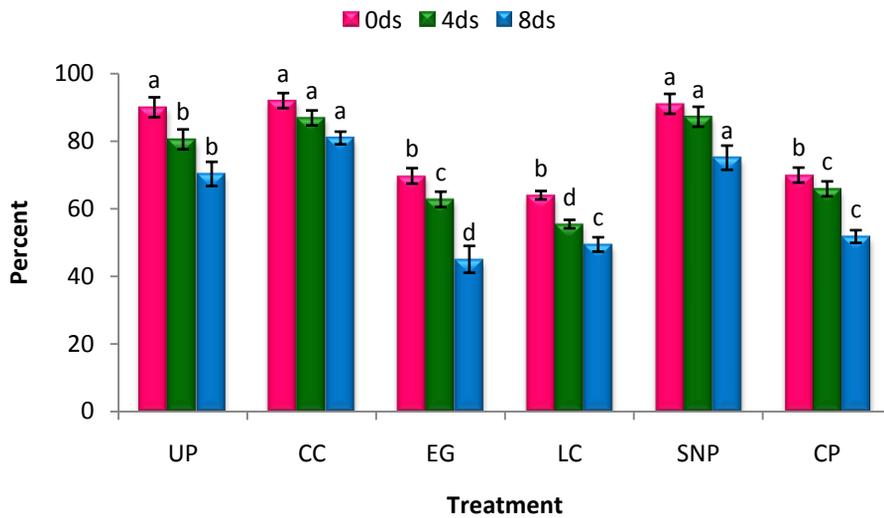
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.2:** Dry weight of fenugreek seedlings primed with different elicitors under saline condition

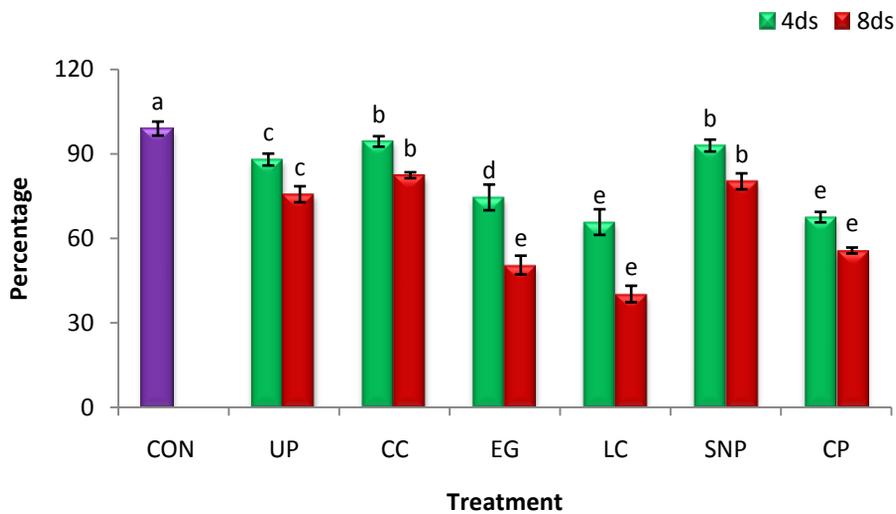
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.3:** Relative water content of fenugreek seedlings primed with different elicitors under salinity stress

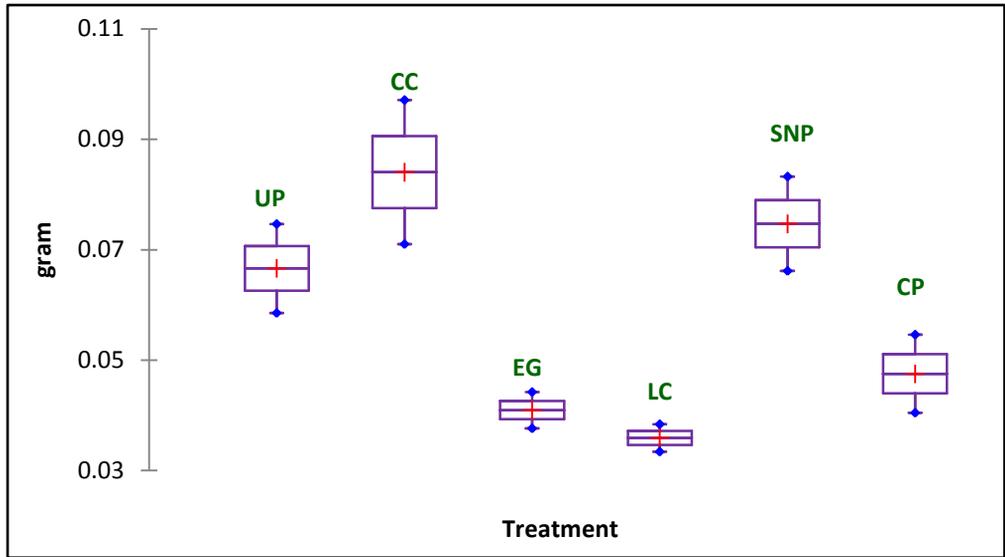
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



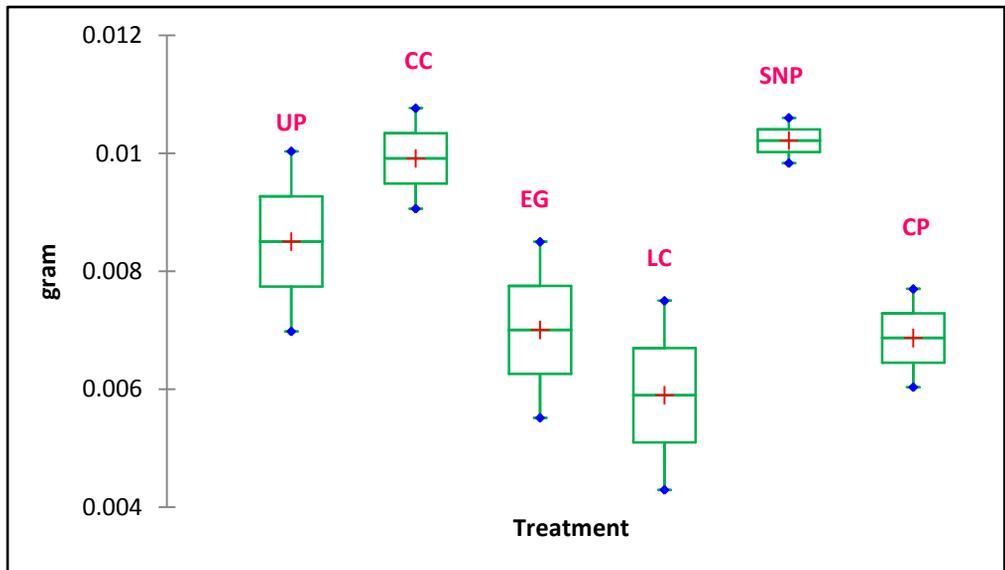
**Figure 6.4:** Stress tolerance index of fenugreek seedlings primed with different elicitors under salinity stress

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

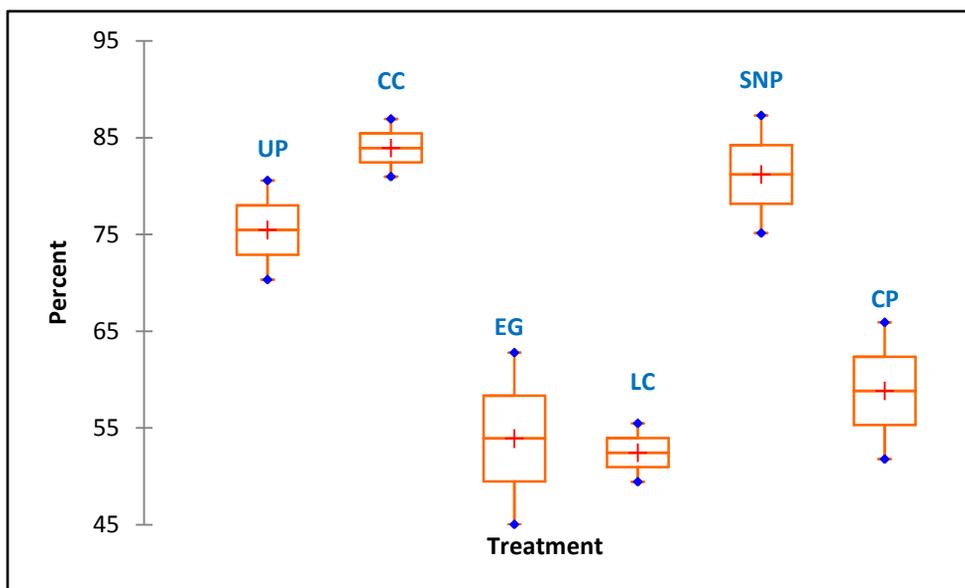


**Figure 6.5:** Box plot depicting the alteration in fresh weight of fenugreek seedlings primed with different elicitors under salinity stress

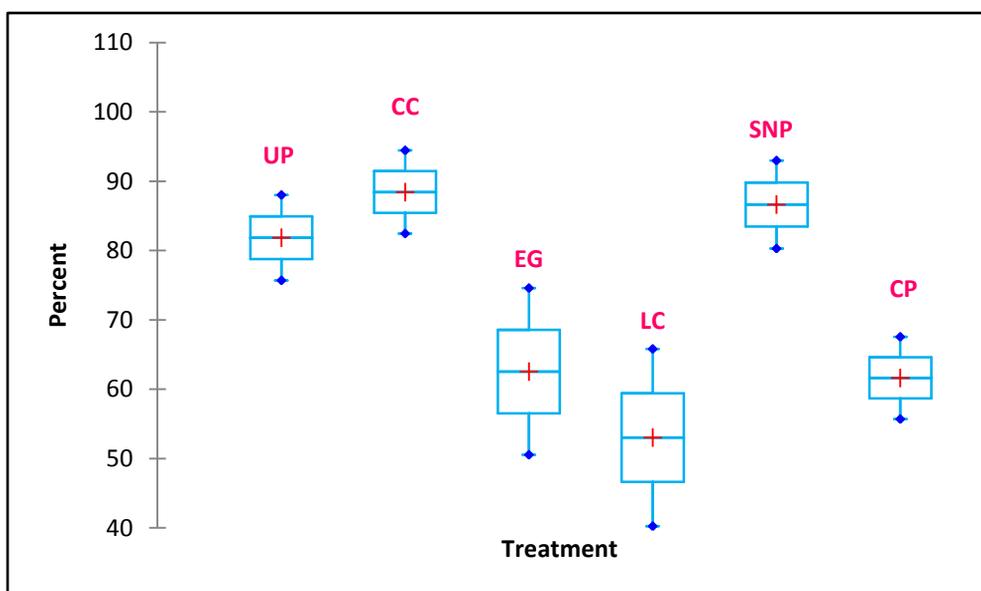


**Figure 6.6:** Box plot depicting the alteration in dry weight of fenugreek seedlings primed with different elicitors under salinity stress

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

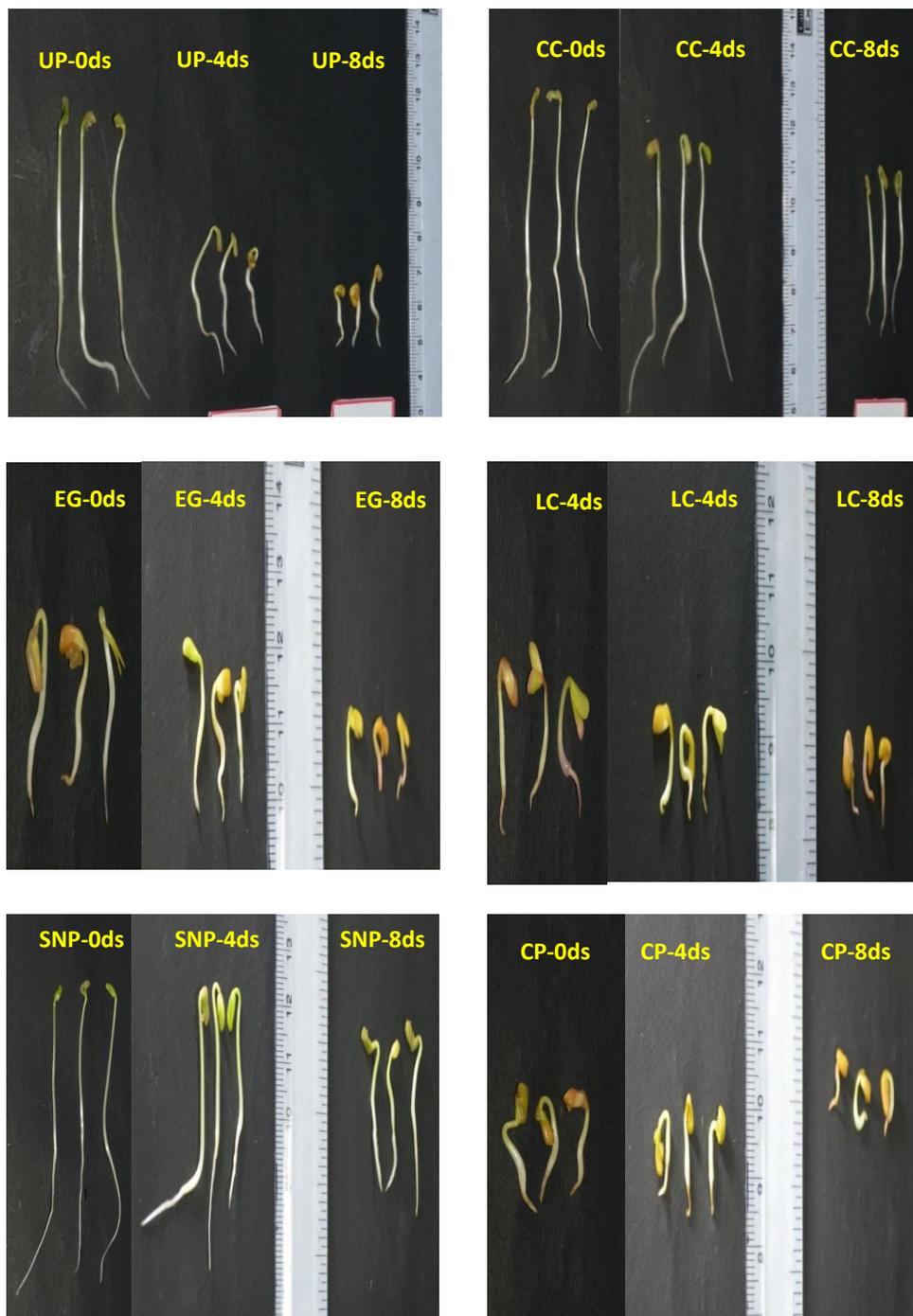


**Figure 6.7:** Box plot depicting the alteration in relative water content of fenugreek seedlings primed with different elicitors under salinity stress



**Figure 6.8:** Box plot depicting the alteration in stress tolerance index of fenugreek seedlings primed with different elicitors under salinity stress

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.9:** The fenugreek seedlings primed with different elicitors under salinity stress on 7<sup>th</sup> day of germination

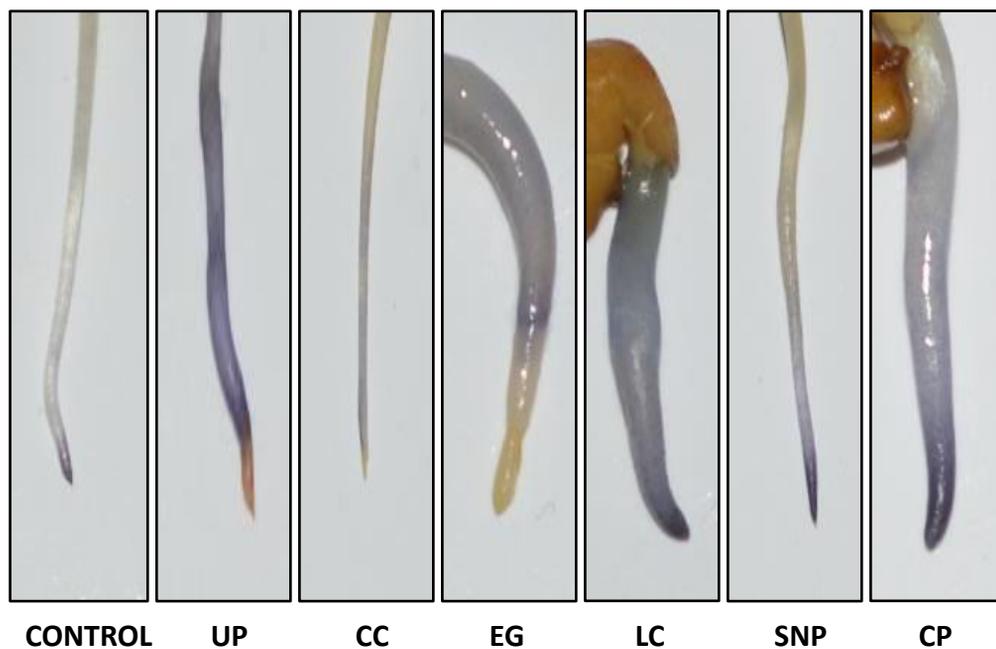
Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

The roots of unprimed and antagonist primed seedlings exhibited darker stain against NBT thus indicating  $O^{\cdot-}$  generation and the accumulation was found to be dose dependent under the influence of salinity, but the intensity of the stain was diminished under the influence of exogenous calcium and nitric oxide priming (Figure 6.10 & 6.11). Likewise, the roots subjected to calcium and nitric oxide priming exhibited lighter stain than those under the influence of saline environment and their antagonists (Figure 6.12 & 6.13), thus suggesting excessive  $H_2O_2$  accumulation in the tissues and the mitigation of same by the signalling molecules (calcium and nitric oxide). Interestingly, the difference in the  $H_2O_2$  accumulation among the various treated seedlings was further evident in the  $H_2O_2$  estimation assay, where the pattern of the  $H_2O_2$  detection was found in accordance of the  $H_2O_2$  content measured in those seedlings, with the seedlings exhibiting darker stain showed higher content of  $H_2O_2$  than those imparting lighter stain was found to contain  $H_2O_2$  in low concentration (Figure 6.18, 6.19 & 6.20). Further the  $H_2O_2$  accumulation in fenugreek seedlings was found to be dose dependent under the influence of salinity stress. The accumulation of these free radicals has been considered as major factor responsible for the disruption of cellular membrane integrity and their peroxidation thus leading to alteration in the cellular functioning (Sharma and Dubey, 2005). Thus, along with the evaluation of the content of these free radicals the membrane integrity and their peroxidation were studied through histochemical detection. The peroxidation of lipid bilayer of the cell membrane is one of the prominent adverse effects of salinity stress in biological system (Ahmad *et al.*, 2014; Hashem *et al.*, 2014). Such damages and injuries of biological membranes due to lipid peroxidation are measured in terms of malondialdehyde content (Hogg and Kalyanaraman, 1999) which indicates the degree of stress experienced by the tissue. Consequently, the membrane fluidity is disrupted leading to ion leakage from the tissue. In agreement to aforesaid statement, it was observed that the amount of MDA was found to increase considerably in response to salinity and further elevation was observed in the seedlings under the influence of antagonists as shown

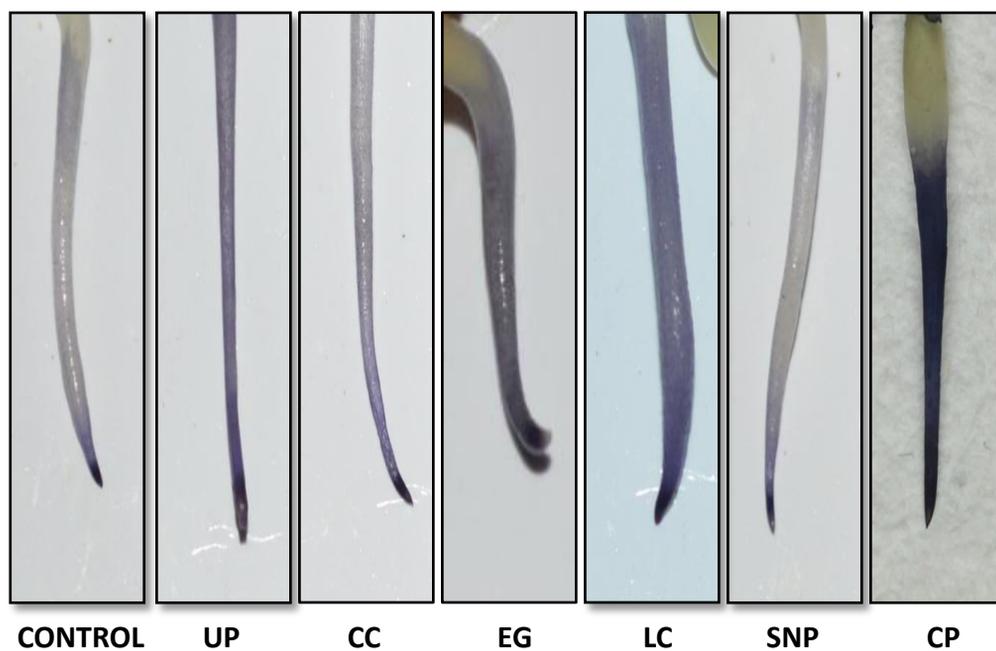
in Figure 6.14 & 6.15. It was observed that the MDA content was accumulated about 2 to 3 fold in the seedlings primed with EG, LC and CP and the accumulation gradually increased with time. Though a significant reduction in MDA content was not observed in the seedlings primed with calcium chloride and sodium nitroprusside during early germination stage but considerably reduction was observed during later stages in comparison to control seedlings (Table 6.3). The increase in MDA content was further confirmed by histochemical detection for lipid peroxidation, where the roots under salinity and antagonists priming stained dark pinkish compared to control and those pre-treated with exogenous calcium and nitric oxide exhibited comparatively lighter stain (Figure 6.14 & 6.15). Further the histochemical detection of plasma membrane integrity revealed that the roots of those under salinity and pre-treated with antagonists imparted darker stain against the Evans blue stain signifying loss of membrane integrity and the effect was dose dependent as well as in concomitant to accumulation of free radicals and degree of lipid peroxidation as mentioned earlier. The membrane integrity of those under the influence of exogenous calcium and nitric oxide was found to be resistant and undisturbed as evident from lighter stain exhibited against the dye (Figure 6.16 & 6.17). It has been earlier cited that calcium ion and nitric oxide serves as a part of signal cascade associated with plant growth and development as well as mitigate the adverse effect of salinity thus preventing oxidative damages of tissue (Arshi *et al.*, 2006; Jaleel *et al.*, 2007; Tian *et al.*, 2015). Likewise, in the present study also seedlings pre-treated with calcium chloride were found to be resistant and exhibited high degree of membrane integrity and low MDA content in concomitant to low amount of ROS localization. Furthermore, in agreement with our result, the protective role of calcium and nitric oxide is also found to be reported in other plant system under salinity stress condition (Bhattacharjee, 2009; Tian *et al.*, 2015). Similarly, other workers have also claimed that disruption of membrane permeability induced by salinity stress in strawberry (Kaya *et al.*, 2002) and tomato (Tuna *et al.*, 2007) was overcome by supplementary calcium. In plant

system calcium is localised mainly in cell wall and vacuole as a calcium store. As cited by Tian *et al.*, (2015)  $\text{Na}^+$  compete with  $\text{Ca}^{2+}$  for membrane binding sites thus presence of higher  $\text{Ca}^{2+}$  level during saline condition could significantly aid in reducing the  $\text{Na}^+$  influx thus protecting the cell membrane from stress mediated damages. Likewise, nitric oxide has also been reported to act as potent inhibitor of lipid peroxidation of cell membrane, thus further preventing ion leakage from the cell system (Hogg and Kalyanaraman, 1997; Rubbo *et al.*, 2006). The reduction of lipid peroxidation and maintenance of plasma membrane integrity under the influence of nitric oxide is documented in earlier studies including various other stress conditions such as UV stress in beans (Shi *et al.*, 2005) and arsenic stress in rice (Singh *et al.*, 2009) including salinity stress (Tian *et al.*, 2015). Such evidences of protective role of calcium and nitric oxide under stress conditions have also been provided by various authors such as Murillo-Amador *et al.* (2006) in cow pea; Gould *et al.*, 2003 in tobacco and Liu *et al.*, 2014 in cotton seedlings. Also the increase in ROS level and membrane injuries in those primed with antagonist agents (EG, LC and CP) further reinforces the active involvement of calcium and nitric oxide in maintaining the stress tolerance.

For studying the effect of exogenous calcium and nitric oxide under the salinity regime on the biochemical status of fenugreek seedlings, various biochemical attributes were taken under consideration. The biochemical attributes estimated in the present study were, proline, glutathione, ascorbate and sugars. It was observed that salt stress has affected the biosynthesis of almost all of these important biochemical molecules stated earlier.

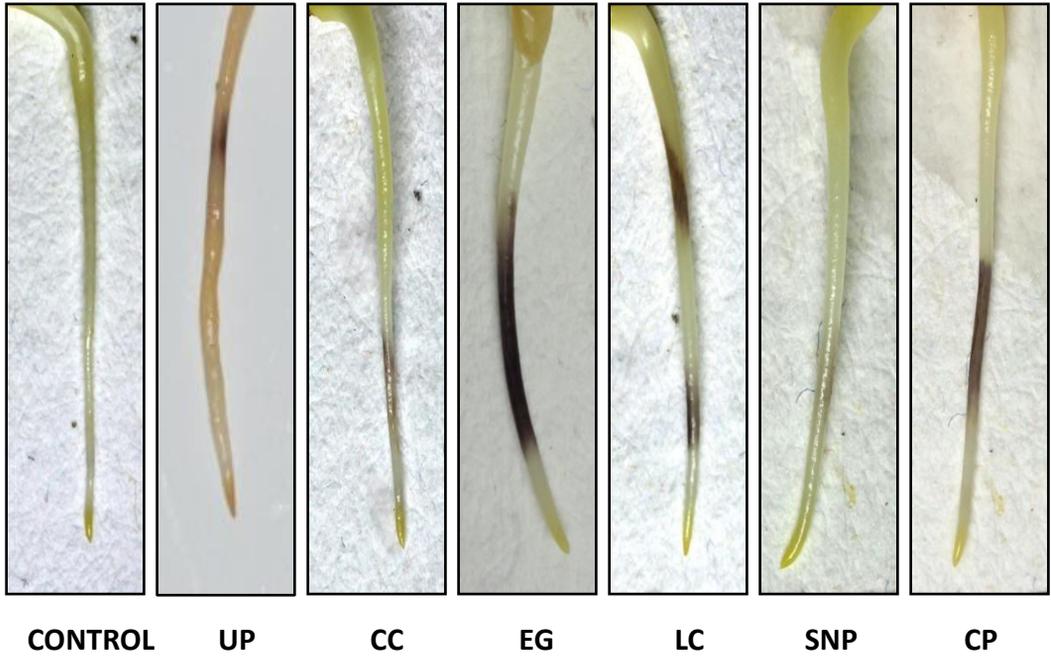


**Figure 6.10:** Histochemical detection of Superoxide localization in the fenugreek seedlings primed with different elicitors under salinity stress (4ds  $m^{-1}$ )

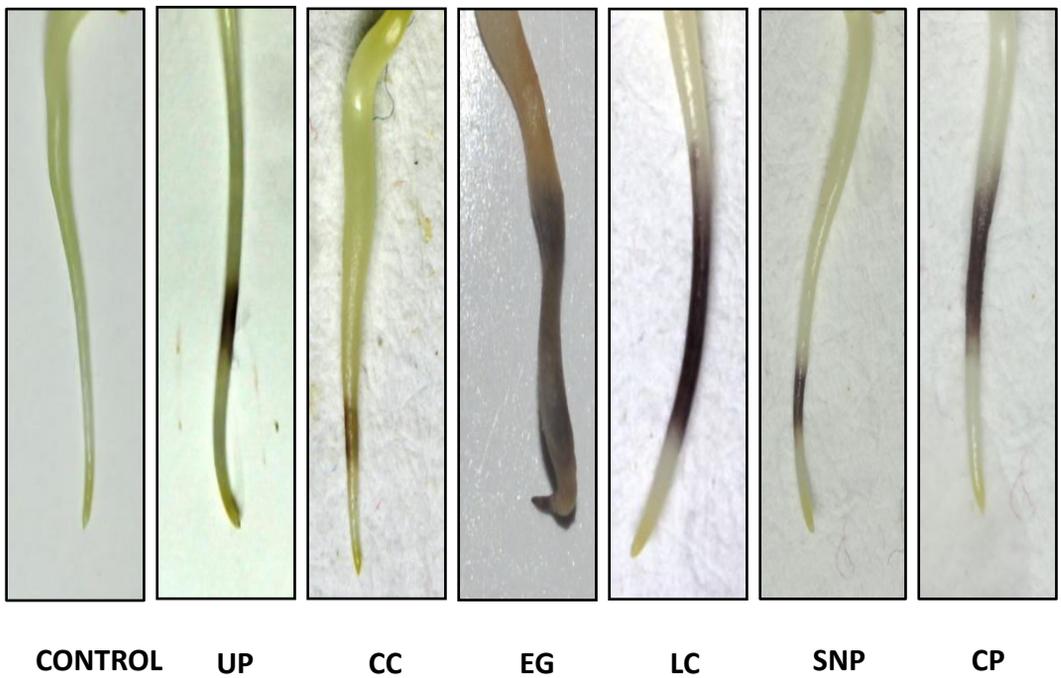


**Figure 6.11:** Histochemical detection of Superoxide localization in the fenugreek seedlings primed with different elicitors under salinity stress (8ds  $m^{-1}$ )

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

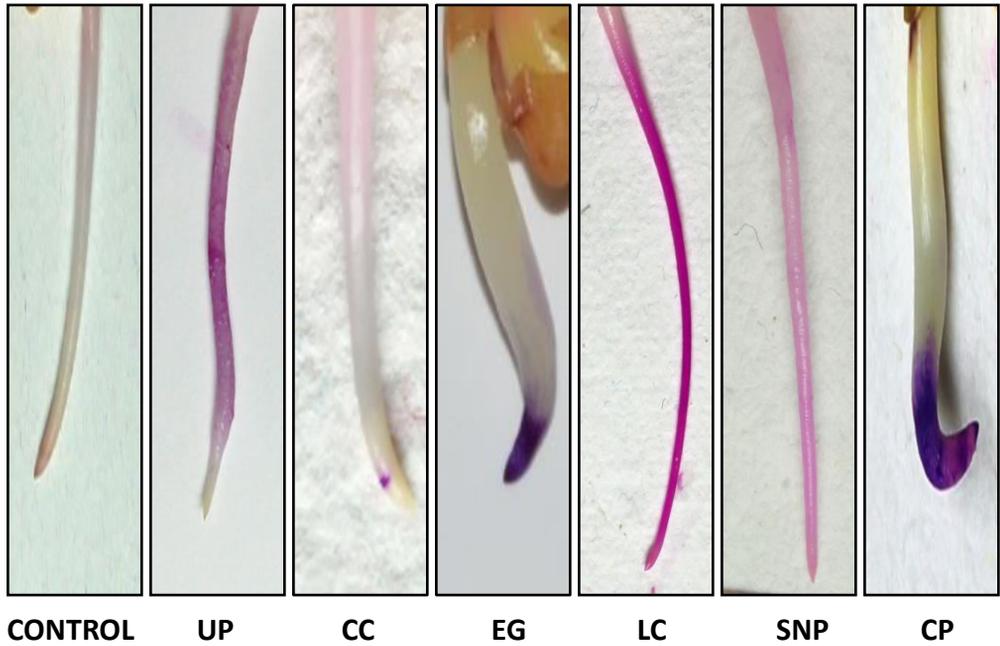


**Figure 6.12:** Histochemical detection of  $H_2O_2$  localization in the fenugreek seedlings primed with different elicitors under salinity stress ( $4ds\ m^{-1}$ )

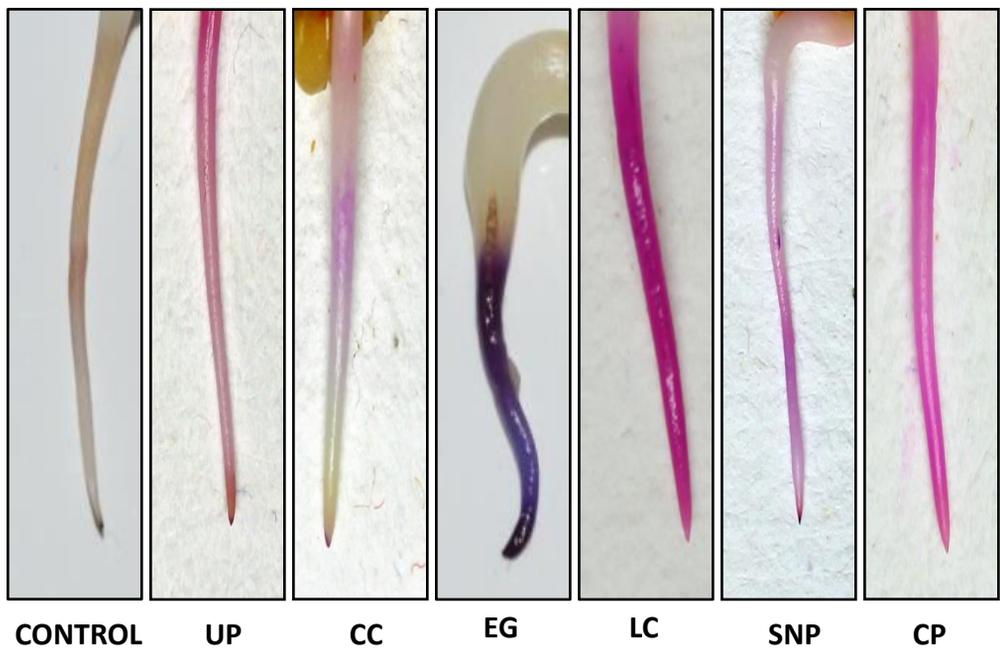


**Figure 6.13:** Histochemical detection of  $H_2O_2$  localization in the fenugreek seedlings primed with different elicitors under salinity stress ( $8ds\ m^{-1}$ )

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

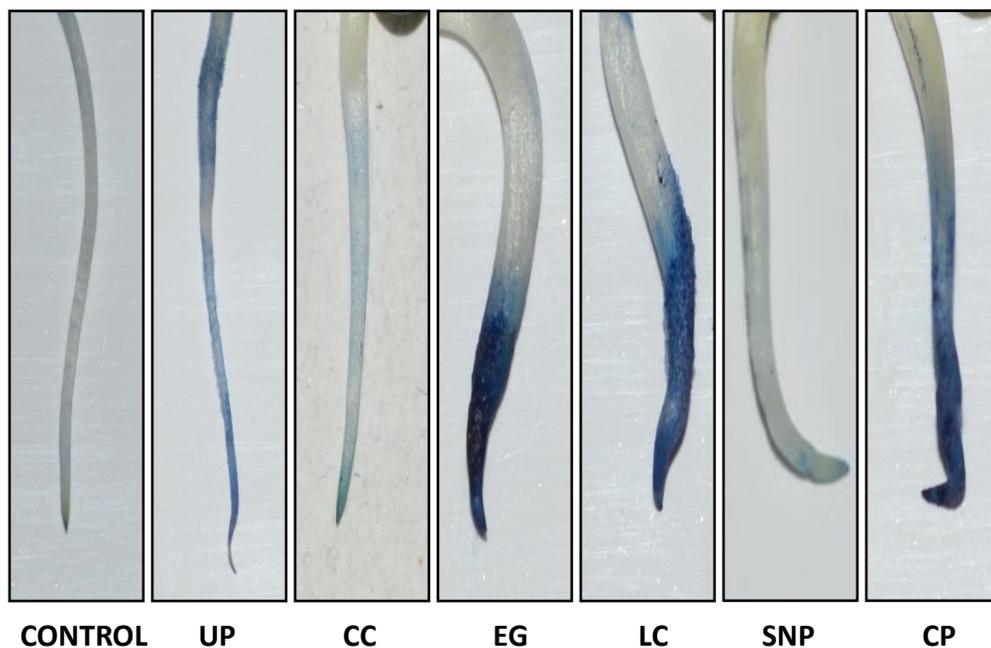


**Figure 6.14:** Histochemical detection of lipid peroxidation in the fenugreek seedlings primed with different elicitors under salinity stress (4ds  $m^{-1}$ )

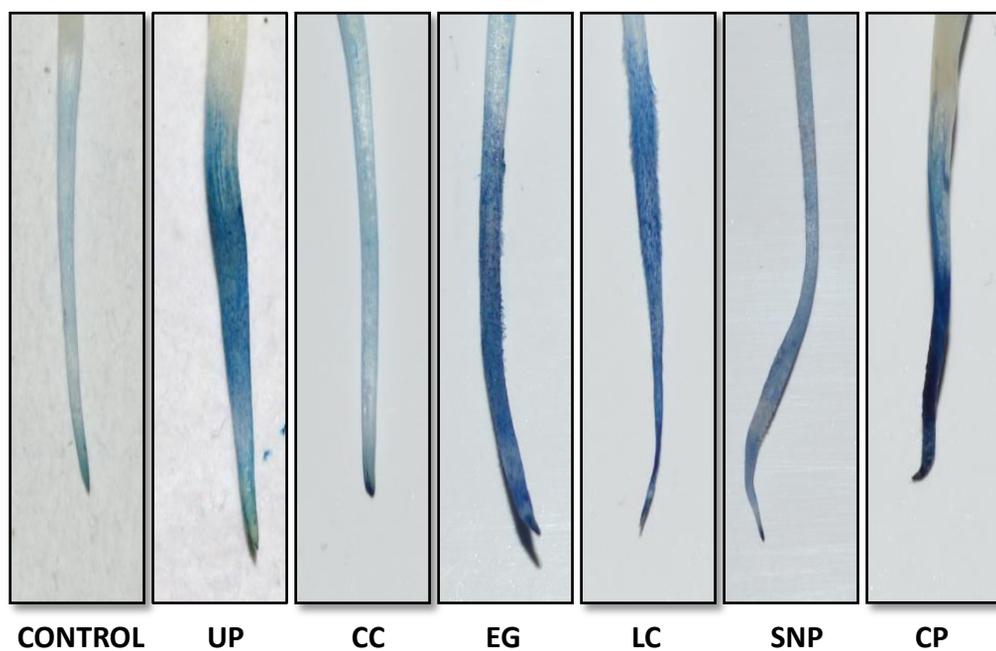


**Figure 6.15:** Histochemical detection of lipid peroxidation in the fenugreek seedlings primed with different elicitors under salinity stress (8ds  $m^{-1}$ )

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

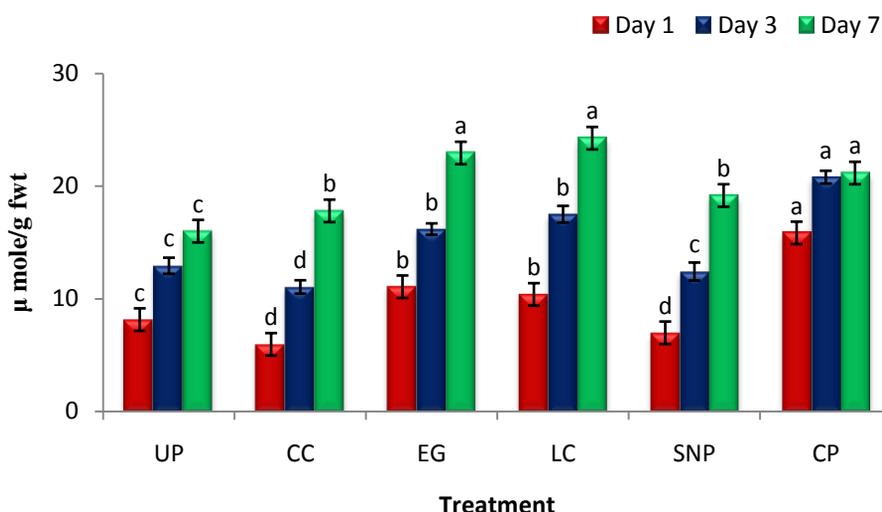


**Figure 6.16:** Histochemical detection of plasma membrane integrity in the fenugreek seedlings primed with different elicitors under salinity stress (4ds  $m^{-1}$ )

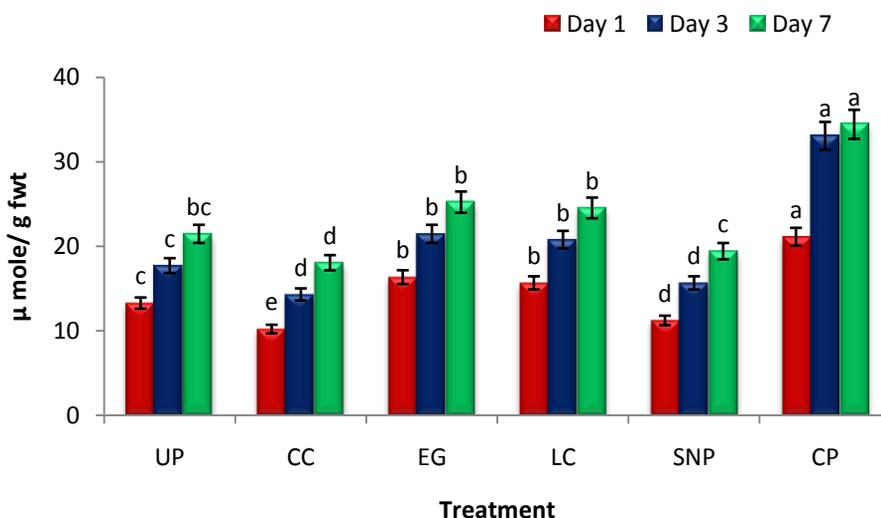


**Figure 6.17:** Histochemical detection of plasma membrane integrity in the fenugreek seedlings primed with different elicitors under salinity stress (8ds  $m^{-1}$ )

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

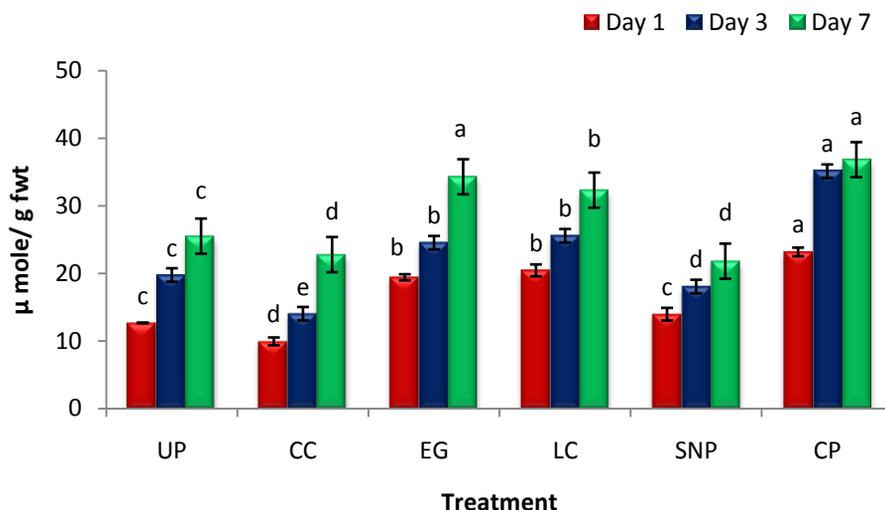


**Figure 6.18:** Effect of different priming agents on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings at 0ds m<sup>-1</sup>  
 Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.19:** Effect of different priming agents on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings at 4ds m<sup>-1</sup>  
 Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.20:** Effect of different priming agents on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings at 8ds m<sup>-1</sup>

Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

**Table 6.2:** ANOVA analysis of effect of different treatment on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>*P-value</i>	<i>F crit</i>
<b>Day of stress</b>	2821.284	6	470.2141	3318.712	1.54E-54	2.323994
<b>Treatment</b>	1560.135	2	780.0676	5505.619	1.5E-51	3.219942
<b>Interaction</b>	96.65362	12	8.054468	56.84743	5.32E-22	1.991013
<b>Within</b>	5.9508	42	0.141686			
<b>Total</b>	4484.024	62				

\*Significant at 0.01 level

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.3:** Effect of different elicitor priming on the MDA content ( $\mu$  mole/ g fwt) of fenugreek seedlings subjected to salinity stress

Treatment	UP	CC	EG	LC	SNP	CP	
<b>Day 1</b>	<b>0ds m<sup>-1</sup></b>	0.163±0.014d	0.159±0.016d	0.187±0.009c	0.198±0.006b	0.156±0.012d	0.201±0.014a
	<b>4ds m<sup>-1</sup></b>	0.298±0.026d	0.232±0.020e	0.338±0.029c	0.368±0.032b	0.253±0.021e	0.407±0.035a
	<b>8ds m<sup>-1</sup></b>	0.327±0.019c	0.296±0.027d	0.418±0.029bc	0.453±0.035b	0.307±0.030d	0.485±0.044a
<b>Day 3</b>	<b>0ds m<sup>-1</sup></b>	0.202±0.024d	0.196±0.018d	0.284±0.02c	0.305±0.019b	0.198±0.015d	0.354±0.022a
	<b>4ds m<sup>-1</sup></b>	0.372±0.019bc	0.325±0.036c	0.394±0.041b	0.398±0.018b	0.343±0.022c	0.506±0.061a
	<b>8ds m<sup>-1</sup></b>	0.487±0.19c	0.417±0.022d	0.503±0.032b	0.539±0.039b	0.445±0.025d	0.608±0.017a
<b>Day 7</b>	<b>0ds m<sup>-1</sup></b>	0.297±0.012d	0.285±0.01d	0.376±0.008c	0.439±0.019b	0.272±0.011d	0.462±0.02a
	<b>4ds m<sup>-1</sup></b>	0.452±0.015d	0.421±0.047e	0.578±0.028c	0.747±0.039b	0.442±0.022de	0.842±0.052a
	<b>8ds m<sup>-1</sup></b>	0.598±0.022d	0.538±0.041e	0.622±0.066c	0.878±0.045b	0.559±0.033e	1.017±0.042a

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.3(a):** ANOVA analysis of effect of different treatment on malondialdehyde content of fenugreek seedlings

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Day of stress	0.980982	6	0.163497	20.18482	6.09E-11*	2.323994
Treatment	1.013009	2	0.506504	62.53139	2.56E-13*	3.219942
Interaction	0.203717	12	0.016976	2.095859	0.038593**	1.991013
Within	0.3402	42	0.0081			
Total	2.537908	62				

\*Significant at 0.01 level; \*\*Significant at 0.05 level

Proline is a proteinogenic amino acid and also functions as a free radical scavenger, an osmolyte and electron sink stabilizer of cell wall components under stress conditions (Matysik *et al.*, 2002). The role of proline in providing the osmotolerance to the plant system during the stress environmental conditions has been widely suggested by various authors (Tateishi *et al.*, 2005; Zhang *et al.*, 2008). The result exhibited that the accumulation of proline was induced by salinity which was found to be further increased under the influence of the signalling molecule. The enhancement in the proline content of unprimed seedlings was about 22% and 33% under 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> condition, respectively with respect to control seedlings. The proline content was increased at 4ds m<sup>-1</sup> but later declined with further increase in the salinity stress 8ds m<sup>-1</sup> in unprimed sets. On the other hand, calcium chloride and SNP primed seeds exhibited significant increase in the accumulation of proline at both 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> condition (Figure 6.22 & 6.23). Likewise, various authors have also reported an enhancement in proline content upon priming with exogenous calcium and nitric oxide of other plants under various stress condition including salinity (Khan *et al.*, 2010; Chaum *et al.*, 2012; Zhang *et al.*, 2008; Ahmad *et al.*, 2016). Accumulation of proline is considered to be one of the most frequently reported defense mechanism that is adapted by plants during salinity stress. In agreement to the statement which claims proline is actively involved in maintenance of water content (Li *et al.*, 2014), it was observed that the relative water content of the seedlings was highly correlated to their proline content. This indicates that the signalling molecules (calcium and nitric oxide) further aids in acquiring this adaptive feature in maintaining the water balance under saline condition. However, there was a huge increase in the accumulation of proline in the seedlings subjected to priming with antagonists (EG, LC and CP) and the probable reason behind such excessive accumulation of proline might be the increase in the degradation of protein molecules (Joshi *et al.*, 2013) in those seedlings due to enormous stress experienced by the seedlings under the salinity stress as well as inhibition in the function of the signalling molecule in presence of their

antagonists. Also the result obtained were in accordance to the above mentioned statement that those seedlings exhibiting excess of proline were found to have reduction in their total protein content than the other treated and control seedlings. Further the accumulation of free proline was increased during the course of germination in each set of seedlings which may be attributed to the prevailing salinity stress on the seedlings during the course of germination.

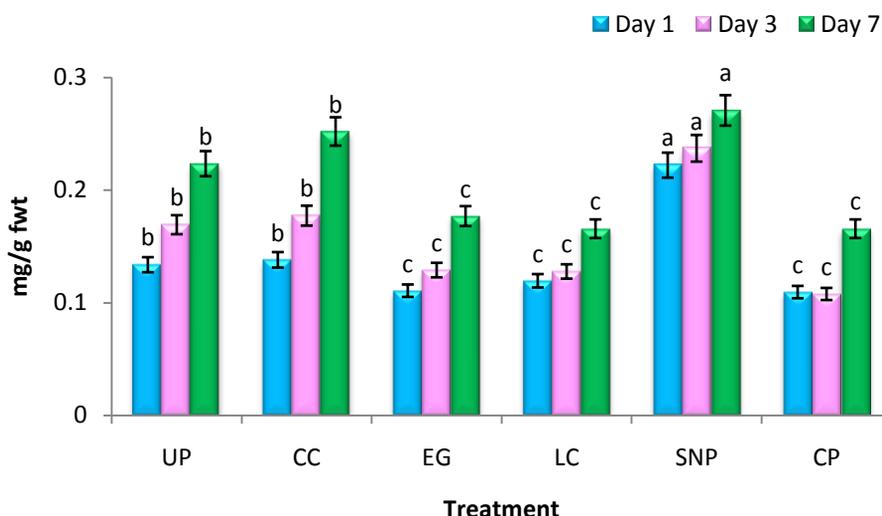
Carbohydrates play a crucial role in the plants survival; plants in their early developmental phase are highly dependent on the carbohydrates. Carbohydrates which are mobilized in the form of soluble sugars such as sucrose, glucose and fructose from source to sink tissues, either serve as substrate for cellular respiration or as osmolytes for maintenance of homeostasis (Gupta and Kaur, 2005) and also reported to be associated with the biosynthesis of secondary metabolites (Hilal *et al.*, 2004). This indicates that under stress condition the metabolism of soluble sugars is a vital process in which both breakdown and synthetic reactions are involved concurrently. Our results showed that the total soluble sugar content was significantly affected by salinity stress which is in agreement with the author who reported that under saline conditions the activity of enzymes involved in the sucrose-starch partitioning are either inhibited or delayed (Rosa *et al.*, 2009) which might lead to retarded growth. Accordingly in our studies the sugar content was reduced in unprimed as well as seedlings subjected to antagonist priming, a maximum loss of about 31% and 41% was recorded in EG primed seedlings; 34% and 39% for LC primed seedlings and 37% and 45% approximately for CP primed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity level respectively; however an increase in the sugar accumulation was observed in unprimed seedlings at lower salinity (4ds m<sup>-1</sup>) which was found to exhibit significant decline at higher salinity level (8ds m<sup>-1</sup>) at later stage (Table 6.5). Likewise, the difference in the morphological parameters in the normal and stressed fenugreek seedlings may be attributed to the difference in their sugar content which was also evident in our present study. Also, it

was observed that under salinity the seeds primed with exogenous calcium and nitric oxide had higher soluble and reducing sugar content than unprimed as well those subjected to antagonists priming (Table 6.6). A maximum loss in reducing sugar content was about 18% and 20% was recorded in EG primed seedlings; 21% and 27% for LC primed seedlings and 28% and 40% for CP primed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity level respectively. On the other hand, an increase in reducing sugar was observed; about 31% and 26% was recorded in calcium chloride priming and 16% and 12% for SNP primed seedlings in comparison to that of unprimed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity level respectively. Similarly, an enhancement in the sugar content under the influence of calcium ion was reported in *Cajanus cajan* (Gill and Sharma, 1993) and *Dioscorea rotundata* during salinity stress (Jaleel *et al.*, 2008). Also the influence of nitric oxide resulted in significant accumulation of soluble sugars in various plant system under stress conditions (Ahmad *et al.*, 2016). The accumulation of carbohydrates in the seedlings during salinity stress must be the defence mechanism of the plant system; during saline condition the water content of the plant body reduced so as a response to such decrease in water level, plant synthesizes soluble sugars and other compounds like proline to reduce their osmotic potential for regulating the loss of water, thus performing osmoregulation (Ahmad *et al.*, 2016). Also it has been reported that the accumulation of such osmolytes have been beneficial in maintaining the optimum turgidity of cells for normal functioning, thus overcoming the stress provoked by salinity (Khan *et al.*, 2012; Abdel Latef and Chaoxing, 2011). Therefore, the accumulation of osmolytes such as carbohydrates and proline under the influence of exogenous calcium and nitric oxide during salinity stress suggests the potential role of these signalling molecules in providing tolerance against salinity to fenugreek seedlings during germination. Moreover, the decrease in the sugar content in unprimed seedlings subjected to higher salinity level (8ds m<sup>-1</sup>) at later stage may due to inhibitory impact on the photosynthetic machinery of prolonged stress condition (Al-Hakimi and Hamada, 2011)

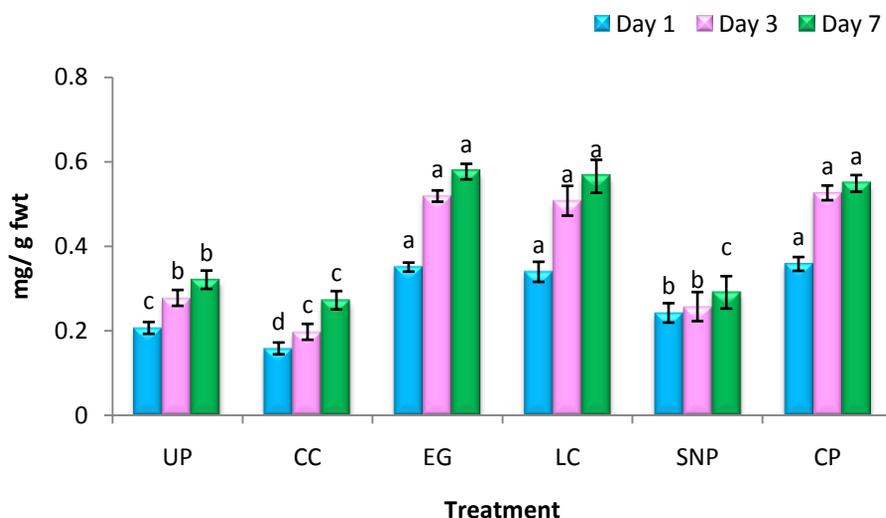
which was in accordance with Aly and Mohamed (2012), who also reported such decline in the sugar accumulation in maize towards higher stress condition.

Plants have evolved various defense mechanisms and strategies for minimizing the adverse effects of these free radicals and stress condition. These defense strategies are known to comprise of both enzymatic and non-enzymatic mechanisms as mentioned earlier. The major enzymes responsible for removal of excess ROS in plant system are CAT, SOD, APX and GR (Evelin and Kapoor, 2014), which are reported to exist in different cellular compartments as isozymes in chloroplast and mitochondria (Apel and Hirt, 2004; Ahmad, 2010). In the present study the non enzymatic antioxidants namely reduced glutathione (GSH) and ascorbate along with enzymatic antioxidants CAT, GPX, GR, GST, SOD, and APX were determined.

Glutathione is known to be an essential factor of thiol pool and actively involved in oxidative stress mitigation (Rennenberg, 1982). The glutathione content was found to be adversely affected by salinity and the antagonists, but the recovery in the glutathione pool during salinity was observed under the influence of exogenous calcium and nitric oxide. Though there was an increase in the glutathione content in the unprimed seedlings but later a decline of about 33% on day 7 was also observed in higher dose of salinity (8ds  $m^{-1}$ ). However under the influence of exogenous calcium and nitric oxide the accumulation of glutathione was found to be further induced; at 4ds  $m^{-1}$  an enhancement of 27% and 24% and at 8ds  $m^{-1}$  34% and 28% of glutathione content was recorded for calcium chloride and SNP primed fenugreek seedlings respectively (Figure 6.25 & 6.26). The well known functions of glutathione in plants are as a major factor in redox chemistry, elimination of heavy metals xenobiotics, serving as antioxidant molecule, regulation of stress defense gene expression, and potential role in alleviation of biotic and abiotic stresses including salinity (Foyer *et al.*, 1997; Gomez *et al.*, 2004; Ball *et al.*, 2004; Ruiz and Blumwald, 2002).

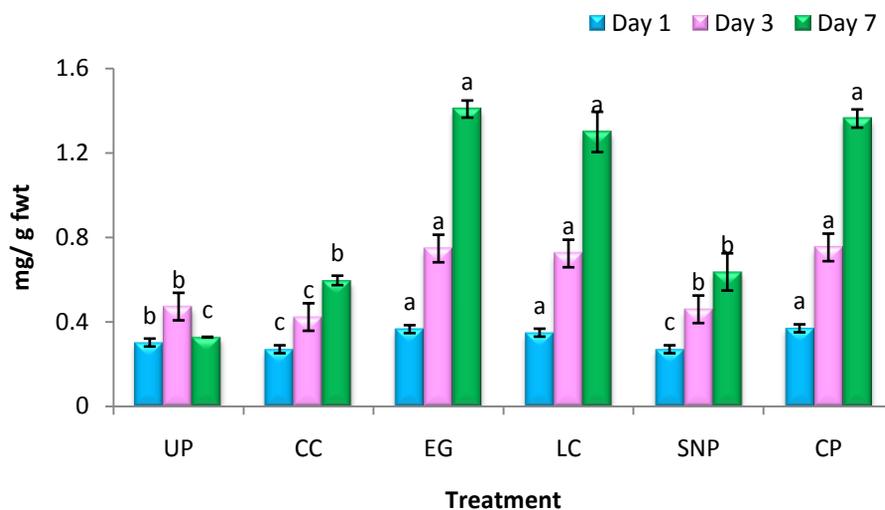


**Figure 6.21:** Effect of different priming agents on proline content of fenugreek seedlings at 0ds  $m^{-1}$ . Results are represented as mean  $\pm$  SEM,  $n=3$ . Values with different letters (a, b, c etc) are significantly ( $p<0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.22:** Effect of different priming agents on proline content of fenugreek seedlings at 4ds  $m^{-1}$ . Results are represented as mean  $\pm$  SEM,  $n=3$ . Values with different letters (a, b, c etc) are significantly ( $p<0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.23:** Effect of different priming agents on proline content of fenugreek seedlings at 8ds m<sup>-1</sup>

Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

**Table 6.4:** ANOVA analysis of effect of different treatment on total proline content of fenugreek seedlings

Source of Variation	SS	df	MS	F	P-value	F crit
Day of stress	3.749492	6	0.624915	295.3286	1.1E-32*	2.323994
Treatment	3.101446	2	1.550723	732.8559	2.21E-33*	3.219942
Interaction	1.876818	12	0.156402	73.91375	3.04E-24*	1.991013
Within	0.088872	42	0.002116			
Total	8.816628	62				

\*Significant at 0.01 level

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.5:** Effect of different elicitor priming on the total soluble sugar content ( $\mu\text{g/ g fwt}$ ) of fenugreek seedlings subjected to salinity stress

Treatment	UP	CC	EG	LC	SNP	CP	
<b>Day 1</b>	0ds m <sup>-1</sup>	157.93±1.46b	166.04±1.15a	143.22±2.04c	145.14±3.12c	164.36±1.74a	136.08±3.04d
	4ds m <sup>-1</sup>	172.51±2.01b	182.10±2.45a	134.17±1.15c	129.37±0.99cd	175.56±2.66b	120.44±1.05d
	8ds m <sup>-1</sup>	178.58±0.92c	189.73±1.92a	131.34±1.06d	126.10±0.76d	182.73±1.92bc	113.68±0.88e
<b>Day 3</b>	0ds m <sup>-1</sup>	171.87±2.16a	184.12±2.92a	145.26±2.24b	141.92±2.12b	174.14±2.74a	138.74±3.16b
	4ds m <sup>-1</sup>	182.44±0.54b	196.49±1.82a	138.53±0.85c	131.55±1.05c	189.46±1.24b	124.79±2.42c
	8ds m <sup>-1</sup>	167.29±0.52c	202.09±1.48a	129.33±2.32d	126.10±0.74d	184.09±1.48b	109.32±1.88e
<b>Day 7</b>	0ds m <sup>-1</sup>	192.05±1.62a	195.06±1.08a	149.54±2.02b	143.63±1.75b	192.18±1.26a	142.06±0.98b
	4ds m <sup>-1</sup>	187.98±0.54c	208.86±2.52a	131.34±1.15d	124.58±1.22d	196.60±2.04b	118.04±2.02e
	8ds m <sup>-1</sup>	158.02±1.16c	195.48±1.34a	109.54±2.14de	113.67±0.72d	179.48±1.34b	103.21±0.98e

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.5(a):** ANOVA analysis of effect of different treatment on total soluble sugar content of fenugreek seedlings

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Day of stress	41467.83	6	6911.304	15396.09	1.62E-68*	2.323994
Treatment	243.8117	2	121.9058	271.5657	9.46E-25*	3.219942
Interaction	2204.625	12	183.7187	409.2643	1.98E-39*	1.991013
Within	18.8538	42	0.4489			
Total	43935.12	62				

\*Significant at 0.01 level

**Table 6.6:** Effect of different elicitor priming on the reducing sugar content ( $\mu\text{g/ g fwt}$ ) of fenugreek seedlings subjected to salinity stress

Treatment	UP	CC	EG	LC	SNP	CP	
<b>Day 1</b>	<b>0ds m<sup>-1</sup></b>	52.25±1.24a	54.16±1.27a	49.22±1.06b	47.15±1.12b	52.96±1.02a	44.26±1.22c
	<b>4ds m<sup>-1</sup></b>	58.43±0.94b	66.98±0.86a	46.84±1.56c	45.38±2.15c	64.96±1.02a	42.12±0.98c
	<b>8ds m<sup>-1</sup></b>	62.93±1.08b	72.92±0.92a	45.76±2.24c	42.78±0.76c	69.66±0.58a	32.82±1.66d
<b>Day 3</b>	<b>0ds m<sup>-1</sup></b>	65.58±0.54b	76.19±1.02a	52.04±0.46c	50.16±0.47c	75.96±0.94a	48.04±0.29d
	<b>4ds m<sup>-1</sup></b>	66.87±0.72b	71.89±1.05a	55.12±1.12c	52.54±0.44c	71.24±0.52a	47.01±1.36c
	<b>8ds m<sup>-1</sup></b>	69.58±0.54b	75.78±0.78a	52.93±1.12c	47.69±0.86c	74.82±0.96a	37.71±1.38d
<b>Day 7</b>	<b>0ds m<sup>-1</sup></b>	72.52±1.56b	85.08±1.28a	56.15±1.85c	54.02±1.92c	79.98±0.92a	49.16±1.76c
	<b>4ds m<sup>-1</sup></b>	63.51±1.28c	82.40±0.88a	42.54±2.06d	39.94±1.44d	73.23±0.66b	44.42±1.15d
	<b>8ds m<sup>-1</sup></b>	60.98±0.48b	79.18±1.36a	40.34±0.96c	35.09±1.44c	74.22±0.58a	35.12±0.96c

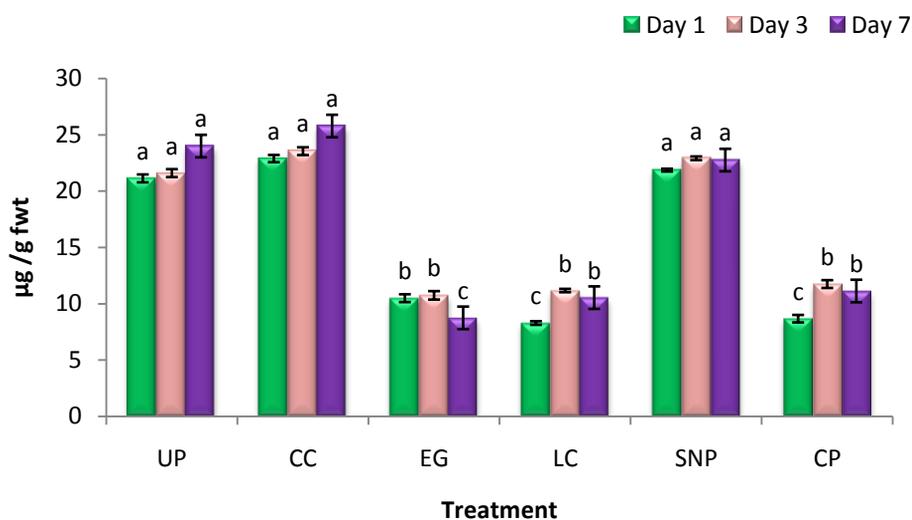
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.6(a):** ANOVA analysis of effect of different treatment on total reducing sugar content of fenugreek seedlings

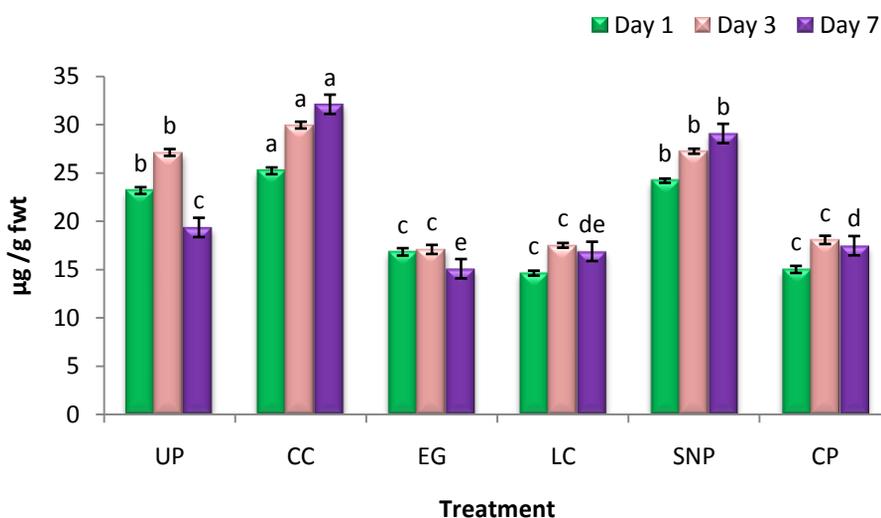
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Day of stress	2337.258	6	389.543	503.0255	1.9E-37*	2.323994
Treatment	714.0475	2	357.0237	461.0327	2.64E-29*	3.219942
Interaction	48.95077	12	4.079231	5.267602	2.54E-05*	1.991013
Within	32.5248	42	0.7744			
Total	3132.781	62				

\*Significant at 0.01 level



**Figure 6.24:** Effect of different priming agents on glutathione content of fenugreek seedlings at 0ds m<sup>-1</sup>

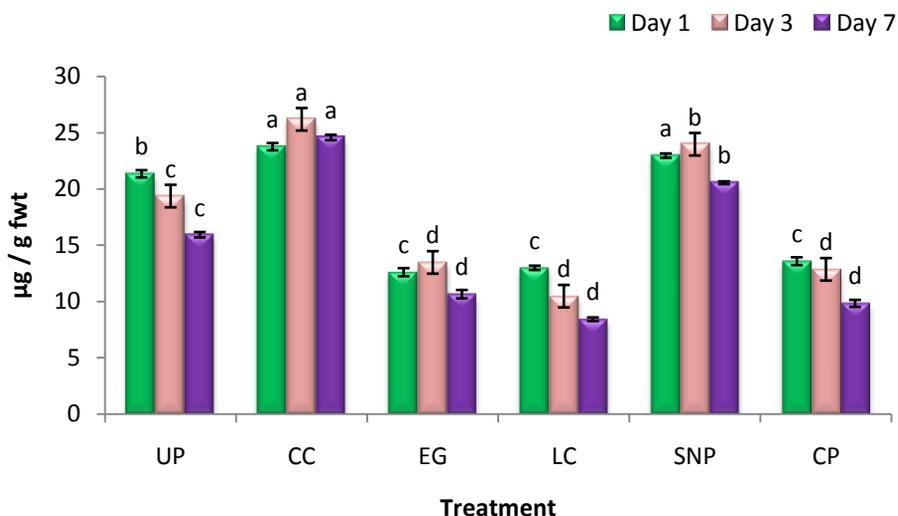
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.25:** Effect of different priming agents on glutathione content of fenugreek seedlings at 4ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.26:** Effect of different priming agents on glutathione content of fenugreek seedlings at 8ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

**Table 6.7:** ANOVA analysis of effect of different treatment on glutathione content of fenugreek seedlings

Source of Variation	SS	df	MS	F	P-value	F crit
Day of stress	1481.323	6	246.8871	1149.076	6.64E-45*	2.323994
Treatments	77.56147	2	38.78073	180.4954	2.38E-21*	3.219942
Interaction	173.7958	12	14.48298	67.4075	1.88E-23*	1.991013
Within	9.024	42	0.214857			
Total	1741.704	62				

\*Significant at 0.01 level

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

GR helps in maintaining the total thiol pool in the cell system which is very much essential for plants. The maintenance of optimum ratio of GSH: GSSG (oxidized glutathione) in the thiol pool is very much important for the plant system for their defense against biotic and abiotic stress conditions (Swamy *et al.*, 2011; Ghanta and Chattopadhyay, 2011). Glutathione is also considered as “master antioxidant” as it serves as a redox buffer and the centre of a complex antioxidant network in plant system thus regulating the intracellular environment (Szalai *et al.*, 2009; Maughan *et al.*, 2010; Ghanta and Chattopadhyay, 2011). The glutathione pool has a significant association with enzymes such as GR and GST as they are involved in glutathione metabolism (Rauser 1999) thus, the activity of these enzymes might be influenced by the glutathione content in the system. Under stress condition glutathione helps in removal of free radicals by being oxidized itself to GSSG form (Noctor and Foyer, 1998). Since highly reduced glutathione pool is essential for functioning of protein actively, the enzyme GR catalyzes the reverse reaction for conversion of GSSG to GSH in presence of NADPH (Ball *et al.*, 2004). Therefore, enzyme GR is important for the plant system for their defence against stress mediated adverse effects in their growth and development. In our study it was observed that the GR activity was significantly reduced under salinity in fenugreek seedlings, the activity of GR enzyme in unprimed seedlings was found to be decreased by 30% and 41% with respect to control seedlings at 4ds  $m^{-1}$  and 8ds  $m^{-1}$  respectively with respect to control. This indicates the activity of GR decreased with increase in the concentration of salt also it was observed that the activity decline during further germination stages (Figure 6.27, 6.28 & 6.29). Such decrease in GR activity was also reported by Kang *et al.*, 2007 in cucumber seedlings subjected to hypoxia stress; further Swamy *et al.*, 2011 observed such reduction in GR activity in fenugreek under cadmium stress along with GSH content. However some authors have also claimed an increase in the activity of GR in barley (Kim *et al.*, 2005) and wheat genotypes (Mandhania *et al.*, 2006) under salinity stress. The application of exogenous calcium and nitric oxide as priming

agents resulted in elevation in the GR activity by 1.5 fold and 2 fold approx. at 4ds m<sup>-1</sup> and 1.6 fold and 1.4 fold approx. at 8ds m<sup>-1</sup>. In agreement to our result a significant induction of GR activity has been observed in *Amaranthus lividus* (Bhattacharjee, 2008) and cool season grasses (Jiang and Huang, 2001) by exogenous calcium application under thermal stress. Interestingly, it was observed that the seedlings with high GSH content exhibited enhanced GR and GST activity.

GST, due to their cytoprotective property is essential for plant defense against stress conditions; the function of GST responsible for its antioxidant activity is the catalysis of GSH conjugation (Marrs, 1996). It is reported that the salt tolerant plants and those having improved seedling growth under stress conditions have exhibited enhanced GR and GST activity (Mandhania *et al.*, 2006; Saha *et al.*, 2015). Similar findings were obtained in the present study, the seedlings with better GR and GST activity exhibited tolerant response towards salinity and hence better growth performance. The application of exogenous calcium and nitric oxide as priming agents resulted in elevation in the GST activity by 23% and 8% at 4ds m<sup>-1</sup> and 22% and 7% at 8ds m<sup>-1</sup> (Table 6.8). As expected the seedlings primed with the antagonists EG, LC and CP displayed negative effects on the activity of GR and GST along with the GSH content, which was in accordance to the findings of Shi *et al.*, (2014) who reported such negative effects of EGTA on the GR activity and GSH content of Bermuda grasses subjected to chilling stress.

For the assessment of the effect of salinity stress on antioxidant system of fenugreek seedlings, the activities of other important antioxidant enzymes were determined. The other antioxidant enzymes considered for our study were CAT, SOD, APX, and NOX. The reactive oxygen species and other free radicals produced in plant system are scavenged by either the antioxidant enzymes and/or water and lipid soluble compounds. Further among these, the antioxidative enzymes are considered to be the most effective against oxidative damage caused by these free radicals (Halliwell and Gutteridge 1992, Foyer and Fletcher

2001). From the result, it was observed that the activities of these enzymes in fenugreek seedlings were significantly altered by the salinity stress as well as the elicitors of calcium and nitric oxide signalling. Interestingly, it was found that the activities of almost all assessed enzymes, the activity significantly increased with the increase in the extent of salinity (4ds m<sup>-1</sup>) and later decreased with further rise in the salt stress (8ds m<sup>-1</sup>).

Catalase is a ubiquitous enzyme in living system having one of the highest turnover rates of all enzymes, it is reported that one molecule of catalase has capacity to convert millions of H<sub>2</sub>O<sub>2</sub> molecules into water and oxygen each second (Chakraborty *et al.*, 2009). It was observed that the activity of CAT enzyme was initially increased in fenugreek seedlings but later declined with prolonged exposure of higher (8ds m<sup>-1</sup>) salinity stress. The CAT activity was found to increase up to day 3 in unprimed and those primed with exogenous calcium and nitric oxide fenugreek seedlings under both 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> but the decline in the activity was much more pronounced in the unprimed seedlings compared to those primed with CC and SNP. Further, the seedlings primed with EG, LC and CP were found to exhibit rapid decline in the CAT activity during the course of germination and higher saline concentration in comparison to control and other primed seedlings (Figure 6.30, 6.31 & 6.32). The application of exogenous nitric oxide and calcium resulted in maximum elevation in the CAT activity by 32% and 8% at 4ds m<sup>-1</sup> and 26% and 14% at 8ds m<sup>-1</sup> respectively with respect to unprimed seedlings. Our results are in accordance to the observations reported by Ahmad *et al.*, 2016 in chick pea, Hayat *et al.*, 2012 in tomato and Dong *et al.*, 2014 in cotton where the NO application significantly increased the CAT activity under salinity stress. The CAT enzymes are actively involved in removal of H<sub>2</sub>O<sub>2</sub> which are produced in huge amount during the process of photorespiration and fatty acid oxidation in the organelles such as peroxisomes and glyoxysomes (Chelikani *et al.*, 2004). Further the elevation in the CAT activity by nitric oxide and calcium application was found to be in accordance with the stress tolerance in plants subjected to chilling, thermal and salinity

stress conditions (Bhattacharjee 2009; Kaya *et al.*, 2015). The significant enhancement in the activity was also further evident in the on gel Native-Page analysis of CAT isozyme activity where variation in the isozyme activity was observed and is discussed in later section.

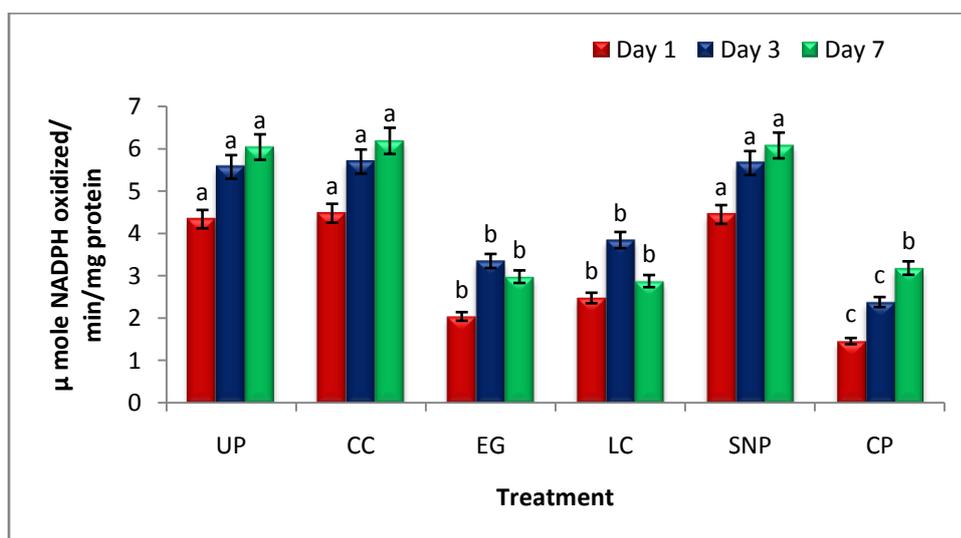
Almost a similar trend was observed in the guaiacol peroxidase (GPX) activity of differently treated and untreated fenugreek seedlings. The activity of GPX was found to be induced in all the seedlings except for those primed with the antagonists of nitric oxide and calcium signalling. The GPX activity on application of SNP and CC was noticed to be induced till 7 days of germination which was significantly higher with respect to the unprimed seedlings (Figure 6.33, 6.34 & 6.35). Further, the seedlings subjected to EG, LC and CP priming exhibited considerable reduction in the GPX activity thus indicating the importance of the presence of nitric oxide and calcium in the cellular system. Similar findings suggesting the elevation of peroxidase activity under the influence of exogenous nitric oxide and calcium were reported by Manai *et al.*, 2014 in tomato and Tian *et al.*, 2015 in wheat during salinity stress. Also, nitric oxide and calcium treatment have been claimed to be beneficial in providing stress tolerance to plants with increase in the peroxidase activity under various stress condition including heavy metal and UV radiations (Singh *et al.*, 2008; Yang *et al.*, 2013). Moreover, exogenous calcium was found to be more effective than nitric oxide in induction of both catalase and peroxidase activity in fenugreek seedlings under salinity stress for longer durations.

Superoxide dismutase (SOD) is the enzyme involved in the first line of antioxidant defence mechanism in cellular systems against reactive oxygen species. It catalyzes the dismutation of superoxide anions into H<sub>2</sub>O<sub>2</sub> which are further scavenged by catalase and peroxidase enzymes (Rybus-Zajac and Kubis, 2010). Our results revealed that initially the SOD activity increased with increase in salt concentration in unprimed as well as CC and SNP primed seedlings but a significant decline was observed during later stage in unprimed seedlings at higher salt concentration (8ds m<sup>-1</sup>) compared to CC and SNP primed seedlings.

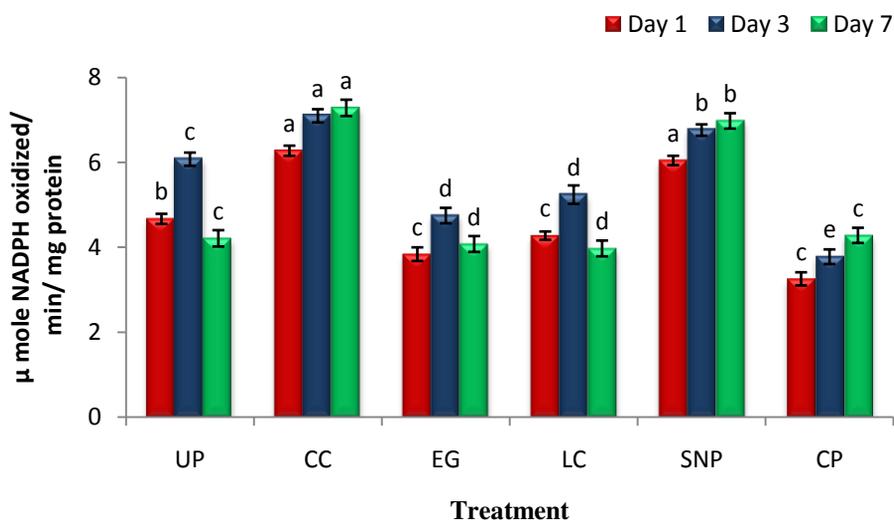
On the other hand the application of antagonist of signalling molecules EG, LC and CP tremendously affected the SOD activity of fenugreek seedlings under salinity stress (Figure 6.36, 6.37 & 6.38); thus, indicating the role of these signalling molecules in induced expression of SOD enzymes during stress conditions. The activity of SOD enzyme was in concomitant with CAT and POD activity thus signifying the complementary roles of these antioxidant enzymes in removal of free radicals. Such parallelism in the activities of SOD and catalase has been reported earlier (Balakumar *et al.*, 1993). Further, similar trend of correlation between elevation in the activities of SOD and peroxidases and salinity stress tolerance was reported in earlier studies (Alscher *et al.*, 2002; Li *et al.*, 2014). Further Bhattacharjee, 2008 in his study demonstrated that the application of exogenous calcium induced the activity of SOD enzyme under heat stress in *Amaranthus lividus* but the application of calcium antagonist like EGTA and LaCl<sub>3</sub> reversed the positive effects of calcium. Also under salinity stress the beneficial effect of nitric oxide and calcium on SOD activity was observed in several plants such as wheat, cluster beans, linseed etc. (Garg *et al.*, 1997; Khan *et al.*, 2010; Tian *et al.*, 2015)

Ascorbate peroxidase (APX) plays an important role in detoxification of free radicals such as hydrogen peroxide using ascorbate as a substrate (Raven, 2000), which indicates the amount of ascorbate regulates the activity of APX. In the present study it was noted that APX activity was greatly influenced by salinity stress which implies the potential role of this enzyme in modulating the stress mediated adversities in plant system. Further, the exogenous calcium and nitric oxide alleviated the stress effects in fenugreek seedlings with increase in the activity of APX (Table 6.9). Such induction of APX activity by these signalling molecules under various stress conditions were also reported by Xu *et al.*, (2013) in *Zoysa japonica*; by Hu *et al.*, (2008) in maize; by Jiang and Huang (2001) in cool season grasses; by Zhou and Guo 2009 in *Stylosanthes guianensis* and by Lin *et al.*, (2012) in cucumber. Moreover it was observed that the amount of ascorbate content was correlated

with the APX activity in the fenugreek seedlings, as such the seedlings with higher ascorbate content exhibited high APX activity and *vice versa*. Further, the trend of the alteration in ascorbate accumulation in differently pre-treated fenugreek seedlings (Table 6.10) was almost in concomitant with the APX activity of the seedlings during saline condition. In agreement to our study, various researchers have also reported a positive correlation between the APX activity and ascorbate content (De Gara *et al.*, 1997; Garneczarska and Wojtyla, 2008). The utilization of ascorbate by APX for removal of H<sub>2</sub>O<sub>2</sub> was also evident in our result as it was observed that the seedlings with enhanced ascorbate content and APX activity showed low accumulation of H<sub>2</sub>O<sub>2</sub>. Further, the effect of these elicitors was evaluated on the protein content of the fenugreek seedlings subjected to different saline conditions. The result demonstrated the protective role of calcium and nitric oxide in synthesis of protein during germination in fenugreek. Whereas, a huge loss in the protein content of the fenugreek seedlings was observed in those untreated and pre-treated with the antagonists of calcium and nitric oxide as presented in Figure 39, 40 and 41.

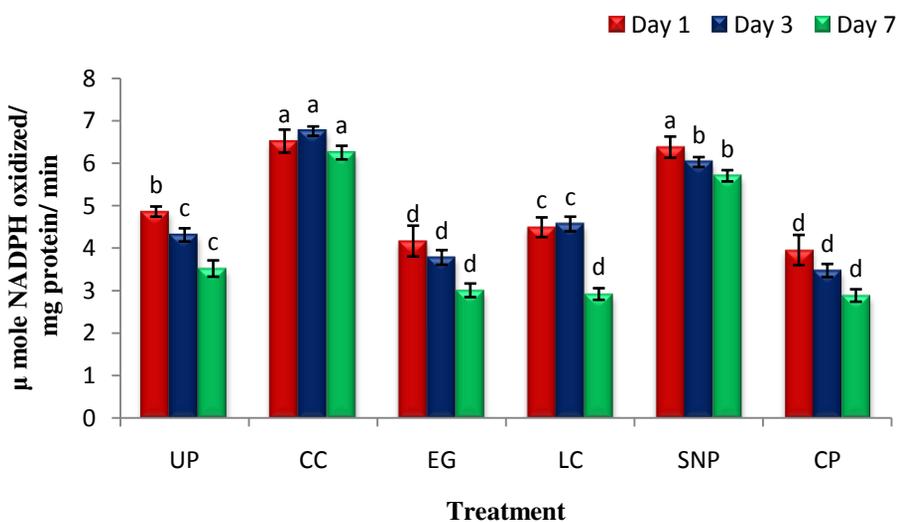


**Figure 6.27:** Effect of different priming agents on glutathione reductase of fenugreek seedlings at Ods m<sup>-1</sup>. Results are represented as mean± SEM, n=3. Values with different letters (a,b,c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test



**Figure 6.28:** Effect of different priming agents on Glutathione reductase activity of fenugreek seedlings at 4ds m<sup>-1</sup> of salinity

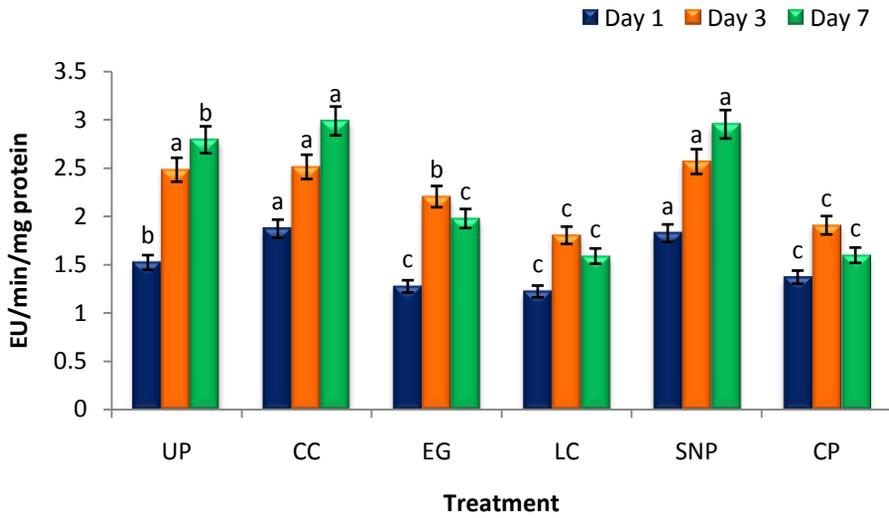
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



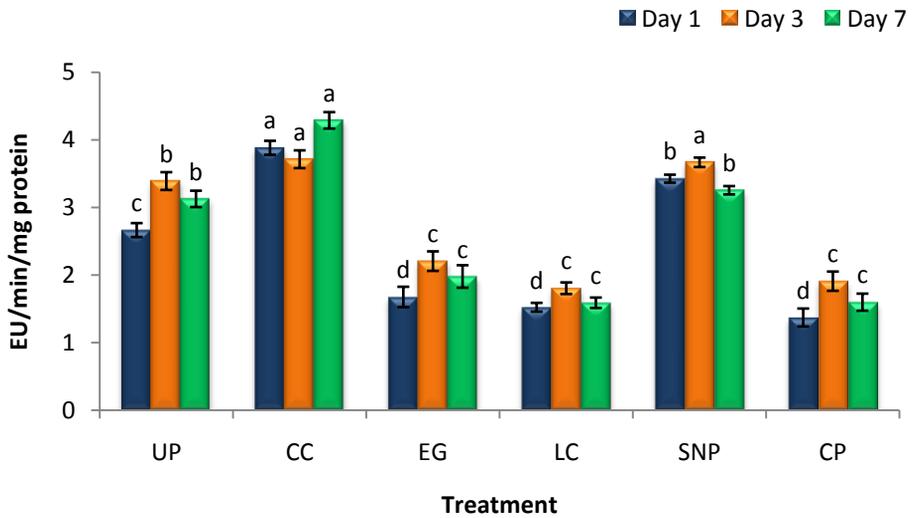
**Figure 6.29:** Effect of different priming agents on Glutathione reductase activity of fenugreek seedlings at 8ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

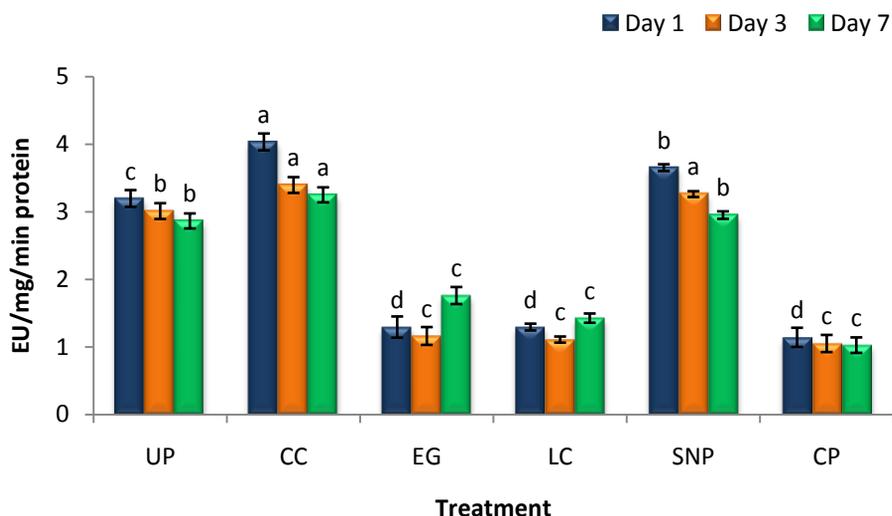


**Figure 6.30:** Effect of different priming agents on catalase activity of fenugreek seedlings at 0ds m<sup>-1</sup>  
 Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)



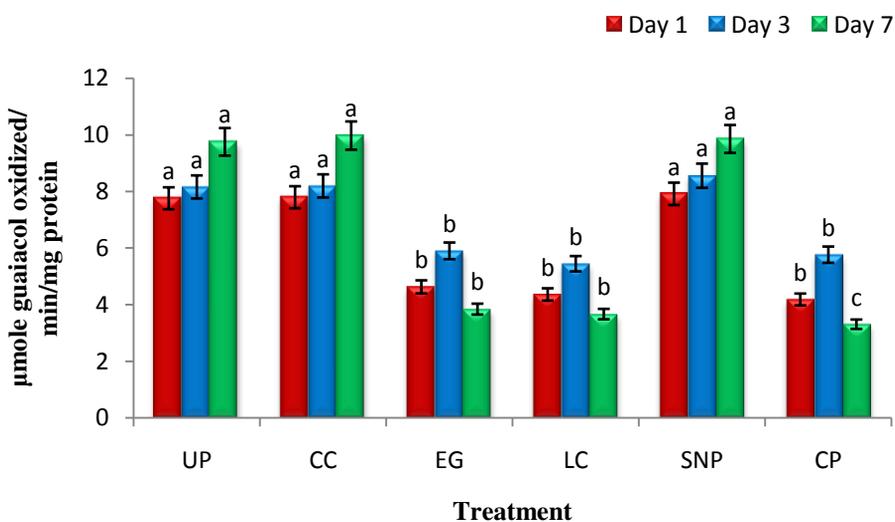
**Figure 6.31:** Effect of different priming agents on catalase activity of fenugreek seedlings at 4ds m<sup>-1</sup> of salinity  
 Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.32:** Effect of different priming agents on catalase activity of fenugreek seedlings at 8ds m<sup>-1</sup> of salinity

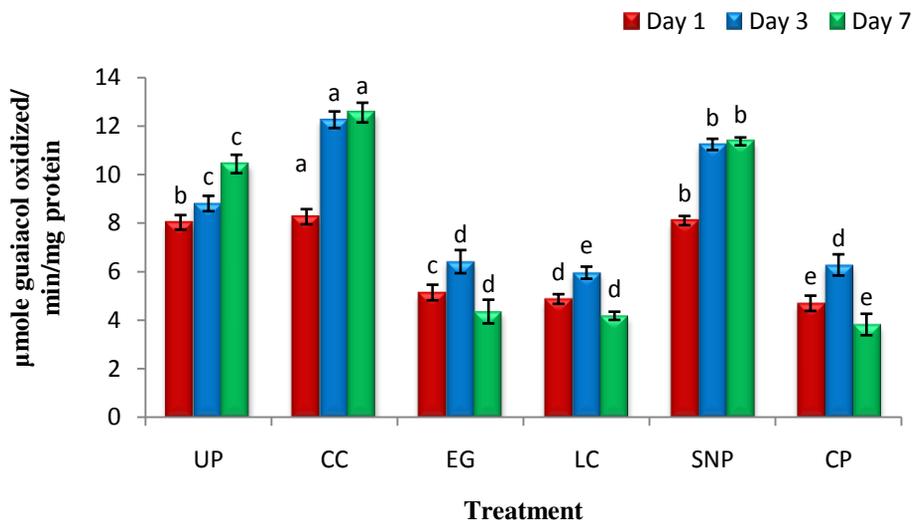
Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



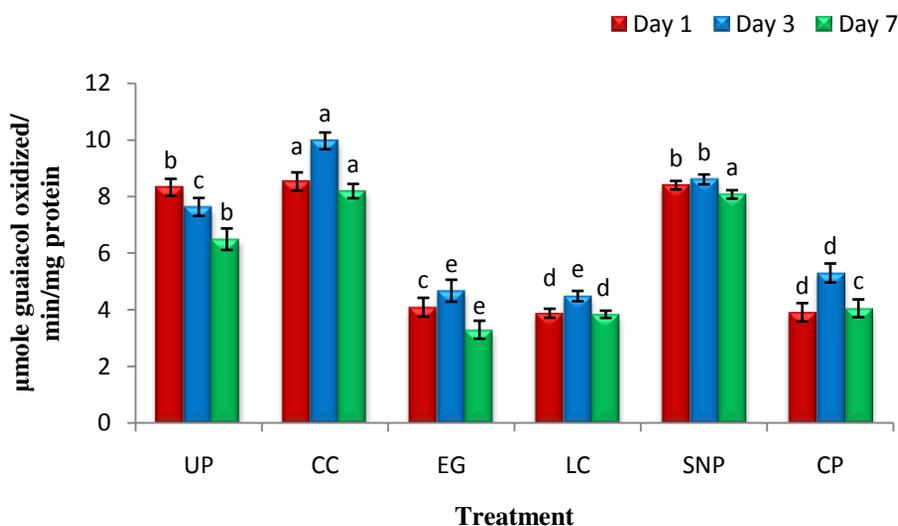
**Figure 6.33:** Effect of different priming agents on guaiacol peroxidase activity of fenugreek seedlings at 0ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

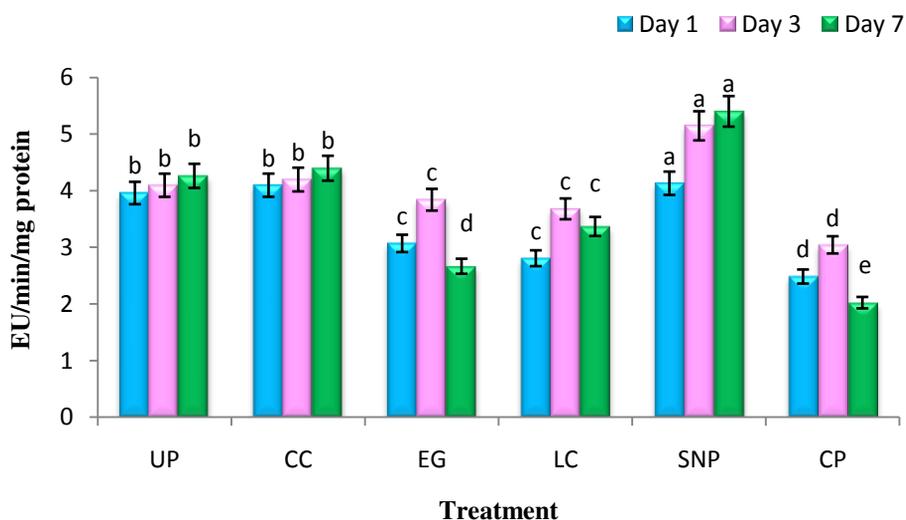


**Figure 6.34:** Effect of different priming agents on guaiacol peroxidase activity of fenugreek seedlings at 4ds m<sup>-1</sup> salinity. Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

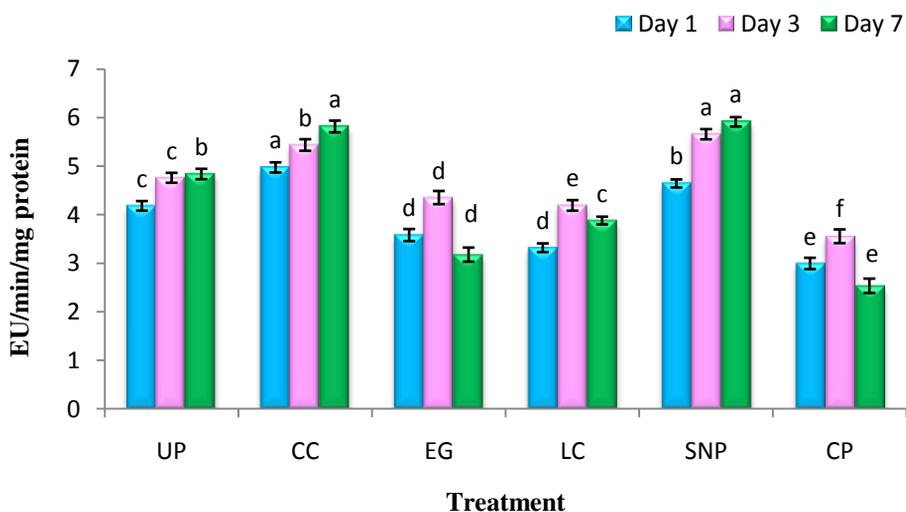


**Figure 6.35:** Effect of different priming agents on guaiacol peroxidase activity of fenugreek seedlings at 8ds m<sup>-1</sup> salinity. Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

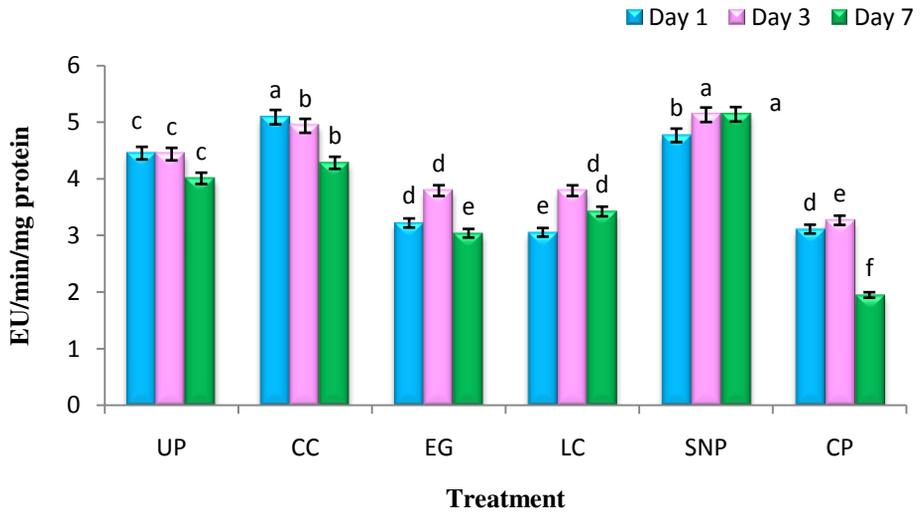


**Figure 6.36:** Effect of different priming agents on superoxide dismutase activity of fenugreek seedlings at 0ds m<sup>-1</sup>. Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)



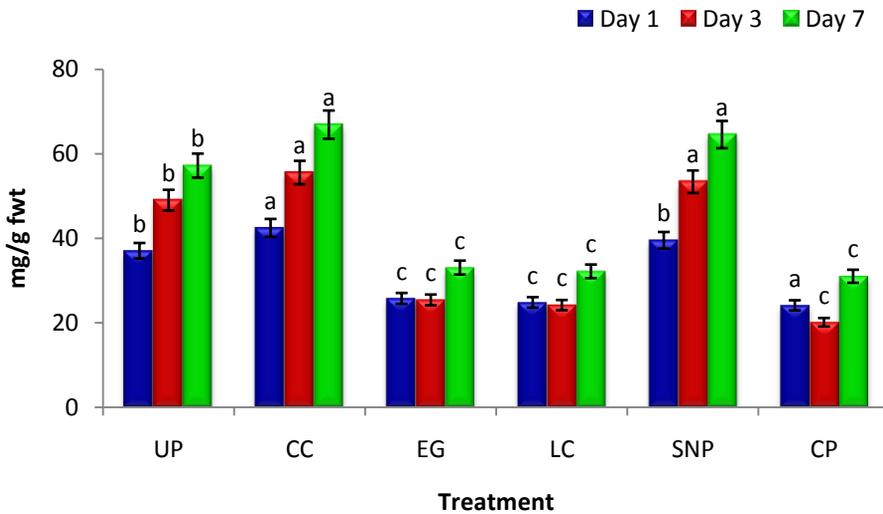
**Figure 6.37:** Effect of different priming agents on superoxide dismutase activity of fenugreek seedlings at 4ds m<sup>-1</sup> salinity. Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.38:** Effect of different priming agents on superoxide dismutase activity of fenugreek seedlings at 8ds m<sup>-1</sup> salinity

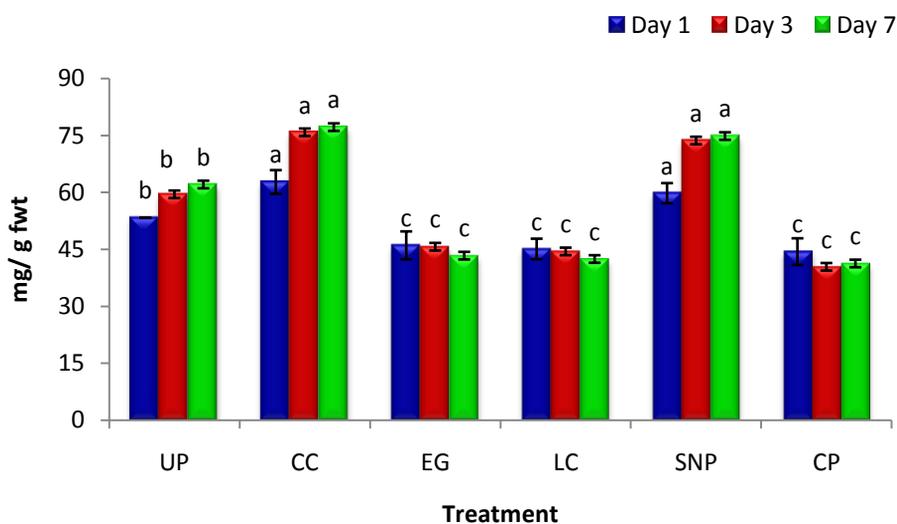
Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.39:** Effect of different priming agents on protein content of fenugreek seedlings at 0ds m<sup>-1</sup>

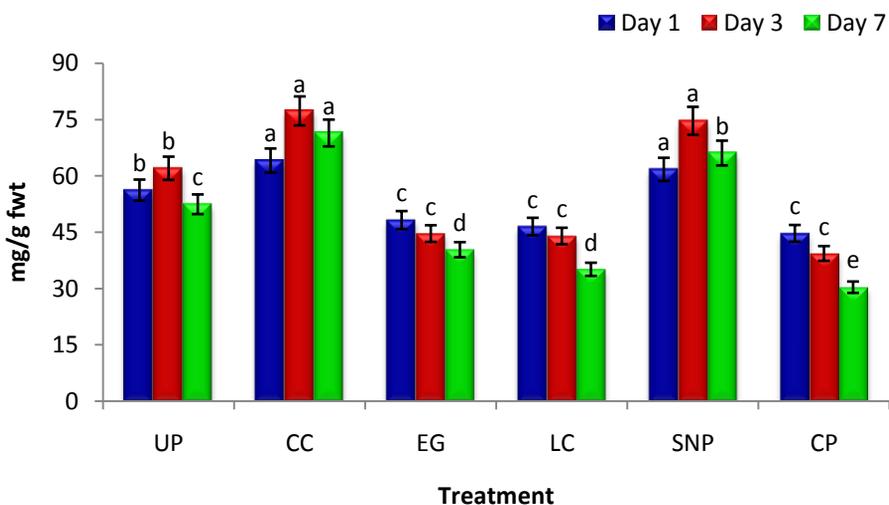
Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.40:** Effect of different priming agents on protein content of fenugreek seedlings at 4ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)



**Figure 6.41:** Effect of different priming agents on protein content of fenugreek seedlings at 8ds m<sup>-1</sup>

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.8:** Effect of different elicitor priming on Glutathione *S*-transferase activity ( $\mu\text{mole CDNB conjugate / min / mg protein}$ ) of fenugreek seedlings subjected to salinity stress

Treatment		UP	CC	EG	LC	SNP	CP
Day 1	0ds m <sup>-1</sup>	5.62±0.12a	5.78±0.08a	4.45±0.10b	4.57±0.09b	5.84±0.08a	4.76±0.11b
	4ds m <sup>-1</sup>	6.05±0.08c	7.47±0.06a	4.97±0.34e	5.19±0.08d	6.44±0.03b	3.05±0.18f
	8ds m <sup>-1</sup>	6.39±0.04b	7.80±0.05a	5.07±0.02c	5.26±0.05c	6.64±0.11b	3.49±0.04d
Day 3	0ds m <sup>-1</sup>	7.22±0.09a	7.36±0.12a	4.96±0.14b	4.87±0.09b	7.43±0.11a	4.98±0.07b
	4ds m <sup>-1</sup>	8.05±0.14c	10.36±0.22a	6.61±0.24d	6.56±0.26d	9.04±0.08b	6.70±0.19d
	8ds m <sup>-1</sup>	6.54±0.13c	7.34±0.05a	5.25±0.07d	3.78±0.14e	7.04±0.02b	6.35±0.08c
Day 7	0ds m <sup>-1</sup>	8.01±0.04a	8.15±0.11a	4.98±0.05b	4.96±0.08b	8.26±0.10a	5.07±0.09b
	4ds m <sup>-1</sup>	8.55±0.07c	10.59±0.12a	4.08±0.16d	3.06±0.22e	9.25±0.05b	4.41±0.13d
	8ds m <sup>-1</sup>	6.75±0.11c	7.56±0.06a	3.69±0.05d	3.42±0.01d	7.18±0.03b	3.87±0.05d

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.9:** Effect of different elicitor priming on Ascorbate peroxidase activity ( $\mu\text{mole ascorbate} / \text{min} / \text{mg protein}$ ) of fenugreek seedlings subjected to salinity stress

Treatment	UP	CC	EG	LC	SNP	CP	
<b>Day 1</b>	<b>0ds m<sup>-1</sup></b>	8.67±0.44b	9.02±0.53a	6.44±0.12c	6.52±0.97c	8.98±0.79a	5.14±0.05d
	<b>4ds m<sup>-1</sup></b>	11.38±0.12b	12.23±0.09a	7.36±0.51c	7.12±0.42c	11.64±0.28b	6.03±0.33d
	<b>8ds m<sup>-1</sup></b>	6.98±0.02b	8.63±0.06a	6.29±0.05c	5.75±0.03d	8.38±0.06a	4.79±0.08e
<b>Day 3</b>	<b>0ds m<sup>-1</sup></b>	9.10±0.05a	9.25±0.92a	6.86±0.15b	6.98±0.04b	9.34±0.95a	5.78±0.19c
	<b>4ds m<sup>-1</sup></b>	12.30±0.09b	13.41±0.02a	7.03±0.01c	7.63±0.04c	13.08±0.06a	6.18±0.02d
	<b>8ds m<sup>-1</sup></b>	7.77±0.08c	9.01±0.04a	6.40±0.02d	6.49±0.05d	8.60±0.02b	4.56±0.04e
<b>Day 7</b>	<b>0ds m<sup>-1</sup></b>	9.85±0.03c	12.33±0.76a	6.87±0.79d	4.98±1.04e	11.64±0.82b	5.08±1.02e
	<b>4ds m<sup>-1</sup></b>	12.65±0.02c	15.95±0.05a	7.41±0.02c	5.46±0.06d	13.12±0.01b	5.62±0.06d
	<b>8ds m<sup>-1</sup></b>	7.43±0.04c	10.69±0.02a	5.91±0.12d	4.34±0.32e	8.92±0.05b	3.98±0.09e

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

**Table 6.10:** Effect of different elicitor priming on the ascorbic acid content ( $\mu\text{g/ g fwt}$ ) of fenugreek seedlings subjected to salinity stress

Treatment	UP	CC	EG	LC	SNP	CP	
<b>Day 1</b>	0ds m <sup>-1</sup>	154.89±0.91b	165.47±0.53a	136.42±1.12c	138.62±0.97c	162.08±0.79a	110.08±2.05d
	4ds m <sup>-1</sup>	202.891±3.39a	203.70±2.74a	163.84±1.96c	162.22±0.84c	180.93±1.34b	145.94±2.66d
	8ds m <sup>-1</sup>	206.95±2.42a	193.94±3.12b	150.83±1.02d	146.75±0.98de	176.86±1.42c	140.25±1.14e
<b>Day 3</b>	0ds m <sup>-1</sup>	158.15±0.86b	168.72±0.92a	136.99±1.15c	135.37±2.04c	163.52±0.95ab	112.59±1.14d
	4ds m <sup>-1</sup>	211.84±0.88c	217.53±0.56a	152.45±2.36d	142.69±1.24d	202.89±0.75b	134.56±1.38e
	8ds m <sup>-1</sup>	195.56±0.66a	196.38±1.02a	147.57±0.85b	135.37±1.42b	189.06±0.92a	120.72±2.78c
<b>Day 7</b>	0ds m <sup>-1</sup>	162.07±0.93b	179.18±0.85a	141.25±0.79c	139.04±1.26c	167.44±0.84ab	124.18±1.37d
	4ds m <sup>-1</sup>	200.45±2.42c	231.36±3.56a	104.46±1.52d	94.69±1.34d	221.60±0.96b	78.43±1.13e
	8ds m <sup>-1</sup>	155.71±2.12b	198.82±0.54a	91.44±1.12c	79.23±0.84d	195.56±1.28a	61.34±0.91e

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) in rows are significantly ( $p < 0.05$ ) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

**Table 6.10(a):** ANOVA analysis of effect of different treatment on ascorbic acid content of fenugreek seedlings under salinity stress (4dsm<sup>-1</sup>) on Day 1, 3 and 7

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Day of stress	83951.05	6	13991.84	43946.2	4.44E-78*	2.323994
Treatment	4075.255	2	2037.628	6399.871	6.41E-53*	3.219942
Interaction	21105.56	12	1758.797	5524.107	4.46E-63*	1.991013
Within	13.3722	42	0.318386			
Total	109145.2	62				

\*Significant at 0.01 level

The plant species and cultivars with tolerant response towards stress conditions have been associated with maintenance of enhanced expression of antioxidant enzymes (Liu and Huang, 2000; Meriga *et al.*, 2004). Parida *et al.*, (2004) have stated that the antioxidant enzyme isoforms can be implemented as specific markers for studying the variance in salt tolerance; hence the electrophoretic study of the isoforms of antioxidant enzymes is essential. In the present study three antioxidant enzymes namely catalase, peroxidase and superoxide dismutase were subjected to on gel electrophoretic analysis of their respective isoforms. Only one isoform of CAT enzyme was detected in the treated as well as untreated fenugreek seedlings under salinity stress. However tremendous difference in the expression of the CAT isozyme was observed among various treated seedlings with respect to unprimed seedlings. The activity of CAT isozyme was found to express more in the seedlings primed with SNP and CC with respect to unprimed seedlings and the reversed effect was observed in the isozymes of seedlings subjected to antagonist priming (Figure 6.42). The difference in the intensity of respective isozymes were estimated in terms of relative density which has been graphically represented in Figure 6.43 which further revealed an increase in the isozyme expression in SNP and CC primed seedlings and reduction in EG, LC and CP respectively, with respect to unprimed seedlings. However in various studies, variation in the number of CAT isoforms was reported previously. A single isoform of CAT was detected by He and Huang (2010) in 'Eagleton' cultivar of Kentucky bluegrass whereas two isoforms were found in 'Brilliant' cultivar indicating difference in the response of the CAT enzyme among cultivars. Moreover four isoforms of CAT were reported by Parida *et al.*, 2004 in *Bruguiera parviflora* subjected to salinity stress. Despite of the variation in the number of CAT isoforms, a considerable difference in the expression of these isoforms were observed which was in accordance to the findings of the present study.

During on gel analysis of peroxidase (POD) isozyme two isozymes were detected in all the treated and untreated seedlings (Figure 6.44). Our findings were in contrary with the

previous workers who have reported multiple isoforms of POD in several plants. Four POD isoforms were reported by Scialabba *et al.*, 2002 in radish; He and Huang (2010) detected 8 isozymes in Kentucky bluegrass; 4 isoforms in safflower by Tayefi-Nasrabadi *et al.*, 2011; three isoforms were found in He and Gao, 2008 in *Chimonanthus praecox* and even 10 isoforms were reported by Zhou *et al.*, 2009 in *Medicago sativa*. However single isoform of POD was also detected in *Euphorbia milli* by Dewir *et al.*, 2006. Such variation in the isoforms of POD in various plant species is in accordance to Ye *et al.*, 1990 and Sreenivasulu *et al.*, 1999 who suggested that the POD isoforms varies among the plants as well as plant parts. Peroxidase besides scavenging harmful active oxygen species namely  $H_2O_2$  is also reported to be involved in the lignification process in plant system (Quiroga *et al.*, 2000). The plants are known to increase lignin synthesis under salinity stress for strengthening their cell wall in order to minimize water (Garcia *et al.*, 1997). Our findings are in accordance with Tayefi-Nasrabadi *et al.*, 2011 who demonstrated that increase in the activity of peroxidase isozyme was associated with mitigation in the water loss in the cell wall of tolerant cultivars under salinity stress. The difference in the intensity of respective isozymes were estimated in terms of relative density which has been graphically represented in Figure 6.45 which further revealed an increase in the isozyme expression in SNP and CC primed seedlings and reduction in EG, LC and CC respectively, with respect to unprimed seedlings. Isoform I was found to be least effected by priming agents during salinity stress as no significant changes in the intensity of the isoform was observed. However, the expression of isoform II to was found to be induced by CC and SNP whereas the antagonists considerably suppressed its expression; thus confirming the beneficial role of the signalling molecules (calcium and nitric oxide) in mitigation of salt stress in plant system.

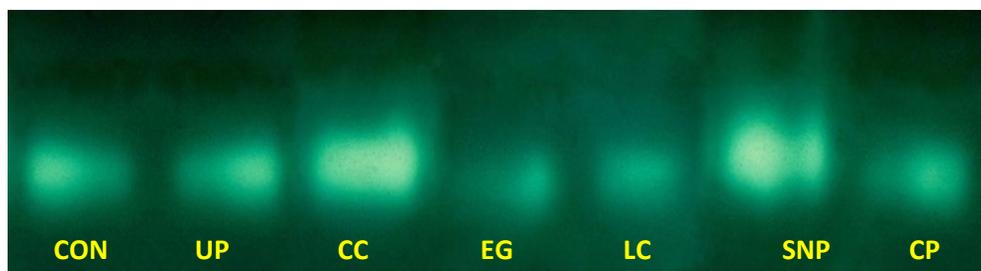
From the native PAGE analysis five isoforms of SOD were detected in all seedlings except four in CP primed seedlings. The isozymes exhibited difference in their expression pattern suggesting variation in their response towards salinity stress (Figure 6.46). In CC

primed seedlings isoform I, II, III and IV exhibited considerable increase in their expression compared to unprimed seedlings. For SNP primed seedlings all the five isoforms were visualized with higher intensity than the unprimed ones. As expected the expression of the isoforms of antagonists primed seedlings reduced as evident from their low intensity. In the EG and LC primed seedlings the expression of isoforms I, III, IV and V were found to be inhibited drastically and negligible change in the intensity of isoform II was observed. In CP primed seedlings there was considerable reduction in the intensity of isoforms I, III and IV and isoform V was almost invisible with isoform II being less affected. Thus a pronounced influence of the signalling molecules calcium and nitric oxide on the expression of SOD isozymes was observed in fenugreek seedlings under salinity stress. The difference in the intensity of respective isozymes were estimated in terms of relative density which has been graphically represented in Figure 6.47 which further revealed an increase in the isozyme expression in SNP and CC primed seedlings and reduction in EG, LC and CP respectively, with respect to unprimed seedlings. Similarly five SOD isoforms were reported by He and Huang (2010) in Kentucky bluegrass and He and Gao, 2008 in *Chimonanthus praecox*. However, like isoforms of other enzymes studied earlier variation in the number of SOD isoforms was also reported in different plant species. In contrary to our findings the number of SOD isoforms varied from 3-10 in various plant systems, for instance Zhou *et al.*, 2009 detected only 3 isoforms of SOD in *Medicago sativa* exposed to Hg induced oxidative stress and seven SOD isozymes were detected by Dewir *et al.*, 2006 in hyperhydric shoots of *Euphorbia milli*. Further five and four isoforms of SOD were reported in leaves and root of cucumber seedlings subjected to salinity stress indicating the variation in the expression pattern of SOD isozymes in different parts of same plant (Du *et al.*, 2010). Such variation in the expression pattern of isoforms supports the fact that antioxidant enzymes exist in multiple isoforms in plant system which responses differentially to different developmental and environmental factors (Scandalios, 1993; Lee and An, 2005).

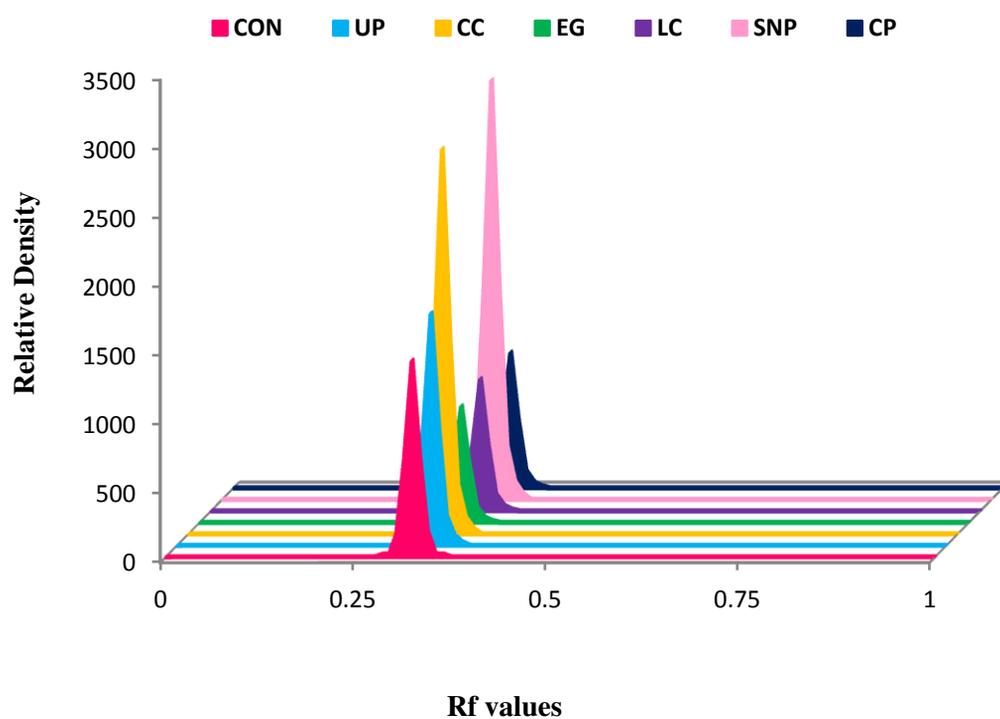
Plasma membrane-bound NADPH oxidase (NOX) is considered to be involved in the regulation of the antioxidant defence mechanism during various stress condition including salinity tolerance in plants (Jiang and Zhang, 2002; Wang *et al.*, 2013; Kaur *et al.*, 2014). Therefore, in the present study the NOX isozyme analysis was performed on native gel to assess the role of calcium and nitric oxide in regulating NOX expression during salinity stress. As a result, four isozymes of NOX were detected in each treated and untreated fenugreek seedlings subjected to salinity stress (Figure 6.48). Similar to other isozymes studied earlier a considerable difference in the expression of NOX isoforms was noted. The difference in the intensity of respective isozymes were estimated in terms of relative density which has been graphically represented in Figure 6.49 which further revealed an increase in the isozyme expression in SNP and CC primed seedlings and reduction in EG, LC and CP respectively, with respect to unprimed seedlings. Isoform I was found to be least affected by the influence of priming agents during salinity stress as no changes in the intensity of the isoform was observed. However the other three isoforms II, III and IV exhibited differential response towards salinity thus indicating the influential role of priming agents on their expression during salinity stress. The expression of NOX isoforms II, III and IV was found to be induced by CC and SNP priming and as expected considerable decline in the intensity of these isoforms were noted in antagonist primed seedlings with respect to unprimed seedlings. Further, isoform IV was almost invisible in the CP primed seedlings indicating the inhibitory effect of the antagonist agents in the expression of NOX enzyme during stress condition. Such variation in the expression of NOX isoforms due to different priming agents suggests well-defined influence of the signalling molecules calcium and nitric oxide on the expression of NOX like earlier mentioned enzymes in fenugreek seedlings under salinity stress.

NOX has been known to be responsible for the production of superoxide anion radical ( $O_2^{\cdot -}$ ) which is further dismutated to  $H_2O_2$  by SOD enzyme (Hao *et al.*, 2006). Also,

H<sub>2</sub>O<sub>2</sub> is considered as the signal for induction of antioxidant defense system in plant system in response to stress conditions (Neill *et al.*, 2002). In agreement to the aforesaid statement it can be stated that the increase in NOX activity leads to increase in O<sub>2</sub><sup>-</sup> production which further activates SOD activity which dismutates O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub> which influxes in the cellular space further activating catalase and peroxidase to overcome the ROS production for removal of H<sub>2</sub>O<sub>2</sub> molecules as suggested by Chang *et al.*, 2012 during their studies. Previously, Xia *et al.*, 2009 also demonstrated that brassinosteroids increased the NOX activity and ROS accumulation thus leading to induction of the defence antioxidant system and further enhancing stress tolerance in *Cucumis sativus*. Furthermore, the induction of the enzymatic oxidative defence mechanism by NADPH oxidase has been reported in *Pluchea indica* and maize leaves during water deficit stress (Jiang and Zhang, 2002; Chang *et al.*, 2012). Likewise considerable enhancement in the activity of NOX enzyme was observed by exogenous calcium and nitric oxide priming in fenugreek seedlings subjected to salinity stress (Figure 6.50, 6.51 & 6.52). Enhancement in the activity of the antioxidant enzymes might be an acclimatisation mechanism regulated by the calcium and nitric oxide in fenugreek seedlings to overcome oxidative stress imposed by salinity stress. Also parallely, better defense potentiality by stress tolerant plants against adverse effect of oxidative stress over stress sensitive ones was reported (Shalata *et al.*, 2001), which was also observed in the present study.

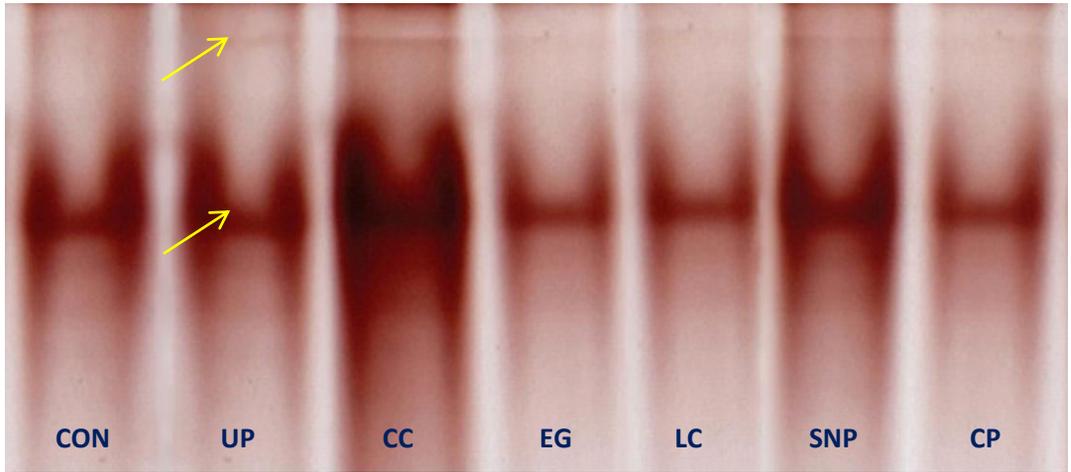


**Figure 6.42:** On gel analysis of Catalase isoform activity in fenugreek seedlings pre-treated with various elicitors under salinity stress

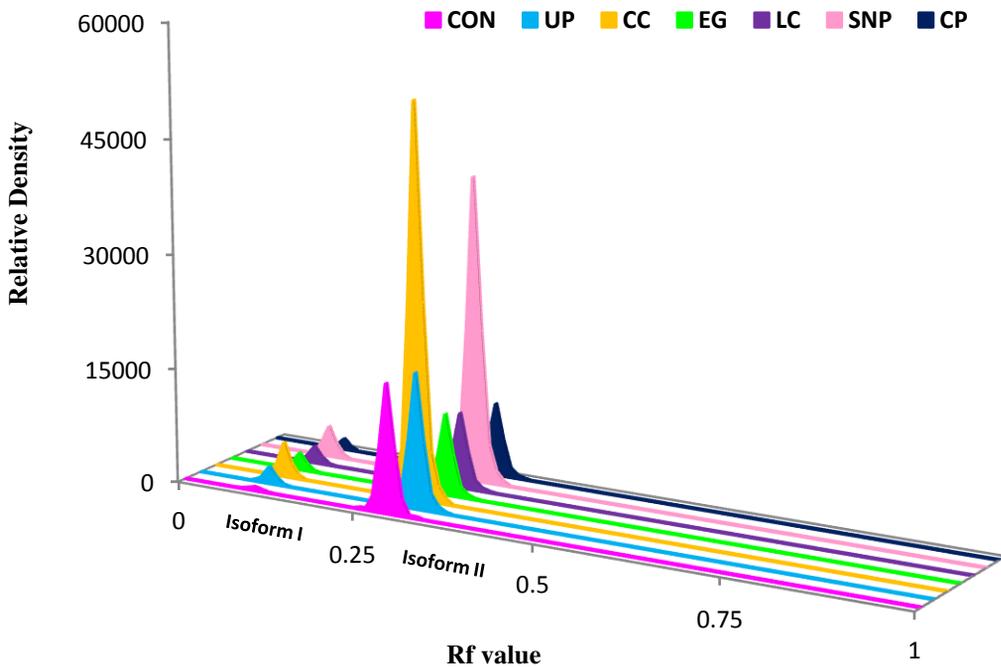


**Figure 6.43:** Chromatogram depicting the relative density of the Catalase isoforms in fenugreek seedlings pre-treated with various elicitors under salinity stress

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

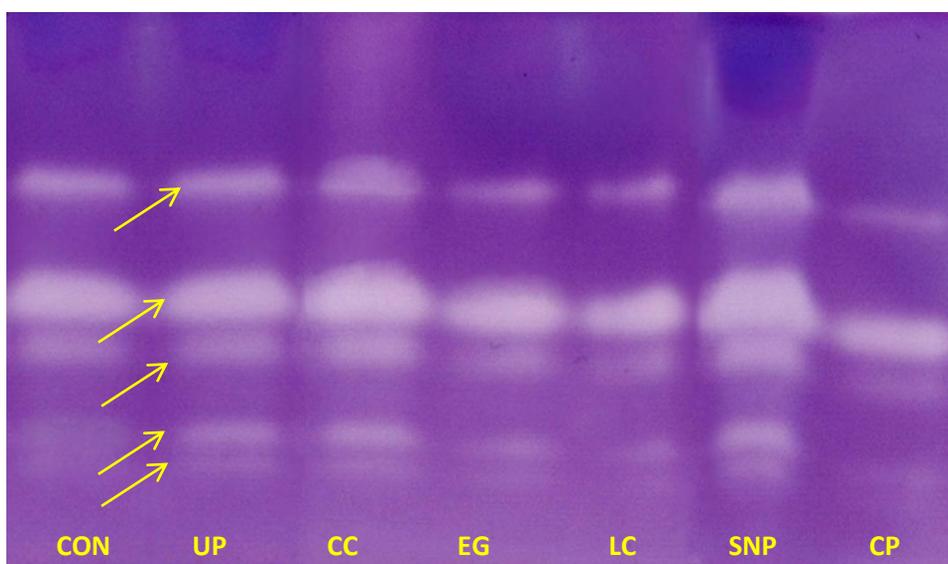


**Figure 6.44:** On gel analysis of POD isoforms activity in fenugreek seedlings pre-treated with various elicitors under salinity stress

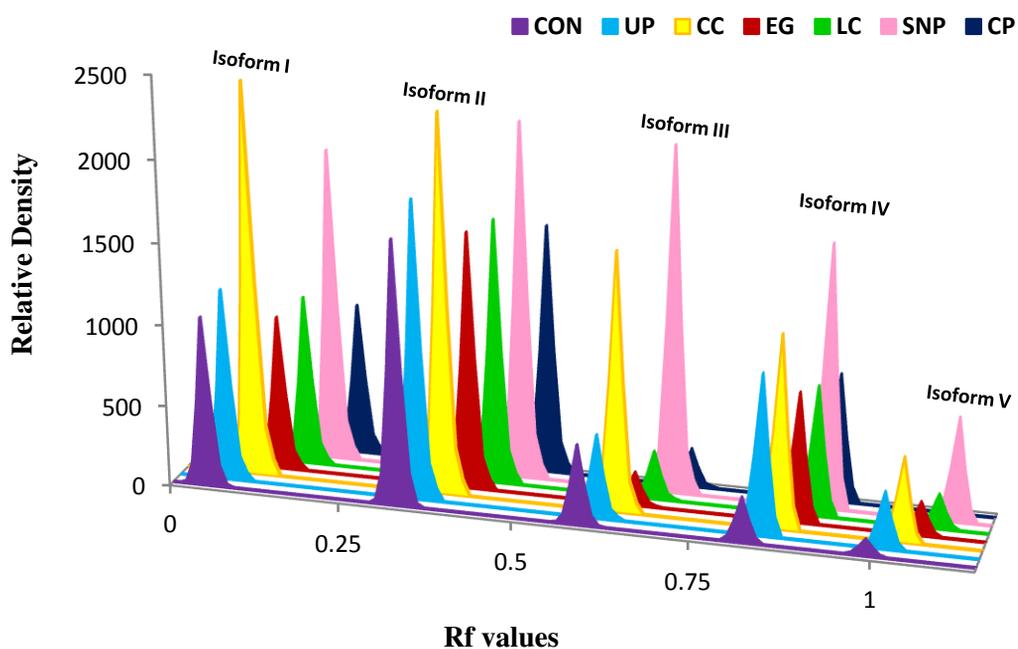


**Figure 6.45:** Chromatogram depicting the relative density of the Peroxidase isoforms in fenugreek seedlings pre-treated with various elicitors under salinity stress

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

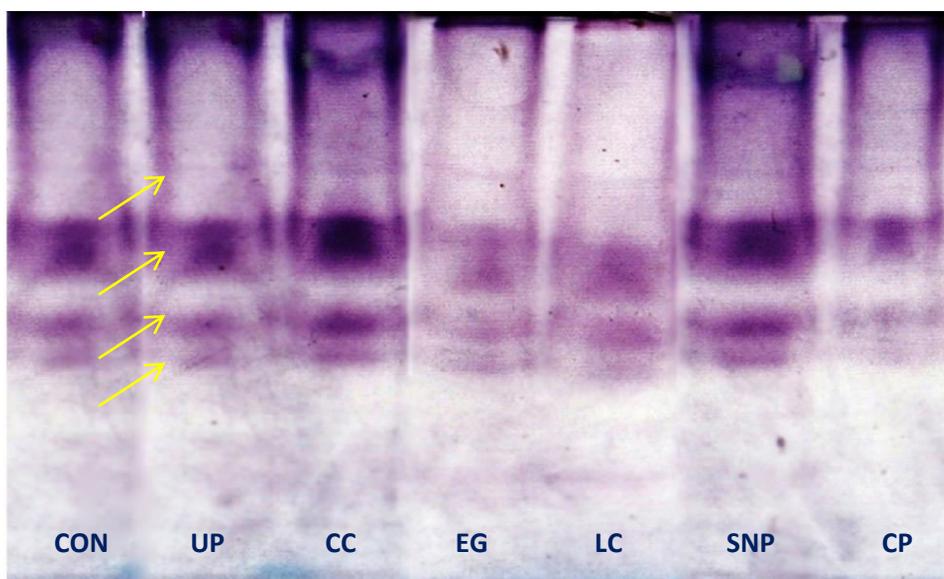


**Figure 6.46:** On gel analysis of SOD isoforms activity in fenugreek seedlings pre-treated with various elicitors under salinity stress.

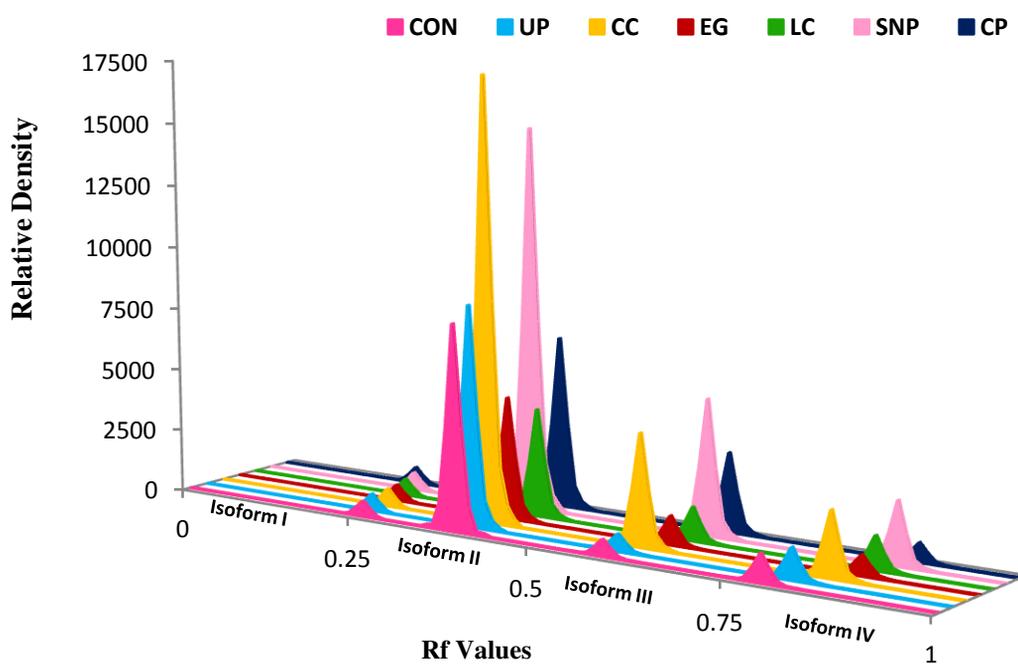


**Figure 6.47:** Chromatogram depicting the relative density of the SOD isoforms in fenugreek seedlings pre-treated with various elicitors under salinity stress.

Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

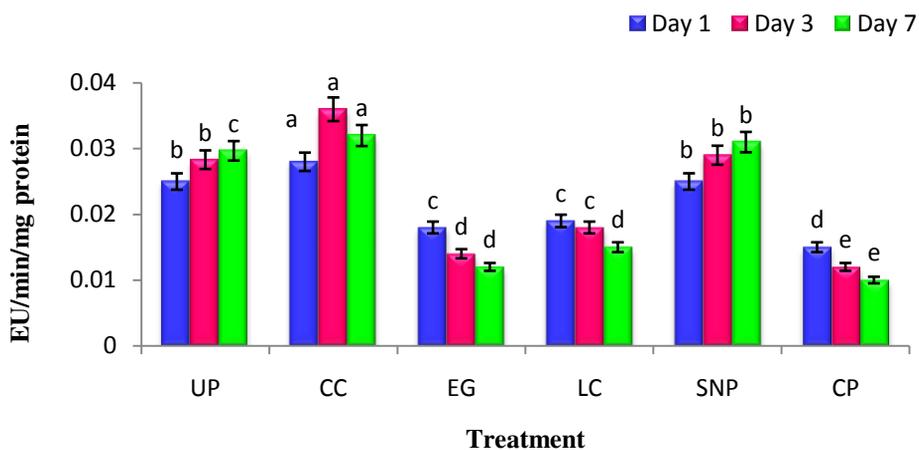


**Figure 6.48:** On gel analysis of NOX isoforms activity in fenugreek seedlings pre-treated with various elicitors under salinity stress

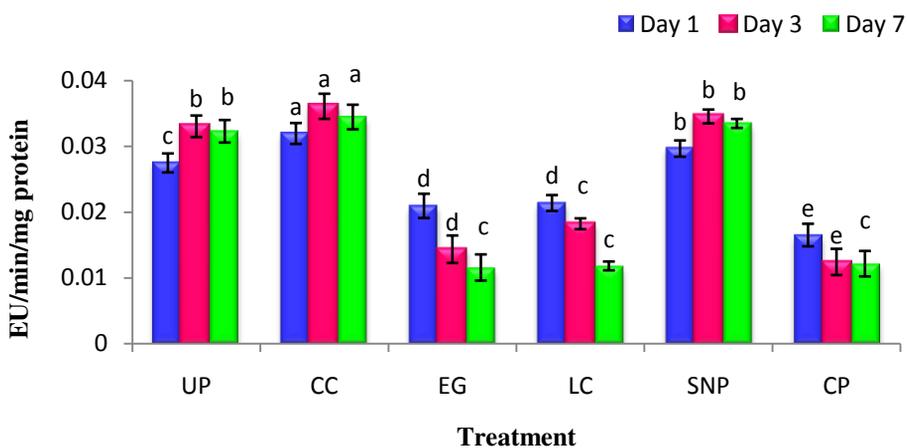


**Figure 6.49:** Chromatogram depicting the relative density of the NOX isoforms in fenugreek seedlings pre-treated with various elicitors under salinity stress

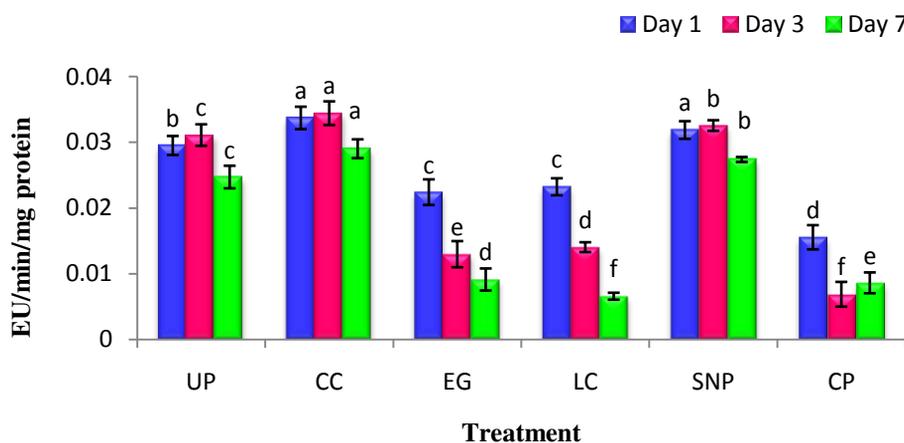
Abbr. used: CON: control; UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.50:** Effect of different priming agents NADPH oxidase activity of fenugreek seedlings at 0ds m<sup>-1</sup>



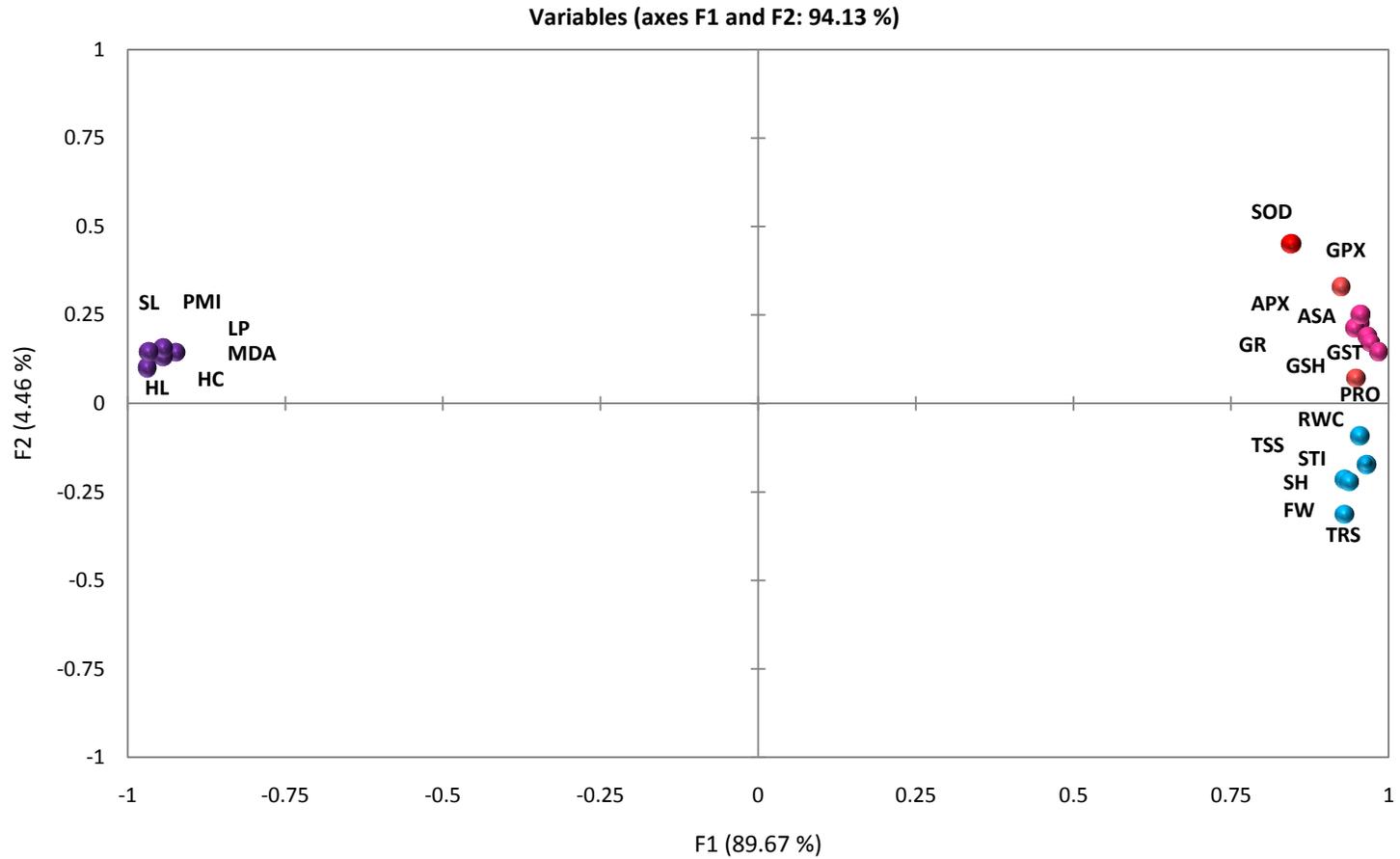
**Figure 6.51:** Effect of different priming agents NADPH oxidase activity of fenugreek seedlings at 4ds m<sup>-1</sup> salinity



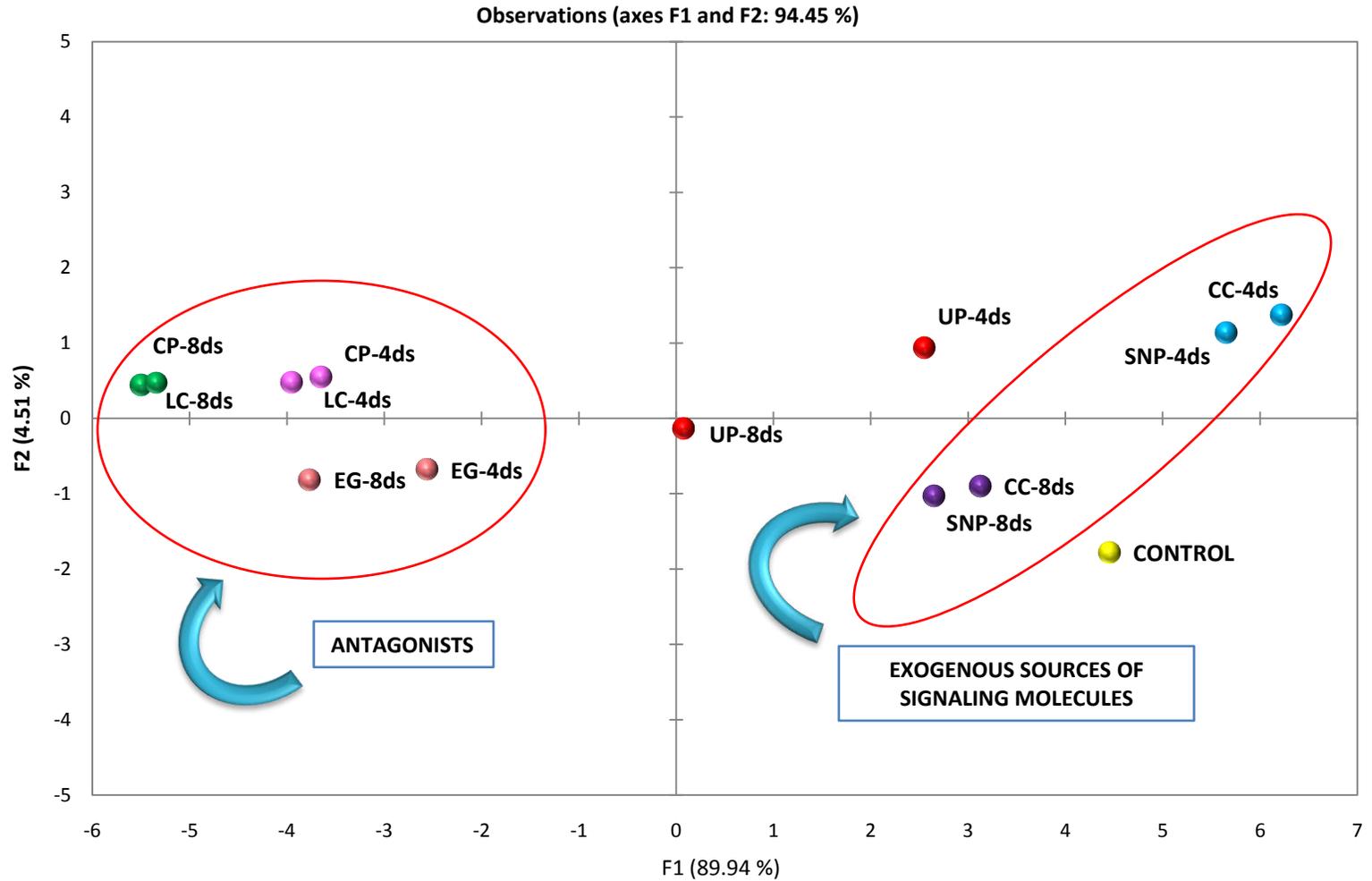
**Figure 6.52:** Effect of different priming agents NADPH oxidase activity of fenugreek seedlings at 8ds m<sup>-1</sup> salinity

Results are represented as mean  $\pm$  SEM, n=3. Values with different letters (a, b, c etc) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT)

Abbr. used: UP: unprimed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO



**Figure 6.53:** Principal component analysis depicting the clustering of highly correlated attributes of fenugreek seedlings under different salinity stress



**Figure 6.54:** Principal component analysis depicting the status of various priming agents on fenugreek seedlings under different salinity stress

Principal component analysis was performed to determine the relation of various biochemical attributes with the antioxidant enzymes and the other morphological and physiological parameters of fenugreek sprouts subjected to salinity stress (Figure 6.53). As a result it was noted that the free radicals were found to be plotted opposite to the antioxidant enzyme activities, growth parameters and other beneficial biochemicals such as glutathione, ascorbate and carbohydrates; thus, indicating their opposite impact on the fenugreek seedlings. Interestingly, the free radicals such as superoxide anion, hydrogen peroxide along with MDA content were clustered with the lipid peroxidation and plasma integrity parameters suggesting their strong impact on the oxidative stress mediated membrane injuries experienced by fenugreek seedlings during stress environment. Further, most of the enzymes were found to form a separate cluster (opposite of free radicals) indicating their collective role in the mitigation of oxidative stress mediated adversities caused by these free radicals. Also, the plot revealed that glutathione and ascorbic acid were highly responsible for the regulation of their associated antioxidant enzymes such as glutathione reductase and glutathione-s-transferase; ascorbate peroxidase respectively.

Moreover biochemicals such as proline and carbohydrates were found to have significant impact on the growth performance of fenugreek seedlings as they were found to be clustered along with growth parameters such as seedling weight, height and relative water content along with stress tolerance index. Though association of growth parameters with carbohydrates were much more stronger than proline which also give an idea of the importance of the accumulation of these osmolytes in cell system for better growth and development during their stress conditions. Further, another plot was obtained depicting the impact of the different priming agents on the fenugreek seedlings during salinity stress (Figure 6.54). It was noted that priming agents CC and SNP were plotted opposite of the antagonists namely EG, LC and CP and the unprimed factor was apparently at the middle of them. Accordingly, it can be inferred that CC and SNP exhibited beneficial role in

mitigation of the stress effects and contrastingly the antagonists (EG, LC and CP) exhibited deteriorating effect on the fenugreek seedlings under salinity stress; thus confirming the potential role of these signalling molecules, calcium and nitric oxide in alleviation of adverse effect of salinity stress in fenugreek seedlings. Interestingly, degree of effect of salinity level on the fenugreek seedlings as well as the mode of action of each priming agents was also evident from the PCA plot as the factors of 4ds  $m^{-1}$  and 8ds  $m^{-1}$  were positioned separately.

In conclusion, our results suggest that priming of fenugreek seeds with exogenous source of nitric oxide and calcium enhanced the morphological and biochemical attributes along with the antioxidant defense system under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seeds which were unprimed and also those primed with the antagonists of these signalling molecules. Therefore, the enhancement in the enzymatic as well as non-enzymatic components might be due to the involvement of calcium and nitric oxide leading to tolerance towards salinity accompanied with better growth and development. Moreover, such differential alteration in the expression of isoforms might aid as markers for studying salinity tolerance in plant systems.

# **CHAPTER-7**

**INTERACTION OF CALCIUM AND NITRIC OXIDE WITH POLYAMINES  
AND ABA DURING GERMINATION UNDER SALINITY STRESS**

## 7.1 INTRODUCTION

The aliphatic polycationic compounds collectively known as polyamines are considered to be essential for growth and development in plant system (Jimenez-Bremont *et al.*, 2014). They are known to have active participation in several fundamental processes namely chromatin condensation, RNA processing, DNA replication, transcription and protein synthesis (Igarashi and Kashiwagi, 2000; Wallace *et al.*, 2003). The most common polyamines found in nature are the putrescine (Put): a diamine, and other higher polyamines are the spermidine (Spd): a triamine and the spermine (Spm): a tetraamine. These polyamines are further reported to play pivotal roles in regulation of cell proliferation, differentiation and morphogenesis (Kusano *et al.*, 2007), fruits and flower development, breaking of dormancy in tubers as well as promote seed germination (Galston *et al.*, 1997; Kusano *et al.*, 2008; Wimalasekera *et al.*, 2011).

Even though the actual mode of action of polyamines still remains obscure, many workers have proposed that their functioning is attributed to the spatial separation of their positive charges under physiological pH (Ruiz-Herrera *et al.*, 1995; Jimenez-Bremont *et al.*, 2014). By virtue of this characteristic feature, they easily bind with the negatively charged biomolecules such as nucleic acids, proteins and phospholipids and further regulate their functioning. Additionally, the presence of polyamines in the cellular system protects these macromolecules from degradation and unwanted modification (D'Agostino *et al.*, 2005).

The plants at the initial stages of their life cycle are very sensitive to environmental stresses they encounter. Further the development and productivity of the plants are tremendously affected by these environmental stresses (Tuteja, 2007; Tuteja and Sopory, 2008). Accordingly, plants are also self-equipped with the efficient defense

mechanisms to cope with such stressful conditions which includes salinity, drought, UV radiation, chill stress and pathogenic infections (Mahajan and Tuteja, 2005; Fujita *et al.*, 2006). Further various authors have suggested polyamines acting as stress messengers in plant system responding to different stress signals (Liu *et al.*., 2007). Therefore, the polyamines are capable of enhancing tolerance to plants against wide range of biotic and abiotic stress conditions (Alcazar *et al.*, 2010; Takahashi and Kakehi, 2010). Due to such crucial role in numerous functional processes, polyamines are considered as a new class of growth modulators which regulates the plant developmental and physiological processes (Kusano, 2007; Gill and Tuteja, 2010).

Another important growth regulator which helps in plant for adaptive responses to abiotic stress is abscisic acid (ABA). Like polyamines, ABA is also well known to be actively involved in regulation of plant responses by various abiotic stresses mentioned earlier (Xiong *et al.*, 2001); Larkindale & Knight 2002). One of the beneficial role of ABA in mitigating stress in plant system is the induction of antioxidant genes expression and enhancement in antioxidant defence systems, including both the enzymatic and non-enzymatic components (Bueno *et al.*., 1998; Bellaire *et al.*., 2000; Jiang & Zhang 2002).

The literature suggests that polyamines exhibit active involvement in various functions similar to nitric oxide and calcium in developmental processes, biotic and abiotic stress tolerance. Considering the aforesaid facts, the present work was designed to study the probable interaction of these signalling molecules with the polyamines and their effect in amelioration of salinity stress in fenugreek seedlings.

## **7.2 MATERIALS AND METHOD**

### **7.2.1 Materials and treatment**

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of sodium nitroprusside (SNP), calcium chloride

(CC), Putrescine (Put), spermidine (Spd), spermine (Spm) and abscisic acid (ABA) in different combinations as mentioned in Table 7.1. For control set, seeds were primed with normal water and placed in a rotary shaker along with the treated seeds. After priming for 24h, the seeds were washed thrice with sterile water and kept in the seed germinator for germination. To provide saline conditions the NaCl at the level of 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> was applied to the seeds for 7days.

**Table 7.1: Different priming agents applied**

Calcium combination		Nitric oxide combination	
UP	H <sub>2</sub> O	UP	H <sub>2</sub> O
CC	CC-5mM	SNP	SNP-5mM
CC+Put	CC-5mM+Put-100µM	SNP+Put	SNP-5mM+Put-100µM
CC+Spd	CC-5mM+Spd-100µM	SNP+Spd	SNP-5mM+Spd-100µM
CC+Spm	CC-5mM+Spm-100µM	SNP+Spm	SNP-5mM+Spm-100µM
CC+ABA	CC-5mM+ABA-100µM	SNP+ABA	SNP-5mM+ABA-100µM

### **7.2.2 Measurement of growth parameters**

Mentioned in section 6.2.2

### **7.2.3 Relative water content**

Mentioned in section 6.2.3

### **7.2.4 Stress tolerance index**

Mentioned in section 6.2.4

### **7.2.5 Quantitative estimation of H<sub>2</sub>O<sub>2</sub>**

Mentioned in section 6.2.6

### **7.2.6 Estimation of lipid peroxidation**

Mentioned in section 6.2.7

### **7.2.7 Determination of glutathione content**

Mentioned in section 6.2.8

### **7.2.8 Determination of ascorbic acid content**

Mentioned in section 6.2.9

### **7.2.9 Estimation of total soluble sugar and reducing sugar content**

Mentioned in section 6.2.10

### **7.2.10 Estimation of proline content**

Mentioned in section 6.2.11

### **7.2.11 Extraction of antioxidant enzymes**

#### **7.2.11.1 Catalase**

Mentioned in section 6.2.13.1

#### **7.2.11.2 Guaiacol Peroxidase**

Mentioned in section 6.2.13.2

#### **7.2.11.3 Superoxide dismutase**

Mentioned in section 6.2.13.3

#### **7.2.11.4 Ascorbate peroxidase**

Mentioned in section 6.2.13.4

#### **7.2.11.5 Glutathione reductase**

Mentioned in section 6.2.13.5

#### **7.2.11.6 NADPH oxidase**

Mentioned in section 6.2.13.6

### **7.2.12 Assay of enzyme activities**

#### **7.2.12.1 Catalase**

Mentioned in section 6.2.14.1

#### **7.2.12.2 Guaiacol Peroxidase**

Mentioned in section 6.2.14.2

#### **7.2.12.3 Superoxide dismutase**

Mentioned in section 6.2.14.3

#### **7.2.12.4 Glutathione reductase**

Mentioned in section 6.2.14.4

#### **7.2.12.5 Ascorbate peroxidase**

Mentioned in section 6.2.14.5

#### **7.2.12.6 NADPH oxidase**

Mentioned in section 6.2.14.7

#### **7.2.13 Statistical analysis**

The data were pooled in triplicate and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing various attributes of different elicited fenugreek sprouts. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the parameters and their standard error of estimates (SEE). Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version

### **7.3 Results and discussion**

After assessing the beneficial role of nitric oxide and calcium in mitigation of salinity stress in fenugreek seedlings, their interactive response with polyamines and ABA was studied. Further, for evaluating the effect of these signalling molecules in association with other regulators, SNP and CC were applied in combination with polyamines and ABA and the fenugreek seeds were primed prior to germination in saline condition. Several authors have proven the effectiveness of the polyamines and ABA in amelioration of stress mediated adversities in numerous plant system (Jiang and Zhang, 2003; Liu *et al.*, 2007; Alcazar *et al.*, 2010). The results revealed that the polyamines and ABA in combination with SNP and CC could enhance the stress tolerance in fenugreek seedlings under salinity stress (Figure

7.1 & 7.2). There was subsequent increase in the fresh weight and dry weight of seedlings pre-treated with all the priming agents with respect to unprimed seedlings. The increase in the fresh weight by addition of ABA to CC was found to be about 12% and 20% (Figure 7.3) and dry weight by 16% and 23% (Figure 2) when compared to CC seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively. Also the fresh weight of SNP+Put primed seedlings was found to be increased by 15% and 18% and dry weight by 11% and 13% approximately (Figure 7.3 & 7.4). Similarly, RWC and STI were enhanced in the seedlings subjected to polyamine and ABA priming in combination with SNP and CC (Figure 7.5 & Figure 7.6). The stress tolerance index of each set of seedlings subjected to salinity was also calculated, the STI of CC+ABA primed was 95.56% and 87.06% and for SNP+Put primed was found to be 94.42% and 85.44% in comparison to CC and SNP primed seedlings exhibiting 94.44% and 82.42% ; 92.98% and 80.42% at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Figure 7.6). The average length of the roots was measured to study the effect on the root elongation; the root length of the CC primed seedlings was 2.7±0.14cm and 1.63±0.19cm, whereas those pre-treated with CC+ABA showed comparatively elongated roots of 2.98±0.12 and 1.91±0.06cm and the roots of SNP+Put were 2.56±0.13 and 1.77±0.06cm long compared to sodium nitroprusside primed seedlings were 2.07±0.12cm and 1.5±0.09cm long at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively (Figure 7.1 & Table 7.2). Similar effect of different priming agents were observed on the length of shoot in fenugreek seedlings; the shoot length of the calcium chloride seedlings was 3.50±0.11cm and 2.30±0.25cm, whereas those pre-treated with CC+ABA showed comparatively elongated shoots 3.74±0.08cm and 2.86±0.15cm (Figure 7.2 & Table 7.3) and the shoots of sodium nitroprusside primed seedlings were 2.57±0.21cm and 2.03±0.12cm long meanwhile those pre-treated with SNP+Put showed comparatively elongated shoots 3.631±0.18cm and 2.52±0.12cm, at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity respectively.

**Table 7.2:** Shoot length, Root length and Seedling length of fenugreek seedlings pre-treated with sodium nitroprusside and its combination with polyamines (Put, Spd and Spm) and ABA on 5<sup>th</sup> day under salinity stress

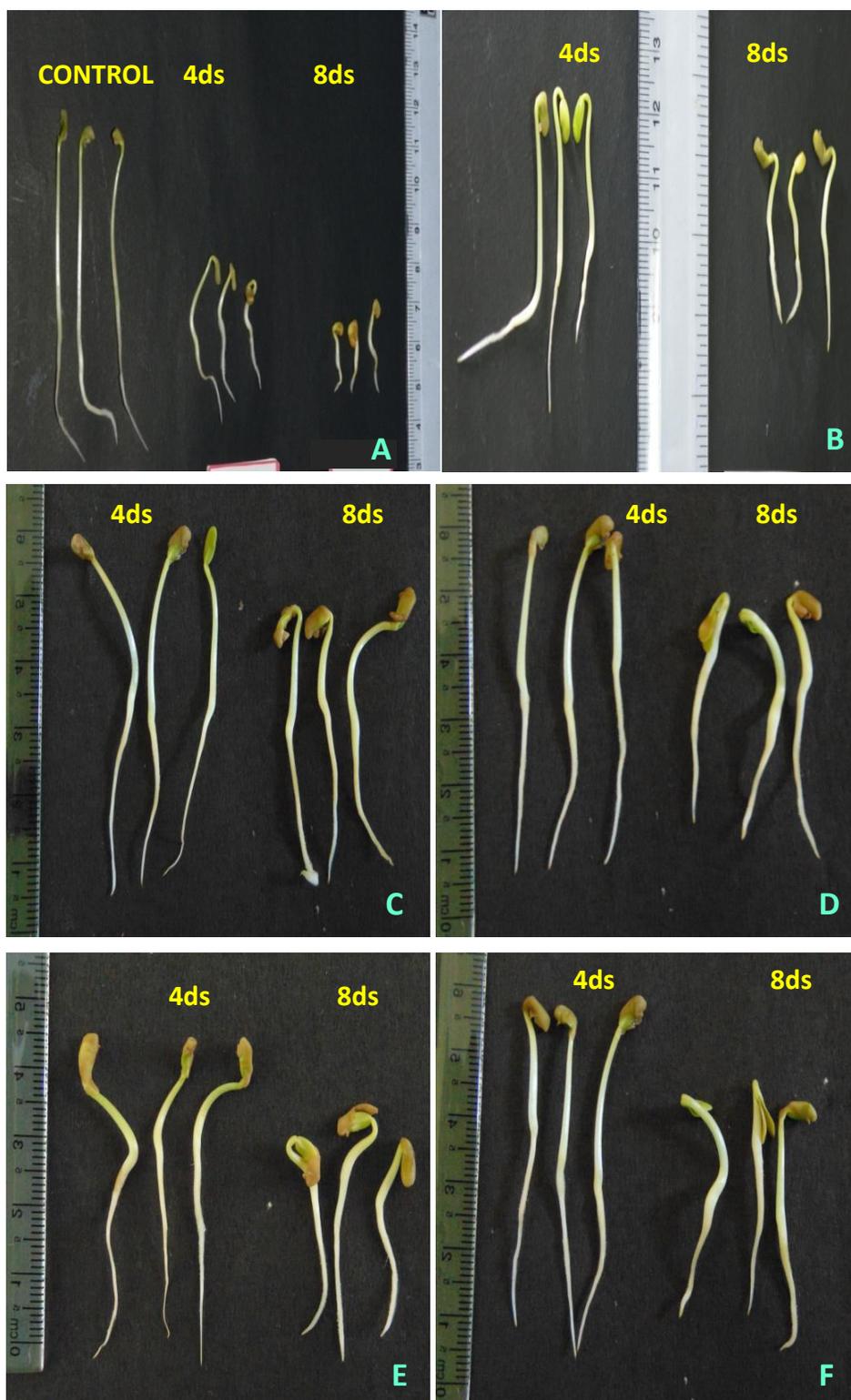
<b>Treatment (Salinity level)</b>	<b>Shoot length (cm)</b>	<b>Root length (cm)</b>	<b>Seedling length (cm)</b>
<b>Control (0ds m<sup>-1</sup>)</b>	3.8±0.12	3.23±0.14	7.03±0.42
<b>UP (4ds m<sup>-1</sup>)</b>	2.03±0.05	1.67±0.05	3.70±0.37
<b>UP (8ds m<sup>-1</sup>)</b>	1.57±0.09	0.97±0.12	2.54±0.29
<b>SNP (4ds m<sup>-1</sup>)</b>	2.57±0.11	2.07±0.08	4.64±0.22
<b>SNP (8ds m<sup>-1</sup>)</b>	2.03±0.12	1.5±0.05	3.53±0.21
<b>SNP+Put (4ds m<sup>-1</sup>)</b>	3.61±0.18	2.56±0.13	6.17±0.33
<b>SNP+Put (8ds m<sup>-1</sup>)</b>	2.52±0.12	1.77±0.06	4.29±0.20
<b>SNP+Spd (4ds m<sup>-1</sup>)</b>	3.48±0.09	2.52±0.07	6.01±0.18
<b>SNP+Spd (8ds m<sup>-1</sup>)</b>	2.45±0.11	1.68±0.09	4.13±0.22
<b>SNP+Spm (4ds m<sup>-1</sup>)</b>	3.32±0.19	2.36±0.12	5.68±0.33
<b>SNP+Spm (8ds m<sup>-1</sup>)</b>	2.37±0.14	1.62±0.07	3.99±0.25
<b>SNP+ABA (4ds m<sup>-1</sup>)</b>	3.04±0.09	2.15±0.08	5.19±0.18
<b>SNP+ABA (8ds m<sup>-1</sup>)</b>	2.18±0.12	1.61±0.10	3.79±0.23

Results are expressed as a mean of 10 seedlings

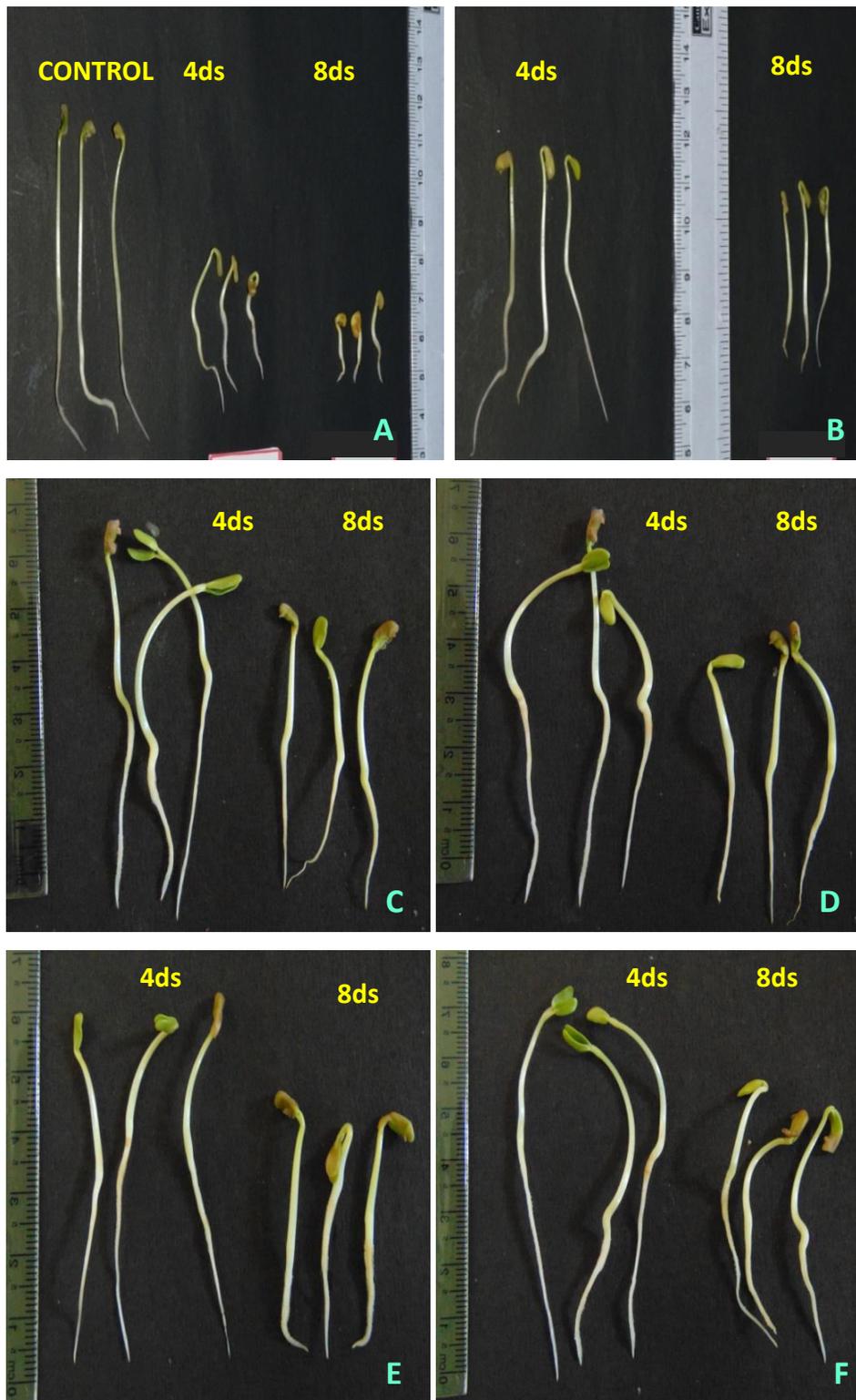
**Table 7.3:** Shoot length, Root length and Seedling length of fenugreek seedlings pre-treated with calcium chloride and its combination with polyamines (Put, Spd and Spm) and ABA on 5<sup>th</sup> day under salinity stress.

<b>Treatment (Salinity level)</b>	<b>Shoot length (cm)</b>	<b>Root length (cm)</b>	<b>Seedling length (cm)</b>
<b>Control</b>	3.8±0.12	3.23±0.14	7.03±0.42
<b>UP (4ds m<sup>-1</sup>)</b>	2.03±0.05	1.67±0.05	3.70±0.37
<b>UP (8ds m<sup>-1</sup>)</b>	1.57±0.09	0.97±0.12	2.54±0.29
<b>CC (4ds m<sup>-1</sup>)</b>	3.50±0.11	2.7±0.14	6.20±0.36
<b>CC (8ds m<sup>-1</sup>)</b>	2.30±0.15	1.63±0.19	3.93±0.45
<b>CC+Put (4ds m<sup>-1</sup>)</b>	3.56±0.10	2.81±0.10	6.37±0.28
<b>CC+Put (8ds m<sup>-1</sup>)</b>	2.52±0.04	1.77±0.12	4.29±0.24
<b>CC+Spd (4ds m<sup>-1</sup>)</b>	3.52±0.11	2.76±0.08	6.28±0.32
<b>CC+Spd (8ds m<sup>-1</sup>)</b>	2.47±0.06	1.70±0.09	4.17±0.23
<b>CC+Spm (4ds m<sup>-1</sup>)</b>	3.50±0.09	2.71±0.05	6.21±0.35
<b>CC+Spm (8ds m<sup>-1</sup>)</b>	2.41±0.10	1.67±0.07	4.08±0.31
<b>CC+ABA (4ds m<sup>-1</sup>)</b>	3.74±0.08	2.98±0.12	6.72±0.26
<b>CC+ABA (8ds m<sup>-1</sup>)</b>	2.86±0.15	1.91±0.06	4.57±0.25

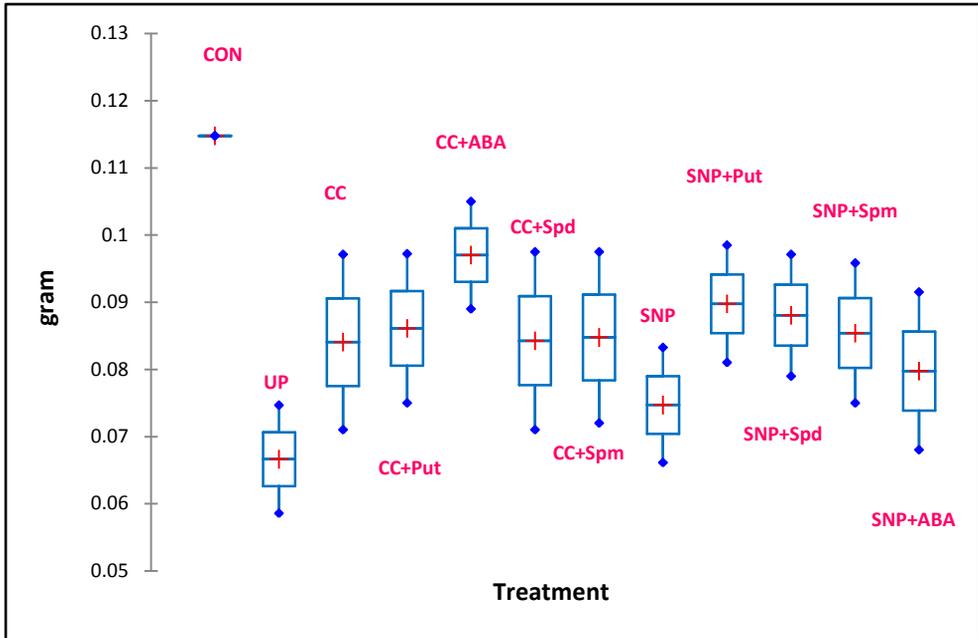
Results are expressed as a mean of 10 seedlings



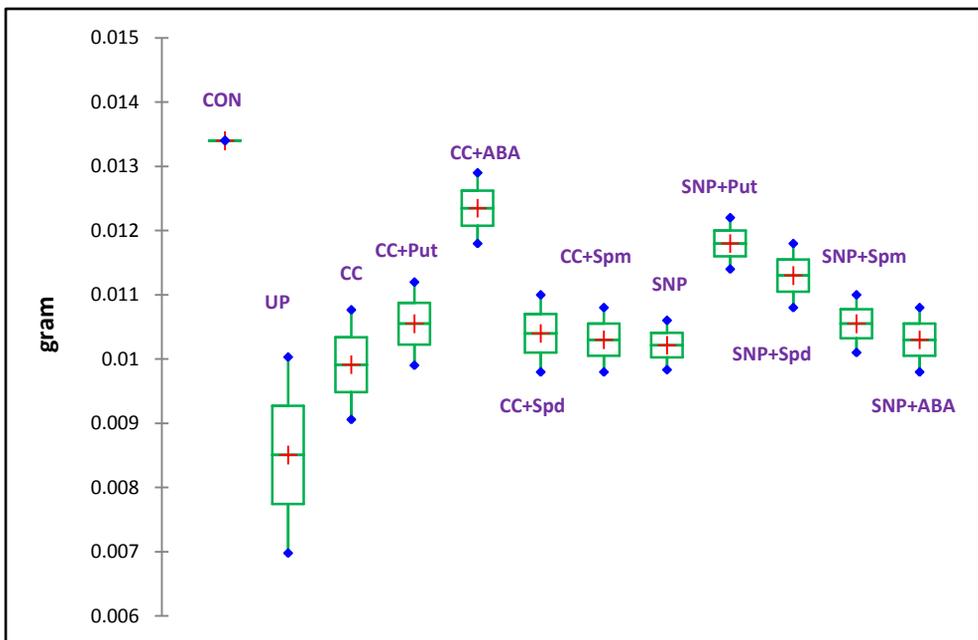
**Figure 7.1:** The growth performance of fenugreek seedlings A) Control and Unprimed B) SNP C) SNP+Put D) SNP+Spd E) SNP+Spm and F) SNP+ABA under salinity stress on 7<sup>th</sup> day of germination



**Figure 7.2:** The growth performance of fenugreek seedlings A) Control and Unprimed B) CC C) CC+Put D) CC+Spd E) CC+Spm and F) CC+ABA under salinity stress on 7<sup>th</sup> day of germination

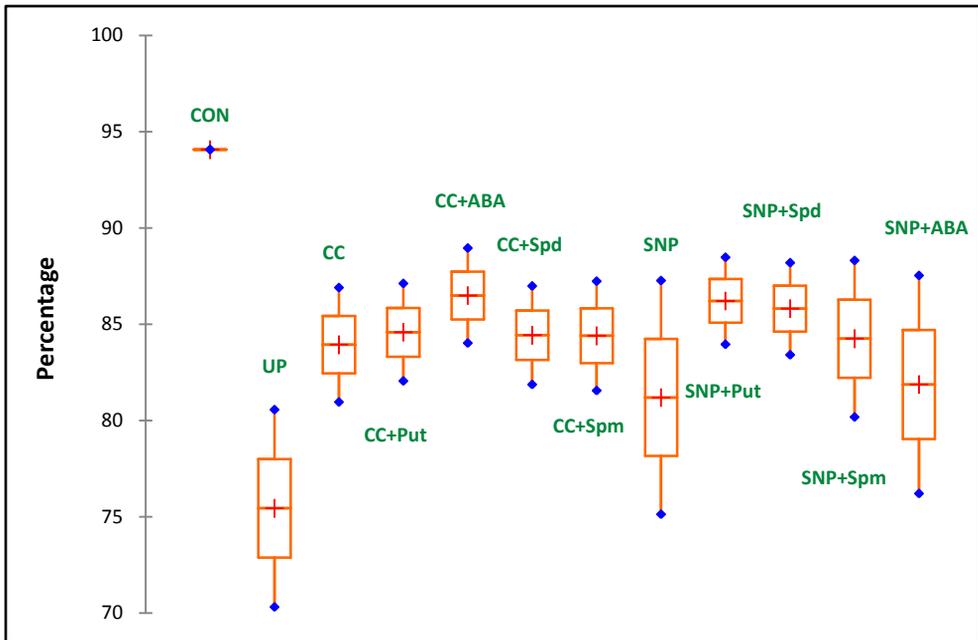


**Figure 7.3:** Fresh weight of fenugreek seedlings primed with different elicitors under salinity stress

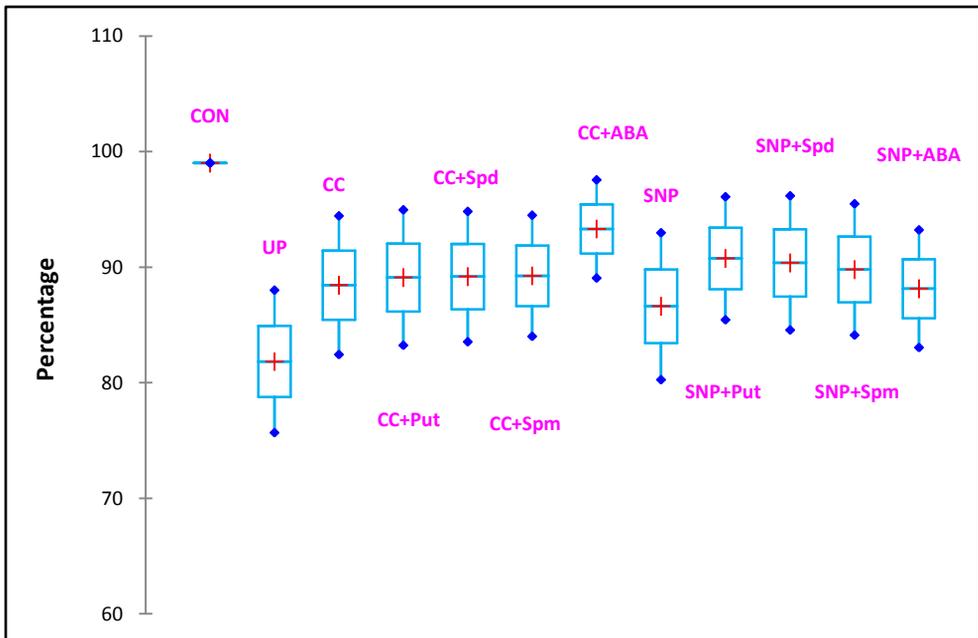


**Figure 7.4:** Dry weight of fenugreek seedlings primed with different elicitors under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid



**Figure 7.5:** Relative water content of fenugreek seedlings primed with different elicitors under salinity stress



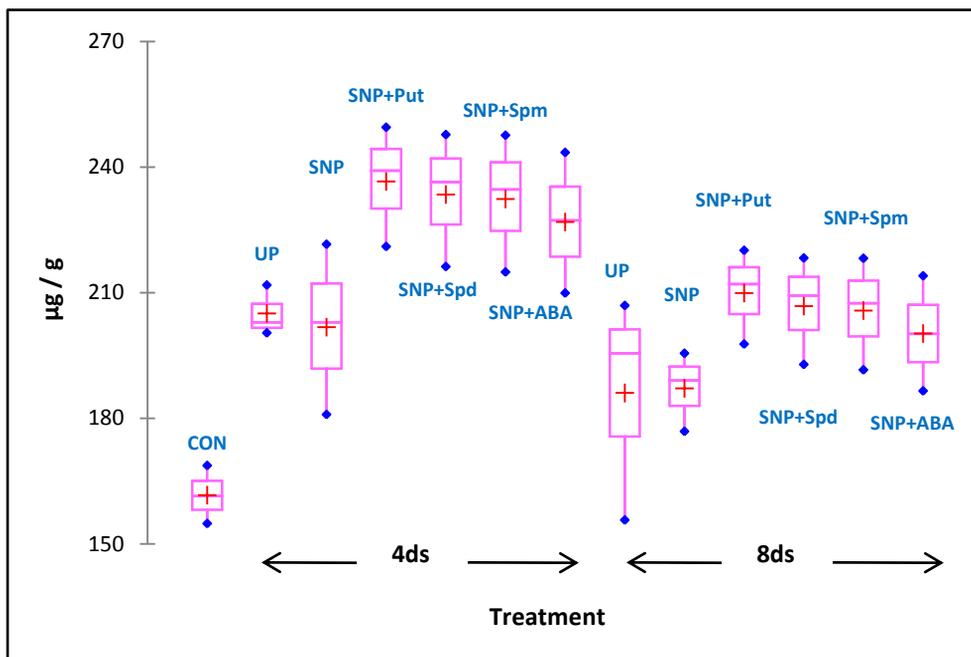
**Figure 7.6:** Stress tolerance index of fenugreek seedlings primed with different elicitors under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

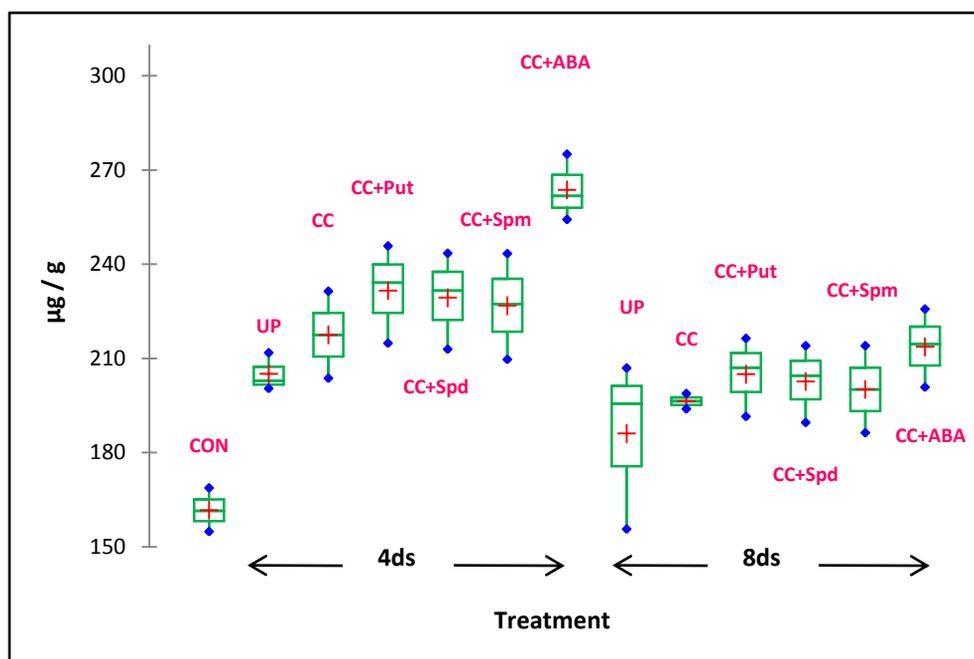
Though enhancement in the growth performance due to other treatments were observed but the effect of CC+ABA was better than the combination of CC and polyamines (Put, Spm and Spd); likewise the SNP+Spd exhibited more effective response than the combination of SNP with other polyamines (Put and Spm) and ABA. The polyamines and ABA are reported to effectively mitigated the deleterious effects resulted by the wide range of abiotic stress including salinity (Gill and Tuteja, 2010; Xue-Xuan *et al.*, 2010). Further, the results suggested that the presence of the polyamines and ABA in priming solution resulted in decline in the accumulation of H<sub>2</sub>O<sub>2</sub> with respect to unprimed seedlings (Figure 7.7 & 7.8). Accordingly, a significant decrease in the H<sub>2</sub>O<sub>2</sub> content was observed when evaluated spectrophotometrically. Likewise, lipid peroxidation was also found to be decreased in the seedlings primed with priming solution containing polyamines and ABA in combination with CC and SNP (Figure 7.9 & 7.10). The accumulation of MDA is known to be a marker for lipid peroxidation in biological system and the result obtained in previous chapter suggested that the lower accumulation of MDA indicates minimal occurrence of lipid peroxidation; thus lesser loss of plasma membrane integrity. It was also mentioned earlier that the seedlings detected with higher amount of H<sub>2</sub>O<sub>2</sub> and MDA content exhibited greater loss in their plasma membrane integrity and vice versa. Accordingly, several authors have suggested that the exogenous application of polyamines have effectively mitigated the stress mediated adversities in plant system (Groppa and Benavides, 2008; Gill and Tuteja, 2010). Furthermore, polyamines have been reported to stabilize cellular membranes in plant system (Borell *et al.*, 1997; Liu *et al.*, 2007) and their involvement in sustaining membrane integrity by protecting them from deteriorating during salinity stress (He *et al.*, 2008).

The polyamines are known for alleviation of salinity stress mediated damages in plant system by inducing antioxidant defence system (Verma and Mishra, 2005; Yamaguchi *et al.*, 2006). Accordingly, during the assessment of the biochemicals such as proline, carbohydrates, glutathione and ascorbic acid, a significant enhancement in the accumulation

of these biochemicals was observed in the fenugreek seedlings under salinity stress. The seedlings pre-treated with CC+ABA showed highest increase in the proline level followed by SNP+Spd with respect to unprimed seedlings. A maximum rise of about 22% and 13% was recorded in CC+ABA primed seedlings; whereas 16% and 8% of rise in total proline content was observed in SNP+Put primed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity level respectively (Figure 7.11 & 7.12). In case of the carbohydrates, a huge loss was observed in their content in the unprimed seedlings during higher salinity stress at later stages. On the other hand the priming agent SNP+Spd exhibited best elicitation in the carbohydrates in seedlings followed by CC+ABA; thus, proving their crucial role in aiding in significant stress tolerance to the seedlings under salinity stress. Accordingly, several authors have proven the enhancement of the osmolytes such as sugar and proline by the application of polyamines and ABA. Gurmani et al., 2011 have proposed the increase in the accumulation of proline and soluble sugar by ABA application in rice under salinity stress. Accordingly in our studies the sugar content was further enhanced by the application of ABA as well as polyamines, a maximum rise of about 7% and 9% was recorded in CC+ABA primed seedlings; whereas 4% and 6% of rise in total soluble sugar was observed in SNP+Put primed seedlings at 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> salinity level respectively (Figure 7.13-7.16). Furthermore, the non-enzymatic antioxidants namely glutathione and ascorbic acid was also evaluated in the present study. As observed under the influence of exogenous polyamines and ABA the accumulation of glutathione was found to be further induced; at 4ds m<sup>-1</sup> an enhancement of 23% and 27% and at 8ds m<sup>-1</sup> 18% and 29% of glutathione content was recorded for CC+ABA and SNP+Put primed fenugreek seedlings respectively (Figure 7.17 & 7.18). The results revealed that CC+ABA exhibited better accumulation of these non-enzymatic antioxidant compounds followed by SNP+Spd in seedlings whereas tremendous loss of glutathione and ascorbate was recorded in the unprimed seedlings under prolonged salinity stress. Apparently similar trend in alteration of ascorbic acid content of fenugreek

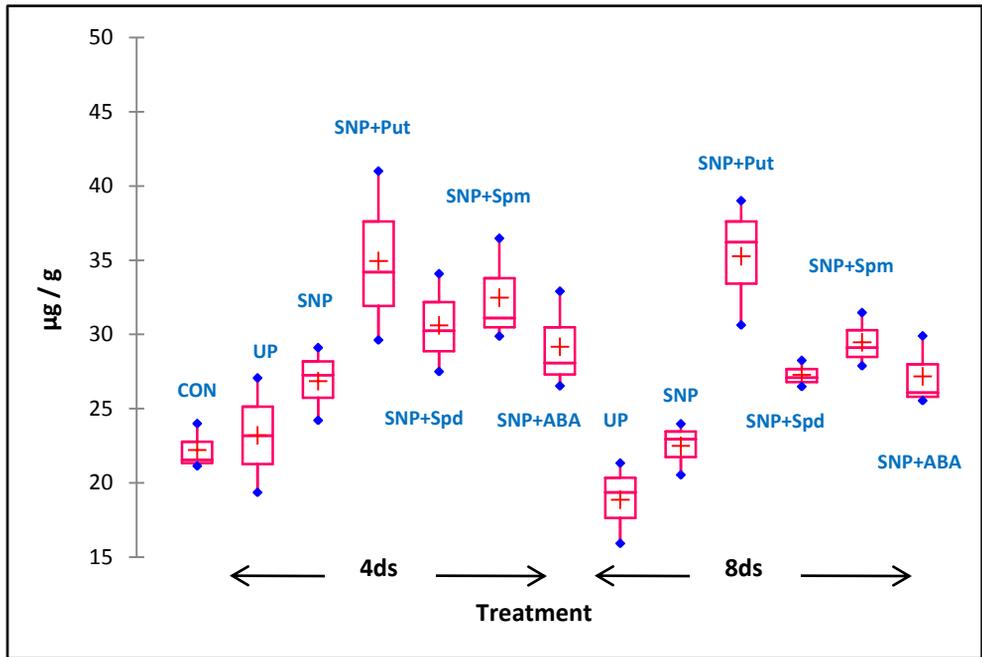


**Figure 7.7:** Effect of application of polyamines and ABA with SNP on ascorbic acid content of fenugreek seedlings primed under salinity stress

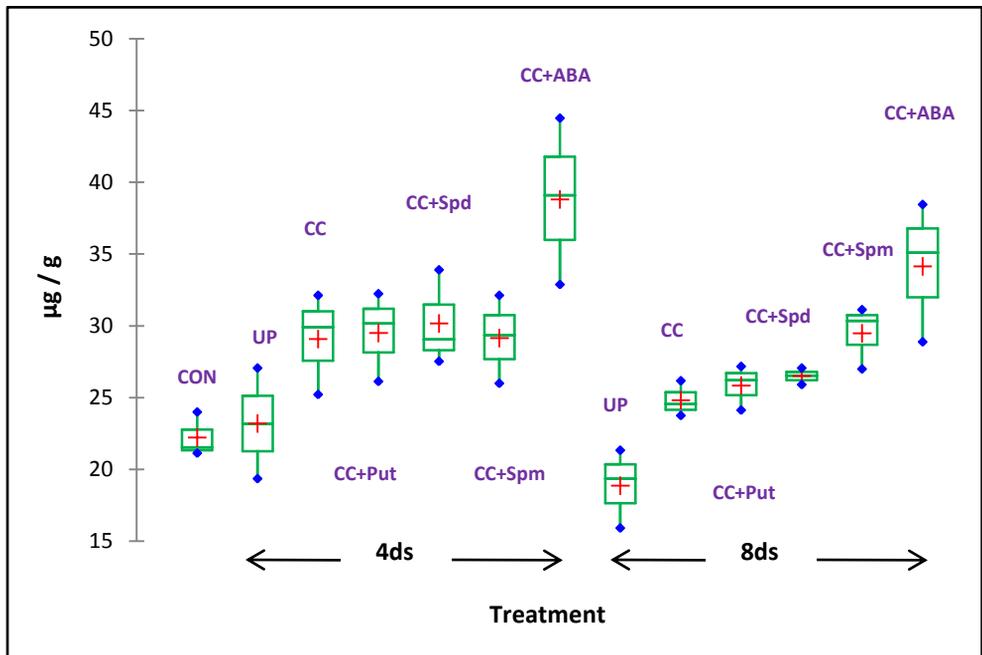


**Figure 7.8:** Effect of application of polyamines and ABA with CC on ascorbic acid content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

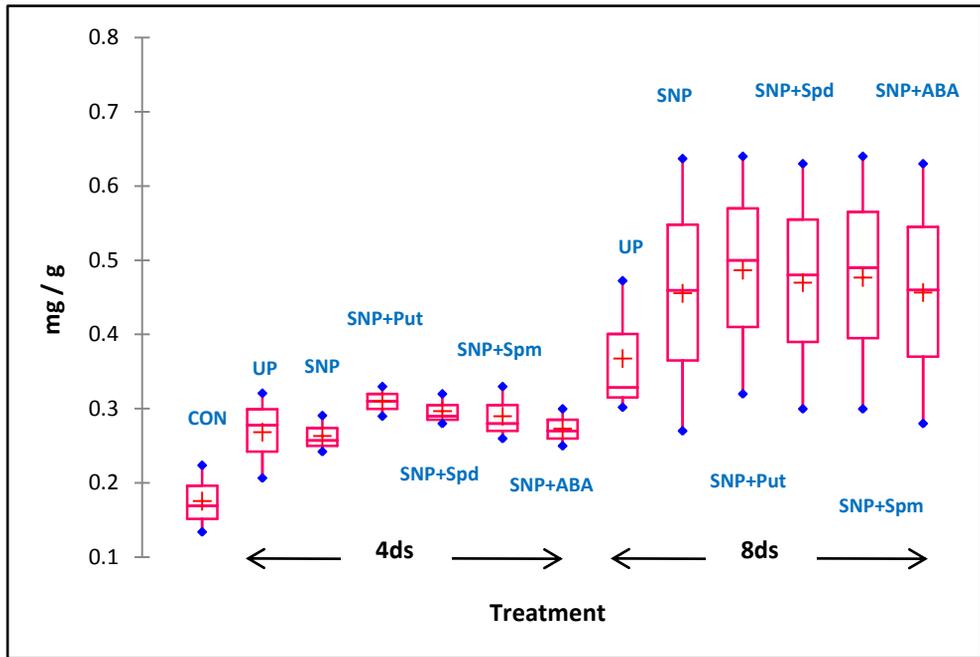


**Figure 7.9:** Effect of application of polyamines and ABA with SNP on glutathione content of fenugreek seedlings primed under salinity stress

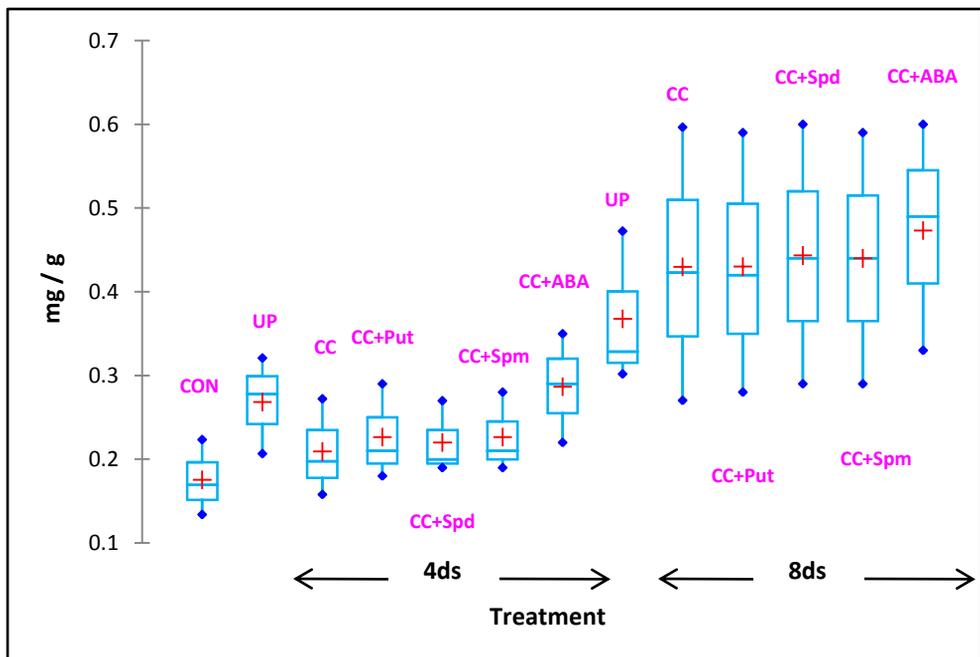


**Figure 7.10:** Effect of application of polyamines and ABA with CC on glutathione content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

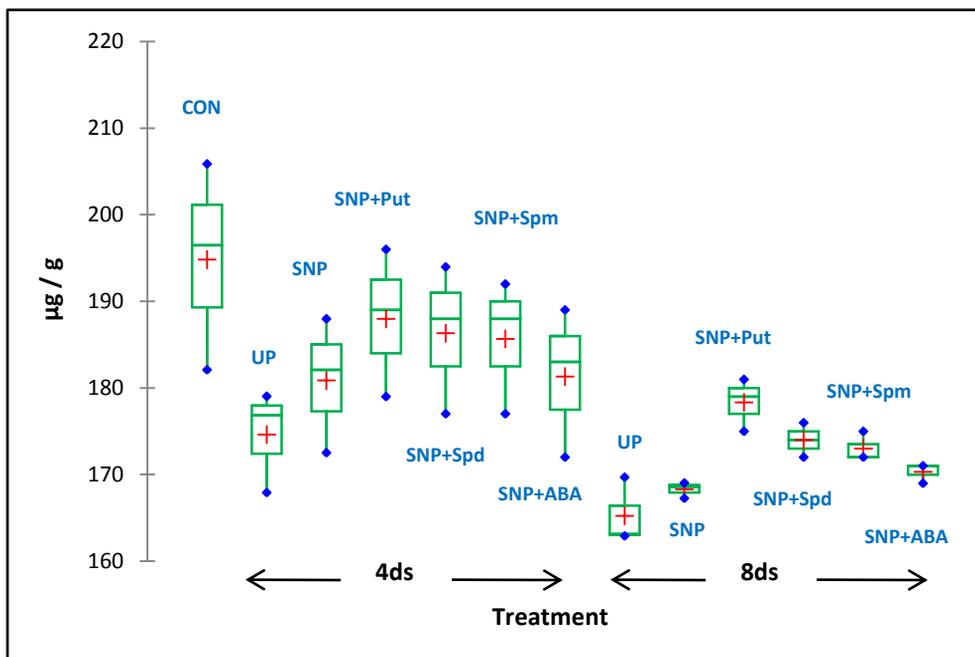


**Figure 7.11:** Effect of application of polyamines and ABA with SNP on proline content of fenugreek seedlings primed under salinity stress

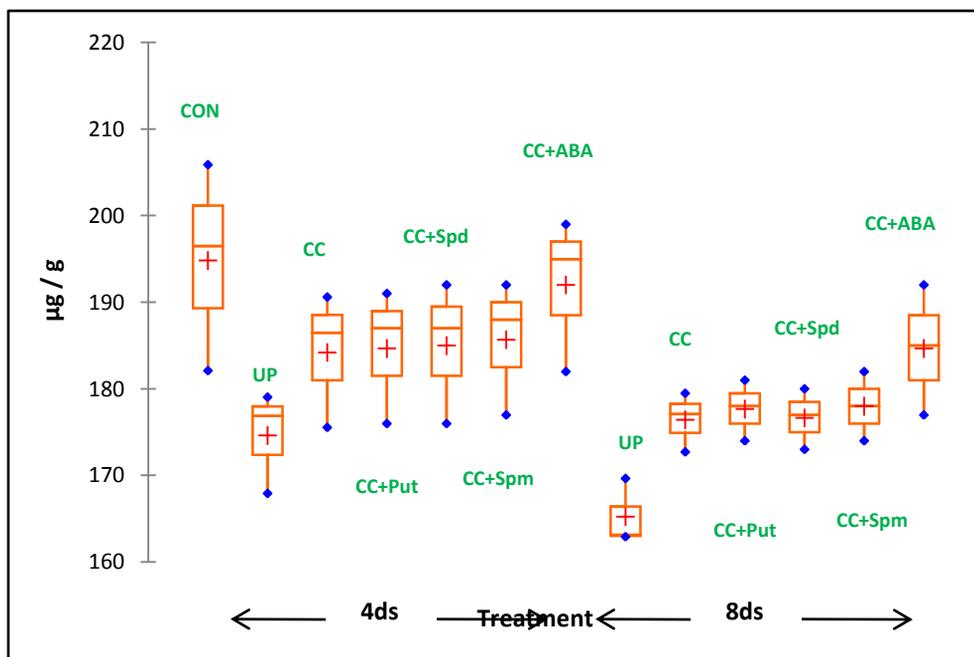


**Figure 7.12:** Effect of application of polyamines and ABA with CC on proline content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

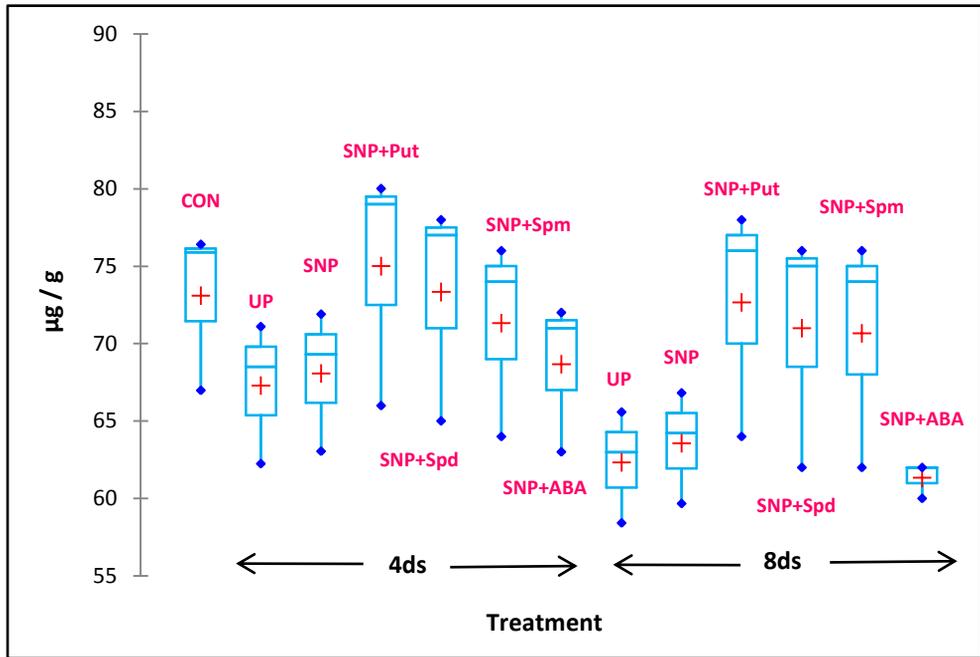


**Figure 7.13:** Effect of application of polyamines and ABA with SNP on total soluble sugar content of fenugreek seedlings primed under salinity stress

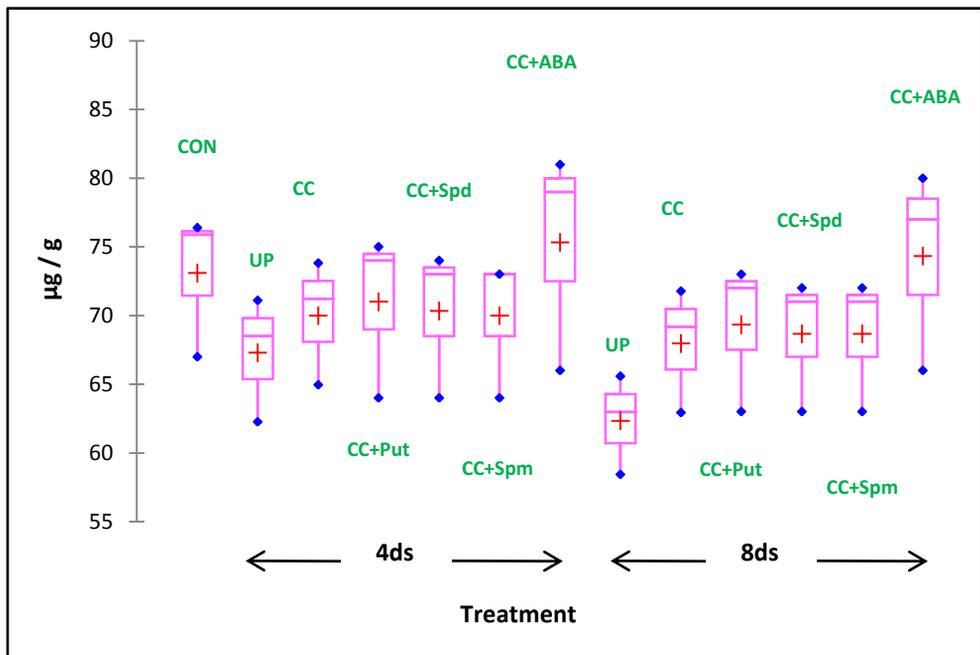


**Figure 7.14:** Effect of application of polyamines and ABA with CC on total soluble sugar content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

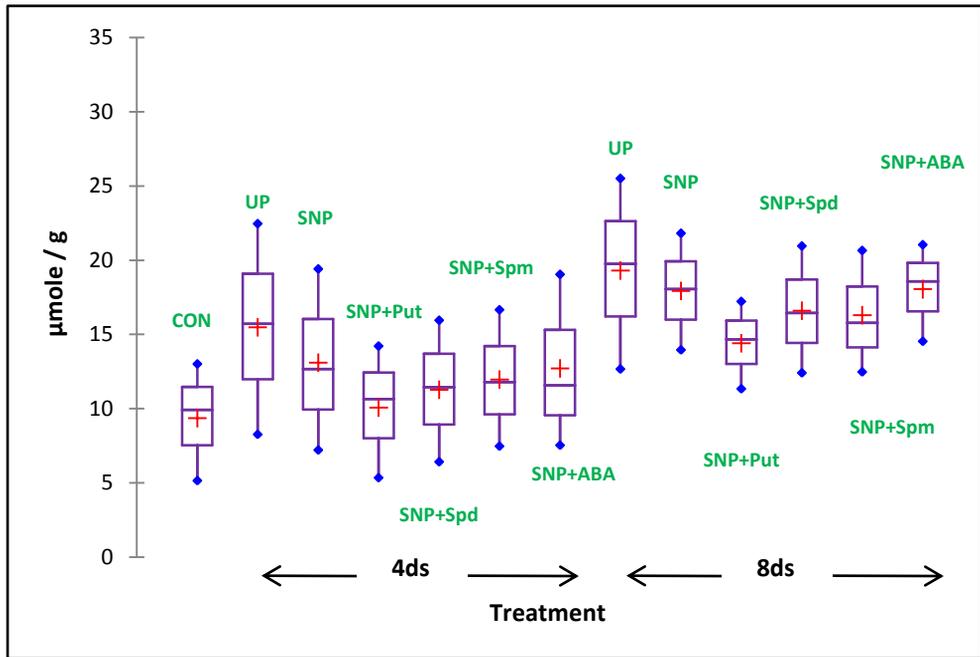


**Figure 7.15:** Effect of application of polyamines and ABA with SNP on total reducing sugar content of fenugreek seedlings primed under salinity stress

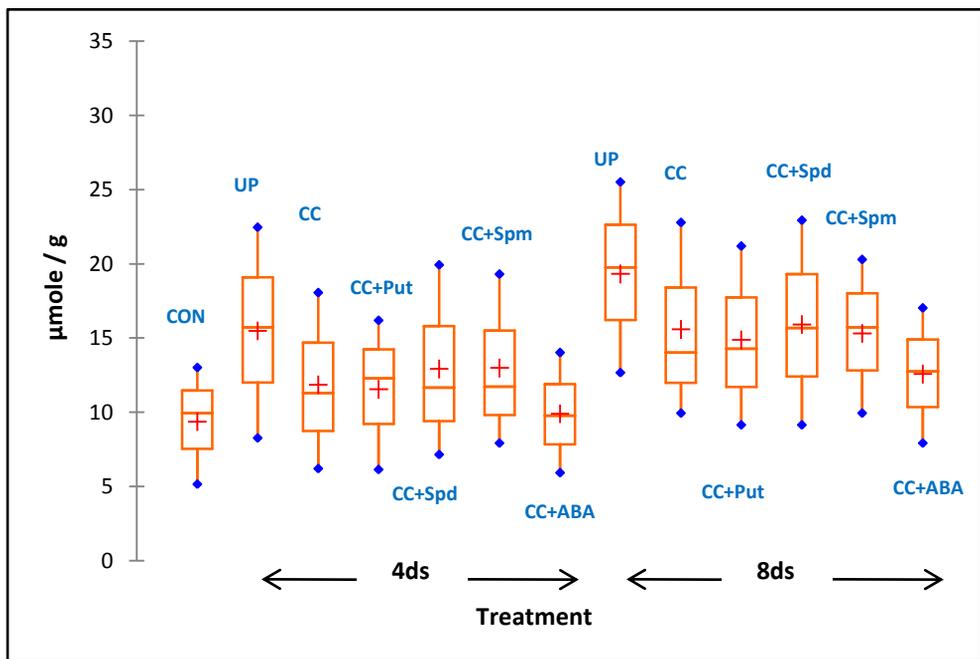


**Figure 7.16:** Effect of application of polyamines and ABA with CC on total reducing sugar content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

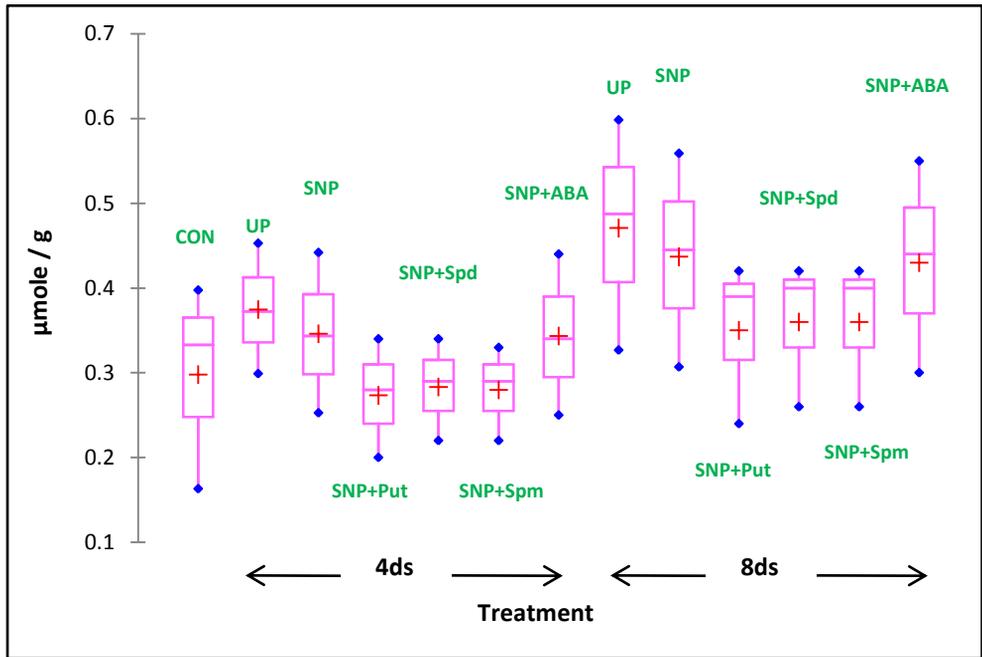


**Figure 7.17:** Effect of application of polyamines and ABA with SNP on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings primed under salinity stress

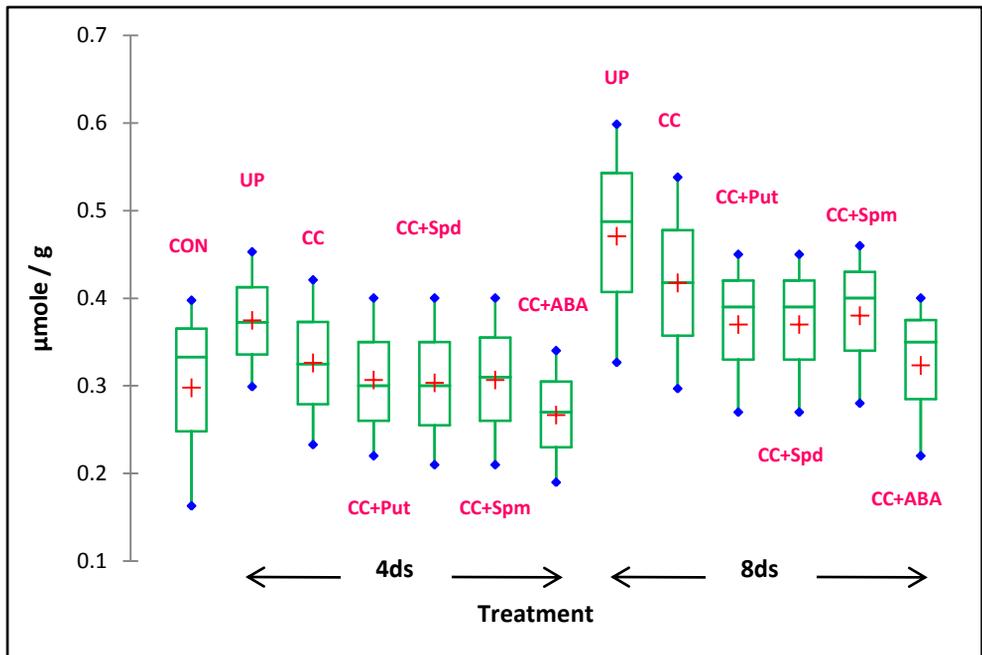


**Figure 7.18:** Effect of application of polyamines and ABA with CC on H<sub>2</sub>O<sub>2</sub> content of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid



**Figure 7.19:** Effect of application of polyamines and ABA with SNP on MDA content of fenugreek seedlings primed under salinity stress

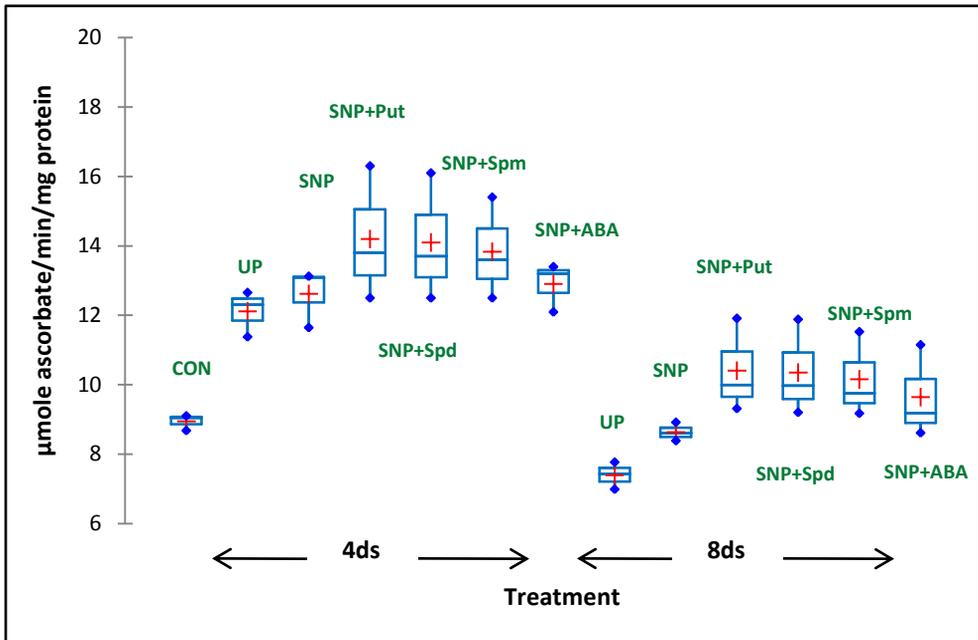


**Figure 7.20:** Effect of application of polyamines and ABA with CC on MDA content of fenugreek seedlings primed under salinity stress

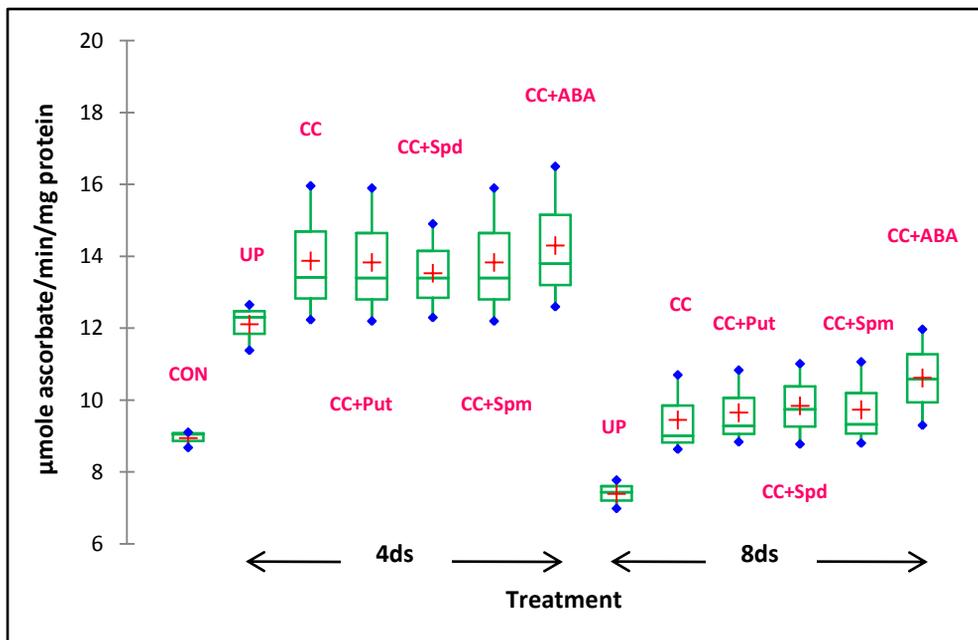
Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

seedlings was found under various pre-treatments during saline condition in our studies. At 4ds  $m^{-1}$  an enhancement of 18% and 12% and at 8ds  $m^{-1}$  15% and 10% of ascorbic acid content was recorded for CC+ABA and SNP+Put primed fenugreek seedlings respectively (Figure 7.19 & 7.20).

After studying the morphological and biochemical parameters, the activities of antioxidative enzymes were assessed. The activity of the most of the antioxidant enzymes determined was found to be remarkably enhanced by the addition of polyamines and ABA in the priming solutions. CAT activity was found to be best elevated by SNP+Put among the SNP combinations (SNP+Put; SNP+Spd; SNP+Spm and SNP+ABA) and CC+ABA among the CC combinations (CC+Put; CC+Spd; CC+Spm and CC+ABA) (Figure 7.21 & 7.22). The application of exogenous nitric oxide and calcium resulted in maximum elevation in the CAT activity by 20% and 27% at 4ds  $m^{-1}$  and 21% and 30% at 8ds  $m^{-1}$  respectively by the CC+ABA and SNP+Put priming. The activity of GPX increased under the influence of all pre-treatments; SNP+Put being the best among the SNP combinations exhibiting 12% and 16% elevation and CC+ABA among the CC combinations exhibiting 9% and 5% at 4ds  $m^{-1}$  and 8ds  $m^{-1}$  respectively (Figure 7.23 & 7.24). On determining the effect of various priming agents on the APX activity of the fenugreek seedlings, it was observed that seedlings primed with CC+ABA exhibited significant elevation of followed SNP+Put 7% and 13% at 4ds  $m^{-1}$  and 4% and 10% at 8ds  $m^{-1}$  respectively (Figure 7.25 & 7.26). Further the activity of SOD was best elicited by SNP+Spm followed by CC+ABA (Figure 7.27 & 7.28). Similarly, SNP+Put and CC+ABA also resulted in increased activity of GR in the fenugreek seedlings under salinity stress. A maximum of 7% and 23% at 4ds  $m^{-1}$  and 12% and 21% at 8ds  $m^{-1}$  enhancement in the GR activity was observed by SNP+Put and CC+ABA priming in fenugreek seedlings (Figure 7.29 & 7.30). The application of polyamines during salinity stress in Virginia pine revealed that putrescine exhibited best enhancement in the activity of antioxidant enzymes such as APOX and SOD among the different polyamines applied

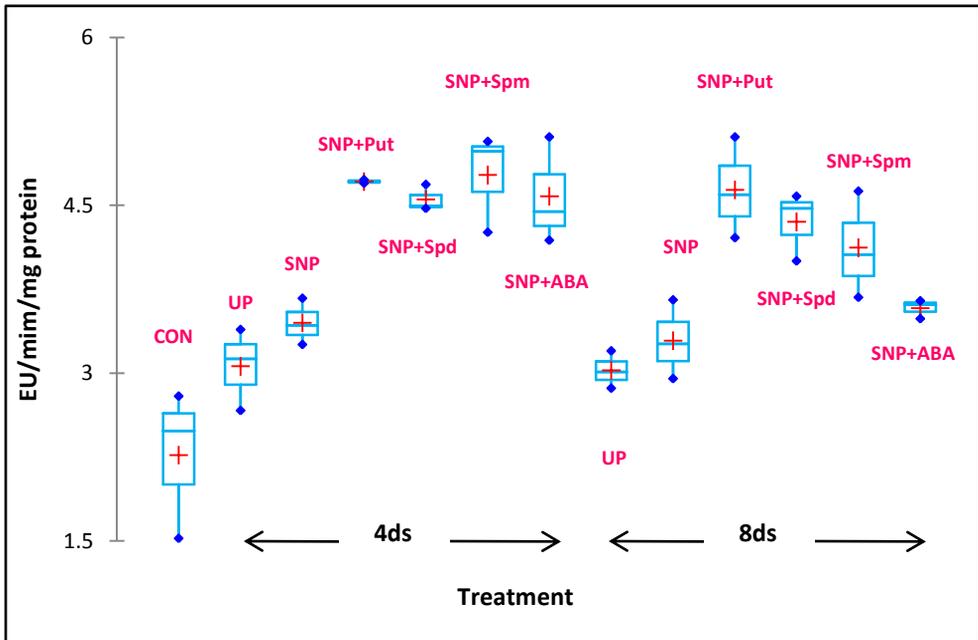


**Figure 7.21:** Effect of application of polyamines and ABA with SNP on ascorbate peroxidase activity of fenugreek seedlings primed under salinity stress

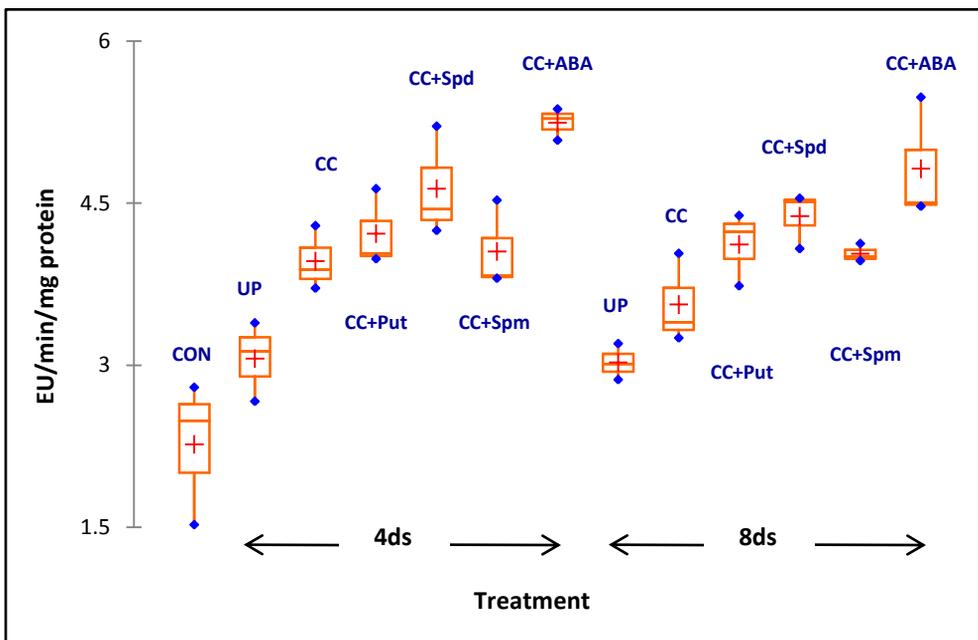


**Figure 7.22:** Effect of application of polyamines and ABA with CC on ascorbate peroxidase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

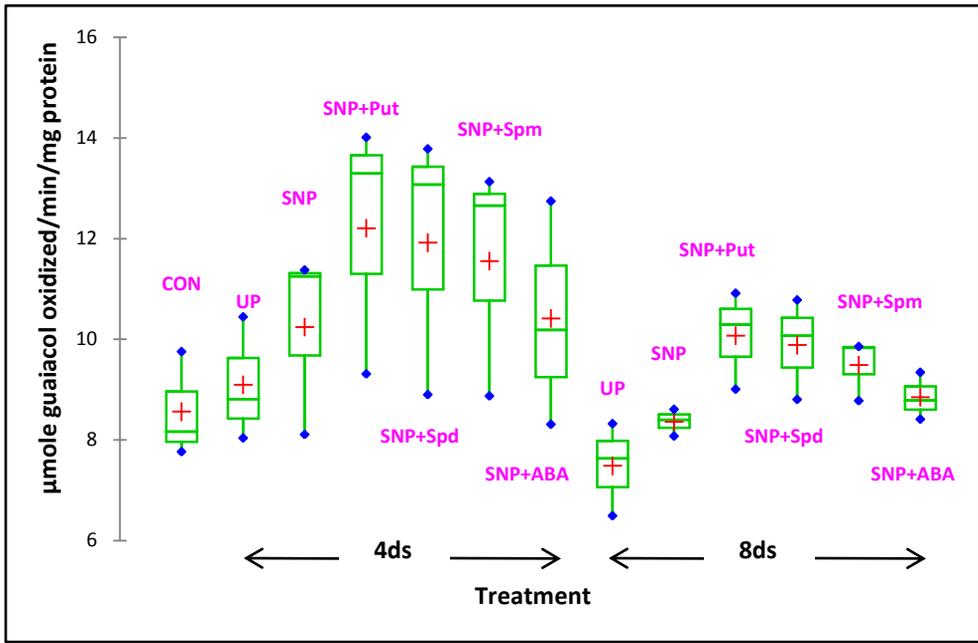


**Figure 7.23:** Effect of application of polyamines and ABA with SNP on catalase activity of fenugreek seedlings primed under salinity stress

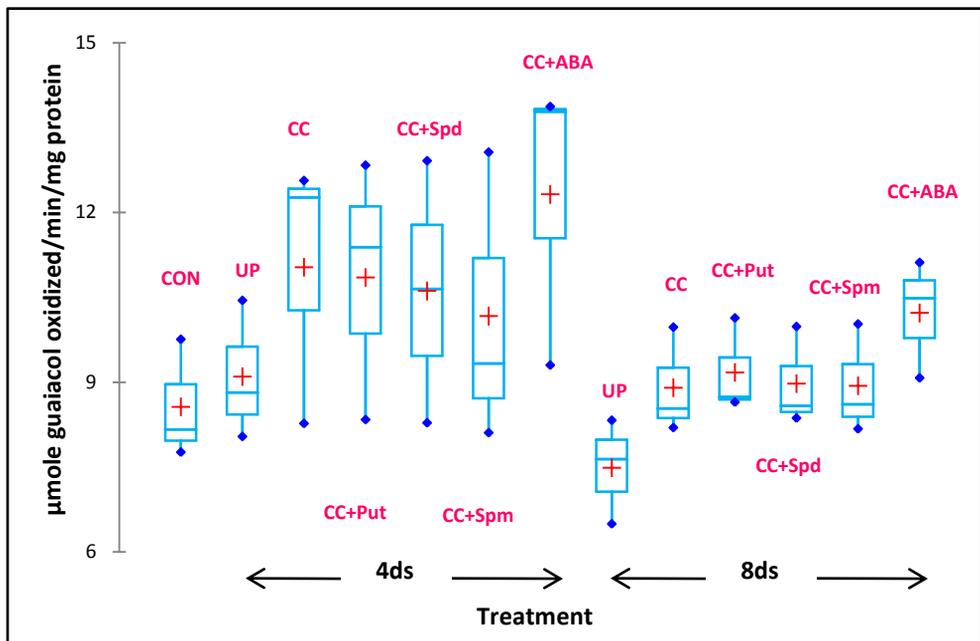


**Figure 7.24:** Effect of application of polyamines and ABA with CC on catalase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

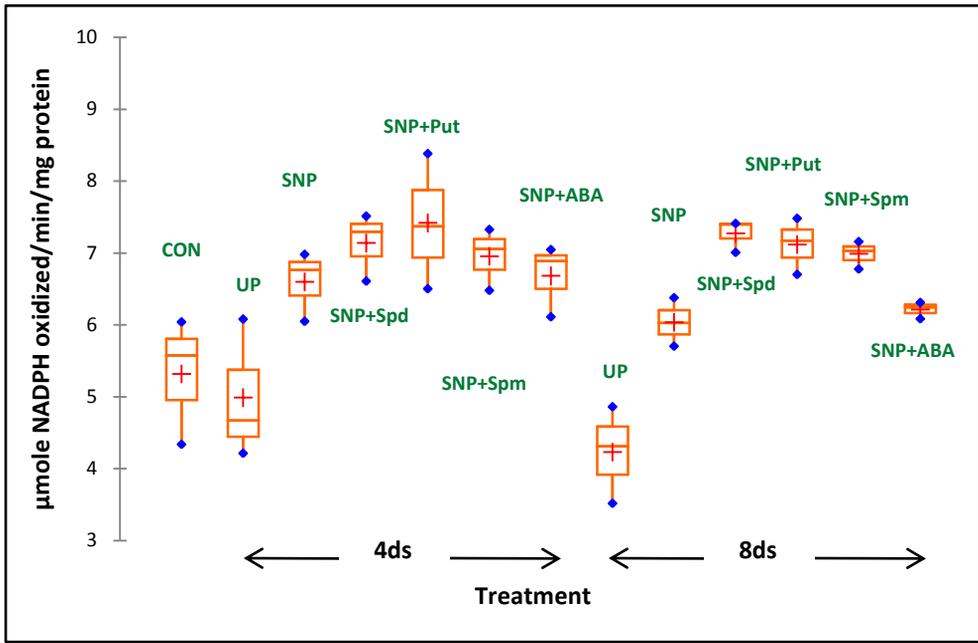


**Figure 7.25:** Effect of application of polyamines and ABA with SNP on guaiacol peroxidase activity of fenugreek seedlings primed under salinity stress

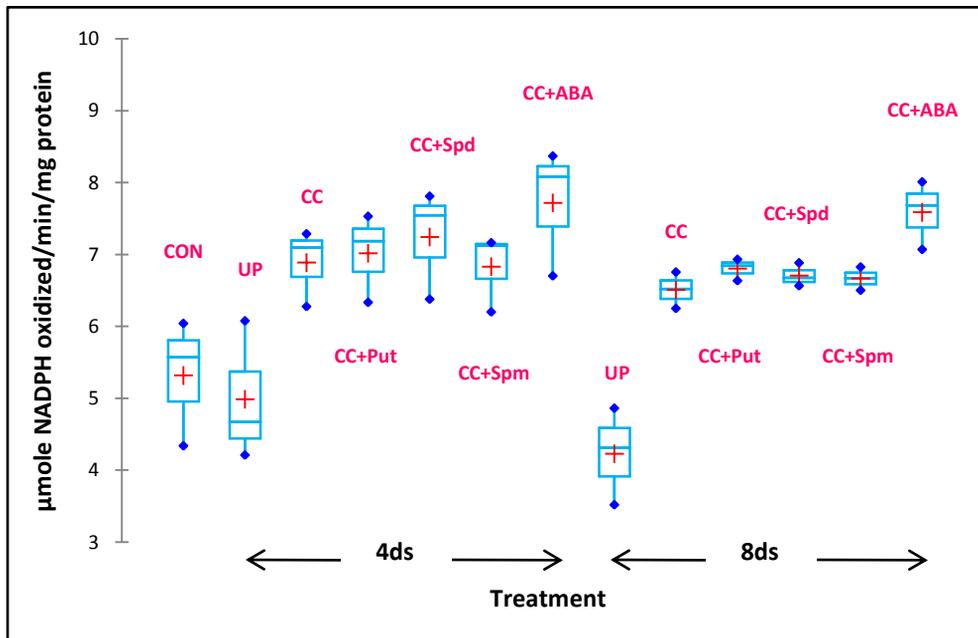


**Figure 7.26:** Effect of application of polyamines and ABA with SNP on guaiacol peroxidase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

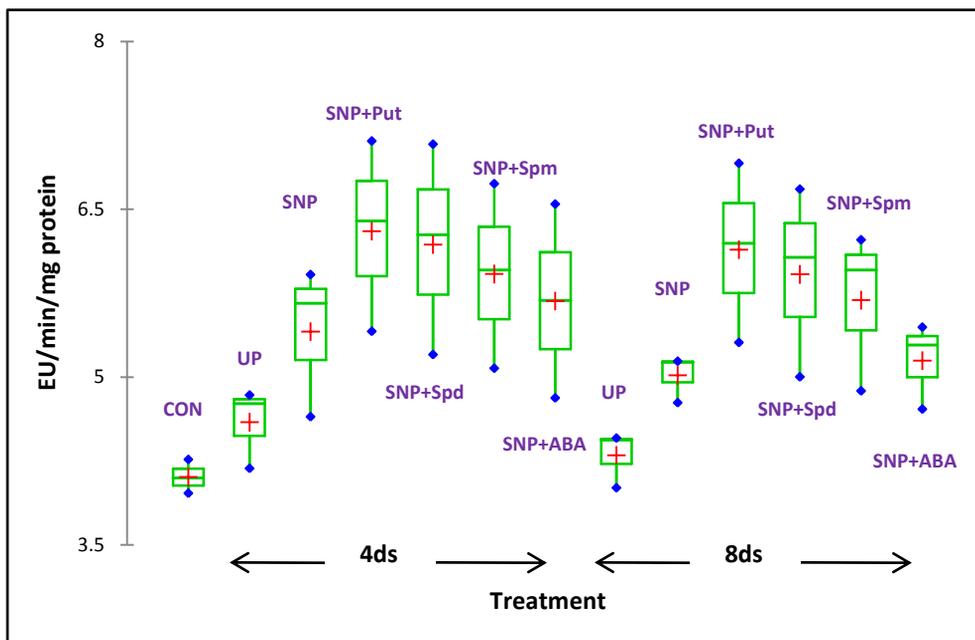


**Figure 7.27:** Effect of application of polyamines and ABA with SNP on glutathione reductase activity of fenugreek seedlings primed under salinity stress

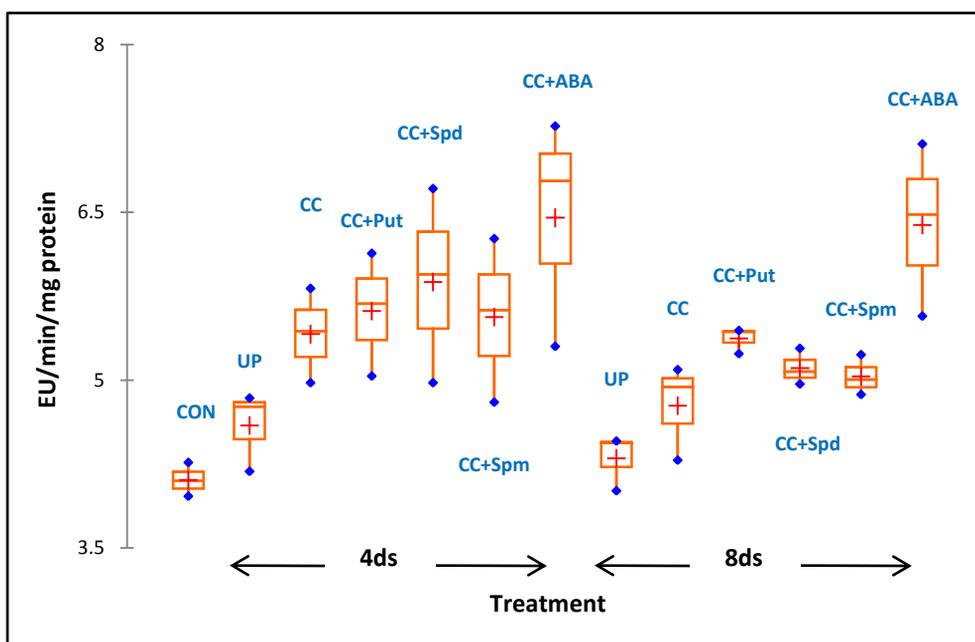


**Figure 7.28:** Effect of application of polyamines and ABA with CC on glutathione reductase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

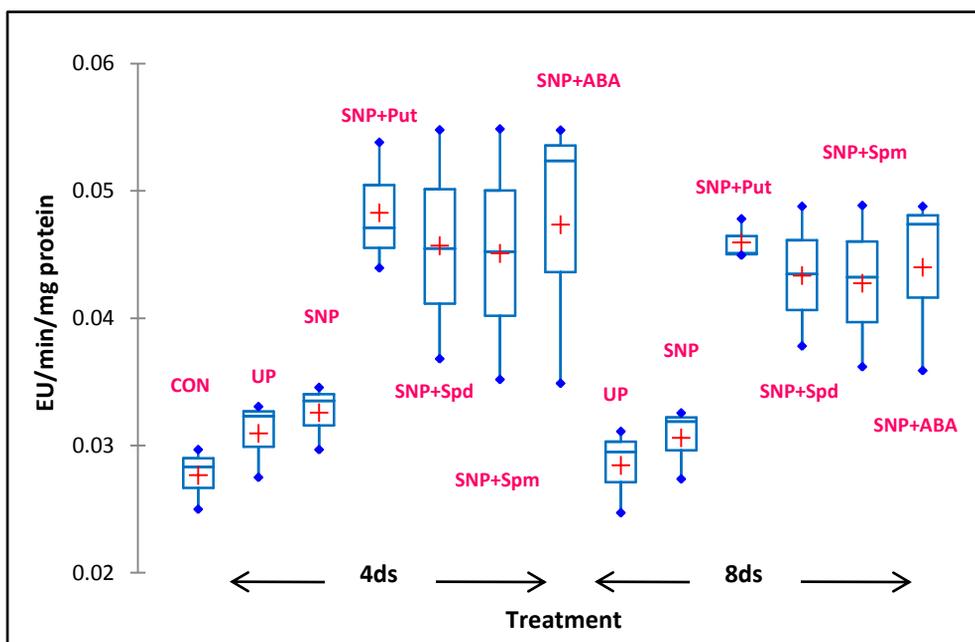


**Figure 7.29:** Effect of application of polyamines and ABA with SNP on superoxide dismutase activity of fenugreek seedlings primed under salinity stress

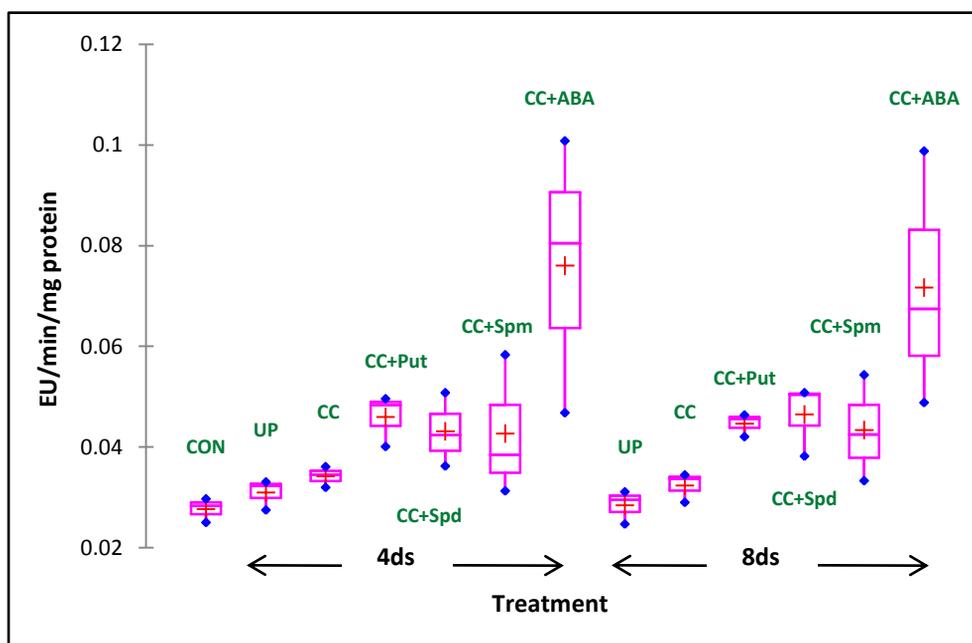


**Figure 7.30:** Effect of application of polyamines and ABA with CC on superoxide dismutase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid

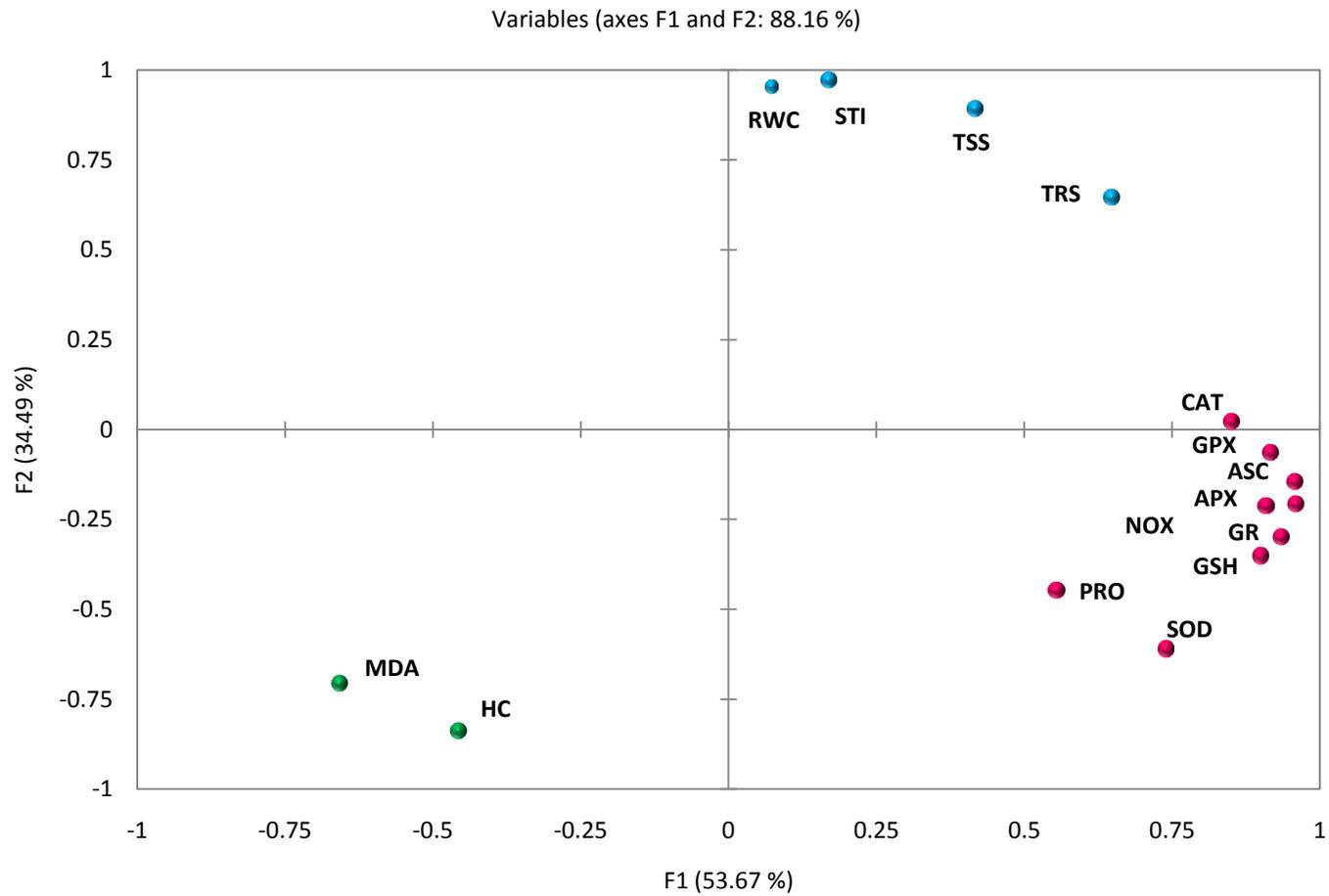


**Figure 7.31:** Effect of application of polyamines and ABA with SNP on NADPH oxidase activity of fenugreek seedlings primed under salinity stress



**Figure 7.32:** Effect of application of polyamines and ABA with CC on NADPH oxidase activity of fenugreek seedlings primed under salinity stress

Abbr. used: CON: control; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO; Put: putrescine; Spd: spermidine; Spm: spermine; ABA: abscisic acid



**Figure 7.33:** Principal component analysis depicting the clustering of highly correlated attributes of fenugreek seedlings under different salinity stress

(Tang and Newton, 2005). Moreover, the interactive role of polyamines and NO during several environmental stresses including salinity has also been reported by Wimalasekara *et al.*, 2011 in their studies. While evaluating the effect of above mentioned priming agents on the NOX activity, it was revealed that priming solution composed of CC and ABA exhibited best enhancement in the NOX activity whereas minimal elevation in the NOX activity was observed in seedlings pre-treated with other priming solutions (Figure 7.31 & 7.32). Earlier, Jiang and Zhang, 2003 have also reported involvement of ABA in enhancement of antioxidant defence enzymes including NOX; further they have suggested an interactive role of calcium and ABA signal transduction in strengthening the antioxidant defence system in maize seedlings. Such enhancement in the antioxidant system by the polyamines and ABA in combination to exogenous nitric oxide and calcium indicates their possible role in boosting the plants' defence mechanism against adversities created during salinity stress.

Principal component analysis was performed to determine the relation of various biochemical attributes with the antioxidant enzymes and the other morphological and physiological parameters of fenugreek sprouts subjected to salinity stress (Figure 7.33). As a result it was noted that the H<sub>2</sub>O<sub>2</sub> and MDA contents were found to be plotted opposite to the antioxidant enzyme activities, growth parameters and other beneficial biochemicals such as glutathione, ascorbate and carbohydrates; thus, indicating their adverse impact on the growth and development of fenugreek seedlings. Further, almost all the enzymatic and non-enzymatic attributes were found to form a separate cluster indicating their collective role in the mitigation of oxidative stress mediated adversities during stress conditions. Moreover the formation of another cluster by the carbohydrates and relative water content along with stress tolerance index suggests important role of the carbohydrates in the maintenance of growth and tolerance against salinity in plant system.

Overall it was observed that combination of CC+ABA and SNP+Put was most effective followed by SNP+Spm in the enhancement of stress tolerance in the fenugreek

seedlings under salinity stress. Whereas the combination of CC with polyamines and the combination of SNP with ABA exhibited least changes in the acclimation of fenugreek seedlings under salinity stress with respect to SNP and CC when applied alone. Such enhancement in the stress tolerance in the fenugreek seedlings indicates a possible interactive role between nitric oxide and polyamines as well as calcium and ABA in cellular system which has also been supported by earlier researches in their respective studies in numerous plant systems under various stress conditions. Our work suggests existence of effective interaction of nitric oxide and polyamines and between calcium and ABA involved in amelioration of salinity stress in plant system.

# **CHAPTER-8**

## **GENERAL DISCUSSION**

The present study deals with the role of two important signalling molecules namely, nitric oxide and calcium ion in enhancement of the therapeutic property of fenugreek seedlings along with their oxidative stress management during germination phase under salinity stress. An extensive review of numerous literatures was done prior to commencement of the research work, which has also been present in the thesis. From the literatures cited, it was found that several researchers have presented wide spectrum of application of the plant, *Trigonella foenum-graecum* including pharmacological as well as folkloric uses among human beings since ancient times. Further, the review highlights the chemistry of free radicals, their types, mode actions and the damages caused to the biological system. The compounds responsible for combating the adversities caused by free radicals and the oxidative stress known as antioxidants have been emphasized as the enhancement of antioxidant activity of fenugreek sprouts being one of the major objectives of the study. Moreover, the review section comprises of the various mode of application of elicitors in enhancement of bioactive metabolites in plants and aiding in tolerance towards various environmental conditions.

Next the present study deals with the alteration in the free radical scavenging activity of fenugreek sprouts under the influence of exogenous sources of calcium and nitric oxide and their antagonists namely, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a nitric oxide scavenger; a calcium chelator: Ethylene glycol-bis(2-aminoethylether)-N,N,N',N, tetra acetic acid and Lanthanum chloride: a calcium channel blocker. In recent times seed sprouting is gaining more significance commercially because it enhances the nutritional value of the seed (Laila and Murtaza, 2014). A large number of chemical changes occur to mobilize the stored carbohydrates and protein reserve into the germinating sprout. Sprouting also removes some anti-nutritive factors such as enzyme inhibitors from the seed that make sprouts safe for consumption (Sangronis and Machado, 2007). Sprouting in fenugreek is known to improve its soluble protein and fibre content and

reduce the phytic, tannic acid and trypsin inhibitors. Additionally, these signal molecules (nitric oxide and calcium) are known to regulate wide range of physiological, biosynthetic and developmental processes. Accordingly, their exogenous sources were applied as priming agents for the elicitation of free radical scavenging activity of fenugreek sprouts. The fenugreek sprouts pre-treated with different priming agents were evaluated for their *in vitro* antioxidant potential through various methods such as DPPH, ABTS, superoxide scavenging activity, metal chelating activity, reducing power, anti-lipid peroxidation and  $\beta$ -carotene protective activity. The results obtained suggested significant increase of free radical scavenging activity by elicited sprouting in fenugreek with exogenous sources of nitric oxide and calcium ion. The reason for efficient medicinal property of the plants is proposed to be the presence of wide range of phytochemicals. Plants synthesize these phytochemicals for fulfilling their self requirements to either achieve specific function or for defence mechanism against adverse conditions (Kasote *et al.*, 2015). Moreover, some of the phytochemicals such as polyphenols, flavonoids, carotenoids, ascorbic acid, and vitamin E have been reported to play important role in human system also. Considering the aforesaid statement, the fenugreek sprouts were further evaluated for their phytochemical contents such as phenol, flavonol, carotene and ascorbic acid. Consequently, the alteration in the accumulation of the above mentioned phytochemicals were assessed through respective method for estimation. As a result, considerable enhancement in the antioxidant activity along with phenolics and ascorbate content was observed by elicited sprouting in fenugreek with exogenous nitric oxide and calcium. In agreement to our findings, Joshi *et al.*, (2013) have also reported increase in phenolics in the seeds of cucumber after priming with calcium chloride. Likewise, a significant enhancement in the accumulation of flavonoids was reported in *Echinacea purpurea* (Wu *et al.*, 2007) and *Ginkgo biloba* (El Beltagi *et al.*, 2015) by nitric oxide elicitation. On the other hand, a huge loss in the phytochemical contents of the fenugreek sprouts was noticed under the influence of the antagonist molecules of nitric oxide

and calcium. Further, the application of nitric oxide and calcium along with their antagonists recovered the loss in the phytochemical contents which reinforces the statement suggesting the role of these signalling molecules in biosynthesis of these metabolites. For further determining the precise relationship among different studied phytochemicals and antioxidant activity of fenugreek sprouts and also their contribution in antioxidant activity, principal component analysis was performed. As expected, a strong association between the phytochemical attributes and the antioxidant activity was evident exhibiting possible cohesiveness among them. Further, similar to our findings such positive correlation between antioxidant and phytochemicals such as phenol, flavonoids, carotene and ascorbate have been suggested by several authors earlier (Wang and Linn, 2000; Rekika *et al.*, 2005; Shin, 2012; Salama *et al.*, 2015). Overall, it was observed that though a significant correlation was established between the phytochemicals and antioxidant activities; on the other hand, insignificant correlation as well as difference in the correlation coefficient was also found between various attributes. This might be due to the variation in the stoichiometry of the reactions between the antiradical components in the sprout extracts and the various radicals, which may be considered as a probable reason for the difference in their scavenging potential of these free radicals.

After successful elicitation of bioactive phytochemicals in the fenugreek sprouts; the well known hypoglycemic property of fenugreek was targeted for further enhancement. Therefore, the pre-treated sprouts were subjected for determination of their *in vitro* anti-diabetic potential through assays involving  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition. The considerable enhancement in the anti-diabetic activity of fenugreek sprouts further induced the idea for the estimation of alkaloids, since these metabolites are considered as one of the major components responsible for the hypoglycemic property of fenugreek. Furthermore, trigonelline is reported to act by regulating cell regeneration, insulin secretion, enzymes associated with glucose metabolism. It is further known to mitigate oxidative stress during

the diabetic complications (Yoshinari *et al.*, 2009; Amaro *et al.*, 2014). Eventually, the elicitation of alkaloids led to purification of the major compound trigonelline, a scientifically proved hypoglycemic agent found in fenugreek. Trigonelline was isolated from the pre-treated fenugreek sprouts and further authenticated by IR and NMR spectroscopy. Overall, it was observed that among all treatments, CC and SNP priming exhibited enhancement in the alkaloid and antidiabetic potential of fenugreek, among which CC was found to exhibit the best result. Such enhancement in the antidiabetic activity and the level of trigonelline in fenugreek has reported by various authors through biotic and abiotic elicitors (Ahmed, 2011; Qaderi, 2016). The therapeutic property of trigonelline is highly attributed to its hypoglycaemic effect, thus the enhancement in the biosynthesis of alkaloids further boosts the antidiabetic potential of plant. The observation of best antidiabetic activity of sprouts during 48h stage indicates that the components including trigonelline responsible for the therapeutic potential were elicited appropriately at this stage. Likewise, various authors have proved that the enhancement in the hypoglycemic activity of fenugreek is considerably associated with increase in the level of bioactive components such as trigonelline and 4-hydroxy leucine (Ahmed *et al.*, 2011; El-Soud *et al.*, 2007; Narender *et al.*, 2006). The impact of nitric oxide scavenger (CP), calcium chelator (EG) and calcium channel blocker (LC) was also assessed on the antidiabetic as well as alkaloid content of the fenugreek sprouts. The effect of these elicitors was found to be deteriorative on both the aspects of the sprouts, i.e. therapeutic as well as phytochemical content. When analyzing the time course of germination, the stimulatory effect of these signal molecules was found to be most pronounced at the early phases of germination i.e. from 24h to 48h and after that the action was declined during further extension of post-germination phases. The work supports the hypothesis that nitric oxide and calcium offer significant role in enhancement of antioxidant activity during the germination phase of fenugreek which may be attributed to significant elicitation of bioactive components by these signal molecules. Thus, this knowledge can be

used to design the priming based sprouting techniques which might have potential application in improving the nutraceutical quality of legume sprouts.

After successful elicitation of bioactive phytochemicals and their related therapeutic properties, the exogenous sources of nitric oxide and calcium were applied for studying their effect on the alteration of various biochemical parameters along with their antioxidant defence system during germination stages. The pre-treated seeds were exposed to saline condition and their various morphological, physiological and biochemical attributes determined. The priming of seeds with various substances such as water, inorganic salts, osmolytes and hormones has been successfully reported as an effective strategy to enhance tolerance under saline conditions. Also the active involvement of these signalling molecules (nitric oxide and calcium) in mitigating the oxidative stress mediated damages during both biotic and abiotic stress led to study their roles in fenugreek seedlings. The seeds primed with the elicitors of nitric oxide and calcium were subjected to saline conditions at the level of 4ds m<sup>-1</sup> and 8ds m<sup>-1</sup> for 7 days. As a result, it was observed that the growth performance of fenugreek seedlings was extensively affected by the saline condition as it was evident by the reduction in the fresh weight and dry weight of the seedlings. A decrease in the reduction of the growth performance was observed in the seedlings subjected to calcium chloride priming. Contrastingly, the decrease in fresh weight was further enhanced in the seedlings pre-treated with the antagonists of these signalling molecules which have been recorded to be as much as 71% of fresh weight by lanthanum chloride and 67% of dry weight. Likewise, the seedling length was also extensively retarded by the scavengers and channel blockers of these signal molecules. Another important parameter, relative water content which is considered as one of the vital factors for the assessment of the extent of salinity induced effects and the degree of tolerance in plants; was calculated. Our findings suggested the positive effect of exogenous calcium and nitric oxide priming on the relative water of fenugreek seedlings under salinity stress. Meanwhile, similar trend of alteration of the

various elicitors were observed on the stress tolerance index of fenugreek seedlings. Our findings were in agreement to previous studies citing the progressive impact of nitric oxide and calcium on several plants including mustard (Zeng *et al.*, 2011), rice (Habib and Ashraf, 2014) chick pea (Ahmad *et al.*, 2016) and linseed (Khan *et al.*, 2010) during stress. Further earlier studies have claimed that the stress tolerant plants exhibit higher RWC than those susceptible ones which are found to lose significant amount of water content from their body parts. Though it is yet to be explored the mechanism of the involvement these signalling molecules in maintenance of RWC in stressed plants; however, Ke *et al.*, (2013) had proposed that NO could decrease solute potential thus enhancing the water potential in plant system under osmotic stress.

The major reactive forms of oxygen also termed as reactive oxygen species such as hydrogen peroxide, and superoxide radicals are known to be the molecules with high toxic potentials to plant tissues (Asada 2006). The accumulation of these free radicals and their probable associated effects: lipid peroxidation and plasma membrane integrity were investigated through histochemical detection as well as spectrophotometric assays. The results revealed a dose dependent effect of salinity on the accumulation of free radicals, lipid peroxidation and loss of plasma membrane integrity of the seedlings. However the seedlings under the influence of exogenous calcium and nitric oxide exhibited significant reduction in the accumulation of free radicals and their adverse effects in comparison to the unprimed seedlings as evident from the minimal appearance of respective stains used for detection. Further, the seedlings pre-treated with EGTA, c-PTIO and lanthanum chloride showed higher degree of deterioration of the plasma membrane integrity as resulted from higher accumulation of free radicals. The membrane integrity of those under the influence of exogenous calcium and nitric oxide was found to be resistant and undisturbed as evident from lighter stain exhibited against the dye. It has been earlier cited that calcium ion and nitric oxide serves as a part of signal cascade associated with plant growth and development

as well as mitigate the adverse effect of salinity thus preventing oxidative damages of tissue (Arshi *et al.*, 2006; Jaleel *et al.*, 2007; Tian *et al.*, 2015). Likewise, in the present study also seedlings pre-treated with calcium chloride were found to be resistant and exhibited high degree of membrane integrity and low MDA content in concomitant to low amount of ROS localization.

Furthermore, in agreement with our result, the protective role of calcium and nitric oxide is also found to be reported in other plant system under salinity stress condition (Bhattacharjee, 2009; Tian *et al.*, 2015). The biochemical attributes estimated in the present study were, proline, glutathione, ascorbate and sugars. It was observed that salt stress has affected the biosynthesis of almost all of these important biomolecules stated earlier. The loss in the accumulation of various osmolytes such as proline and carbohydrates which are considered to be essential factor for the maintenance of better physiological and morphological parameters of plant body, were minimized by the exogenous supply of nitric oxide and calcium in the priming solutions. Also it has been reported that the accumulation of such osmolytes have been beneficial in maintaining the optimum turgidity of cells for normal functioning, thus overcoming the stress provoked by salinity (Khan *et al.*, 2012; Abdel Latef and Chaoxing, 2011). Therefore, the accumulation of osmolytes such as carbohydrates and proline under the influence of exogenous calcium and nitric oxide during salinity stress suggests the potential role of these signalling molecules in providing tolerance against salinity to fenugreek seedlings during germination.

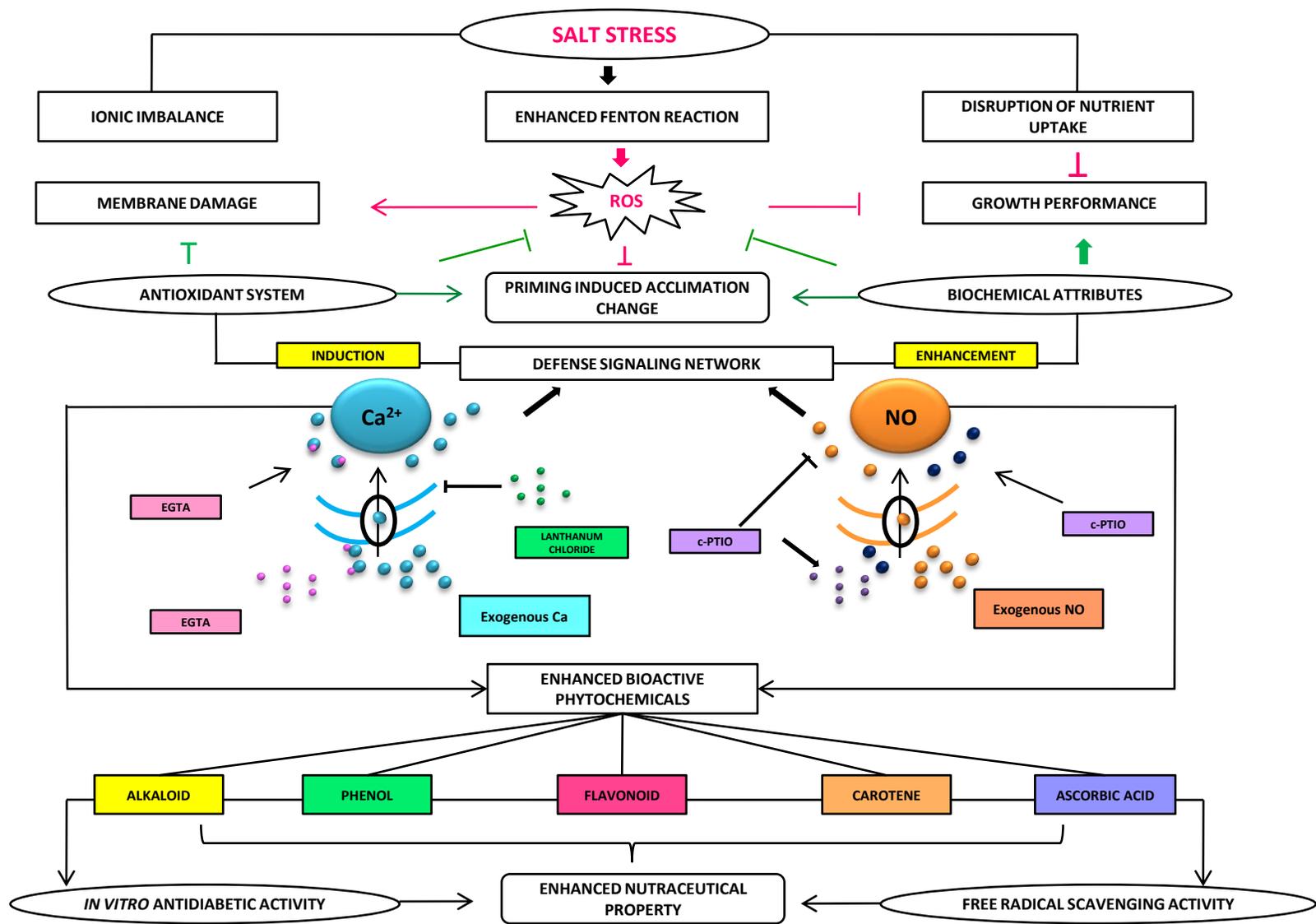
Plants have evolved various defense mechanisms and strategies for minimizing the adverse effects of these free radicals and stress condition. These defense strategies are known to comprise of both enzymatic and non-enzymatic mechanisms as mentioned earlier. The major enzymes responsible for removal of excess ROS in plant system are CAT, SOD, APX and GR, which are reported to exist in different cellular compartments as isozymes in chloroplast

and mitochondria. In the present study the non enzymatic antioxidants namely reduced glutathione (GSH) and ascorbate along with enzymatic antioxidants CAT, GPX, GR, GST, SOD, and APX were determined. The glutathione content was found to be adversely affected by salinity and the antagonists, but the recovery in the glutathione pool during salinity was observed under the influence of exogenous calcium and nitric oxide. Though there was an increase in the glutathione content in the unprimed seedlings but later a decline of about 33% on day 7 was also observed in higher dose of salinity (8ds m<sup>-1</sup>).

However under the influence of exogenous calcium and nitric oxide the accumulation of glutathione was found to be further induced; at 4ds m<sup>-1</sup> an enhancement of 27% and 24% and at 8ds m<sup>-1</sup> 34% and 28% of glutathione content was recorded for calcium chloride and SNP primed fenugreek seedlings respectively. Meanwhile, it was observed that the seedlings with high GSH content exhibited enhanced GR and GST activity, thus indicating the requirement of optimum level of glutathione for the better expression of these antioxidant enzymes. Similar findings were recorded for the ascorbate content and the ascorbate peroxidase activity of fenugreek seedlings, suggesting the role of ascorbate for modulation of APX enzyme. Furthermore, the other antioxidant enzymes CAT, POD and SOD were also positively induced by the application of exogenous nitric oxide and calcium as priming agents. In agreement to our findings, numerous scientifically proven reports on the role of these signalling molecules (nitric oxide and calcium) in mitigation of adversities of stress conditions in plants, have been provided by the researchers all over the world (Jaleel *et al.*, 2007; Khan *et al.*, 2009; Hayat *et al.*, 2012; Xu *et al.*, 2013; Dong *et al.*, 2014; Hameed *et al.*, 2015; Sharma and Dhanda, 2015; Ahmad *et al.*, 2016). After spectrophotometric estimation of various antioxidant enzymes, the alteration in the isozymes of four enzymes, CAT, POX, SOD and NOX were studied in native polyacrylamide gel electrophoresis. Accordingly, pronounced difference in the expression of the isozymes of the studied enzymes was observed due to respective response of the seedlings under the

influence of various elicitors during salinity stress. Most of the isoforms were induced by SNP and calcium chloride whereas negative impact of the antagonist molecules was observed on the expression of the isoforms of the studied enzymes. Such variation in the expression pattern of isoforms supports the fact that antioxidant enzymes exist in multiple isoforms in plant system which responses differentially to different developmental and environmental factors (Scandalios, 1993; Lee and An, 2005).

Principal component analysis was performed to determine the relation of various biochemical attributes with the antioxidant enzymes and the other morphological and physiological parameters of fenugreek sprouts subjected to salinity stress. It was revealed that, biochemicals such as proline and carbohydrates were found to have significant impact on the growth performance of fenugreek seedlings as they were found to be clustered along with growth parameters such as seedling weight, height and relative water content along with stress tolerance index. Also, the plot revealed that glutathione and ascorbic acid were highly responsible for the regulation of their associated antioxidant enzymes such as glutathione reductase and glutathione-s-transferase; ascorbate peroxidase respectively. Further, most of the enzymes were found to form a separate cluster (opposite of free radicals) indicating their collective role in the mitigation of oxidative stress mediated adversities caused by these free radicals. In a nut shell, our results suggest that priming of fenugreek seeds with exogenous source of nitric oxide and calcium enhanced the morphological and biochemical attributes along with the antioxidant defense system under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seeds which were unprimed and also those primed with the antagonists of these signalling molecules. Therefore, the enhancement in the enzymatic as well as non-enzymatic components might be due to the involvement of calcium and nitric oxide leading to tolerance towards salinity accompanied with better growth and development. Additionally, an uninterrupted influx of these signal molecules is very much essential for the



**Figure 8.1:** Schematic representation of involvement of nitric oxide and calcium in enhancement of nutraceutical property and oxidative stress management in fenugreek

better nutraceutical quality and growth of fenugreek seedlings, which can be hypothesized on the basis of the tremendous deterioration of the therapeutic potential as well as oxidative stress management status of the fenugreek seedlings revealed in the present study (Figure 8.1).

Lastly, to study the interactive role of nitric oxide and calcium with the polyamines and other growth regulators in amelioration of salinity stress; the polyamines (Putrescine, Spermidine and Spermine) and ABA were added to the priming solution along with SNP and calcium chloride. It was observed that combination of CC+ABA and SNP + Putrescine was most effective followed by SNP + Spermine in the enhancement of stress tolerance in the fenugreek seedlings under salinity stress. Such enhancement in the stress tolerance in the fenugreek seedlings indicate a possible interactive role between nitric oxide and polyamines as well as calcium and ABA in cellular system which has also been supported by earlier researches in their respective studies in numerous plant systems under various stress conditions. Our work suggests the existence of effective interaction of nitric oxide and polyamines and between calcium and ABA involved in amelioration of salinity stress in plant system.

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## **Appendix -A**

## CHEMICALS USED

### A

$\alpha$ -amylase enzyme  
 $\alpha$ -glucosidase  
Abscisic acid  
ABTS [2,2' azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)]  
Acryl amide  
Aluminium chloride  
Ammonium per sulphate  
Anthrone reagent  
Ascorbic acid

### B

$\beta$ -Carotene  
Benzidine  
Bis-acrylamide  
Bovine serum albumin  
Bromocresol Green  
Bromophenol blue

### C

Calcium chloride  
1- Chloro-2,4- dinitrobenzene  
Chloroform  
c-PTIO[2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide ]  
Copper sulphate

### D

Dichloromethane  
DNS (3,5-dinitrosalicylic acid) reagent  
Dinitrophenyl hydrazine  
Dipotassium hydrogen phosphate  
Disodium hydrogen phosphate  
5,5'-dithiobis-(2-nitrobenzoic acid)  
DPPH (2,2-diphenyl-1-picrylhydrazyl)  
Dragendroff's reagent

### E

Ethanol  
Ethyl Acetate  
Ethylenediamine tetraacetic acid  
Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N', tetra acetic acid  
Evans blue

### F

FeCl<sub>2</sub>  
FeCl<sub>3</sub>  
Fehling's solution I (A)  
Fehling's solution II (B)  
Ferrozine  
FeSO<sub>4</sub>, 7H<sub>2</sub>O  
Folin-Ciocalteu reagent

### G

Gallic acid  
Glacial acetic acid  
Glutathione  
Glutathione reductase  
Glycerol  
Glycine  
Guaiacol

### H

Hydrochloric acid  
Hydrogen peroxide

### L

Lanthanum chloride  
Linoleic acid  
Liquid nitrogen

**M**

Methanol  
 Methanol-d<sub>4</sub>  
 Methionine

**N**

Naphylethylenediamine dihydrochloride  
 Nicotinamide-adenine dinucleotide phosphate (NADPH)  
 Ninhydrin  
 Nitro-blue tetrazolium  
 Nujol

**O**

Oxidized glutathione

**P**

Petroleum ether  
 Phenazine methosulphate  
*p*-nitrophenol- $\alpha$ -D-glucopyranoside  
 Polyvinyl pyrrolidone  
 Potassium dihydrogen phosphate  
 Potassium bromide  
 Potassium ferricyanide  
 Potassium hydroxide  
 Potassium iodide  
 Potassium persulfate  
 Potassium sulphite  
 Proline  
 Putrescine

**Q**

Quercetine

**R**

Rochelle salt  
 Riboflavin

**S**

Schiff's reagent  
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 Sodium dodecyl sulfate  
 Sodium dihydrogen phosphate  
 Sodium hydroxide  
 Sodium nitroprusside  
 Spermidine  
 Spermine  
 Starch  
 Sulfanilamide  
 Sulfosalicylic acid  
 Sulphuric acid

**T**

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 Thiourea  
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 Trichloroacetic acid  
 Tween 20  
 Trigonelline  
 Tris base  
 Tris-HCl  
 Triton

**X**

XTT [3'-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate]

**Z**

Zinc dust

## **Appendix - B**

## ABBREVIATION AND SYMBOLS USED

°C	Degree centigrade	CBL	Calcineurin B-like protein
'OH	Hydroxyl	CC	Calcium chloride
<sup>1</sup> O <sub>2</sub>	Singlet oxygen	CDPK	Ca <sup>2+</sup> - dependent protein kinases
AA	Alpha amylase	CML	Calmodulin like protein
AAE	Ascorbic acid equivalent	CON	Control
abs.	Absorbance	COX	Cytochrome <i>c</i> oxidase
	2,2' azinobis-(3-	COSY	correlated spectroscopy
ABTS	ethylbenzthiazoline-6-sulfonic acid)	CP	c-PTIO
			c-PTIO[2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide ]
AG	Alpha glucosidase	c-PTIO	
AGE	advanced glycation end-products		
ALP	Anti-lipid peroxidation	DCM	Dichloromethane
ANOVA	Analysis of variance	DMRT	Duncan's Multiple Range Test
APOX	Ascorbate peroxidase		
APS	Ammonium persulphate	DNPH	Dinitrophenyl hydrazine
ASC	Ascorbic acid content	DPPH	2,2-diphenyl-1-picrylhydrazyl
ATP	Adenosine triphosphate	DR	Degradation rate
BCB	β-carotene bleaching		
BCE	β-carotene equivalent	DTNB	5-5'- dithiobis-2-nitrobenzoic acid
BHA	Butylated hydroxyanisole	dS	deciSiemens
BHT	Butylated hydroxytoluene	EDTA	Ethylenediaminetetraacetic acid
Ca	Calcium	ET	Electron transfer
CaM	Calmodulin	EG	EGTA
CAT	Catalase	Fe <sup>2+</sup>	Ferrous ions

FRAP	Ferric reducing antioxidant power	MC	Metal chelating
FWT	Fresh weight tissue	MDA	Malondialdehyde
g	gram	METH	Methanol
GAE	Gallic acid equivalent	mg	Milligram
GPX	Guaiacol peroxidase	min	Minute
GR	Glutathione reductase	ml	Millilitre
GSH	Glutathione	MVSP	Multivariate Statistical Package
GSSG	Glutathione oxidized	NA	Not detected/ not applicable
GST	Glutathione-S-transferase	NADH	Nicotinamide-adenine dinucleotide reduced
IR	Infra red	NADPH	Nicotinamide-adenine dinucleotide phosphate
h	Hour	NBT	Nitro-blue tetrazolium
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide	NMR	Nuclear magnetic resonance
HAT	Hydrogen atom transfer	NO	Nitric oxide
HC	H <sub>2</sub> O <sub>2</sub> content	NO <sub>2</sub> <sup>•</sup>	Nitrogen dioxide
HETCOR	heteronuclear correlation	NOX	NADPH oxidase
HYDRO	Hydroprimed	NR	Nitrate reductase
HL	H <sub>2</sub> O <sub>2</sub> localisation	O <sup>2•-</sup>	Superoxide
IC50	50% Inhibition concentration	O <sub>3</sub>	Ozone
LC	Lanthanum chloride	OD	Optical density
LOO <sup>•</sup>	Lipid peroxy	OG	octyl gallate
LOOH	Lipid peroxide	OH	Hydroxyl radical
LP	Lipid peroxidation	ONOO <sup>-</sup>	Peroxynitrite
M	Molar	ORAC	Oxygen radical absorbance capacity
mg	milligram	PAGE	Polyacrylamide gel
mM	millimolar		

	electrophoresis		
PC	Principal component	SPSS	Statistical package for the Social sciences
PCA	principal component analysis	STI	stress tolerance index
PG	propyl gallate	TBA	Thiobarbituric acid
PKC	Protein kinase C	TBARS	Thiobarbituric acid reactive substances
PMI	Plasma membrane integrity	TCA	Trichloroacetic acid
PMS	Phenazine methosulphate	TCC	Total carotene content
pNPG	p-nitrophenylglucopyranoside	TEAC	Trolox equivalent antioxidant capacity
POD	Peroxidase	TEMED	N,N,N',N'- Tetramethylethylenediamine
Put	Putrescine	TFC	Total flavonoid content
QE	Quercetine equivalent	TLC	Thin Layer Chromatography
RNS	Reactive nitrogen species	TOCSY	Total correlation spectroscopy
RO <sup>•</sup>	Alkoxy	TPC	Total phenol content
ROO <sup>•</sup>	Peroxy	TPTZ	2,4,6-tripyridyl-s-triazine
ROS	Reactive oxygen species	TRAP	Total radical antioxidant trapping
RP	Reducing power	TRS	Total reducing sugar
rpm	Revolutions per minute	TSS	Total soluble sugar
RWC	Relative water content	μl	Microliter
SDS	Sodium dodecyl sulfate	μM	Micro molar
SEE	Standard error of estimates	UP	unprimed
SL	Superoxide localisation	UV	Ultra violet
SNP	Sodium nitroprusside	XOR	Xanthine oxidoreductase
SO	Superoxide		
SOD	Superoxide dismutase		
Spd	Spremidine		
Spm	Spermine		

## **APPENDIX-C**

### **List of Publications**

1. Mandal P and **Gupta SK**. Improvement of antioxidant activity and related compounds in fenugreek sprouts through nitric oxide priming. *International Journal of Pharmaceutical Sciences Review and Research*. 2014; 26(1): 249-257
2. **Gupta SK** and Mandal P. Involvement of calcium ion in enhancement of antioxidant and antidiabetic potential of fenugreek sprouts. *Free Radicals and Antioxidants*. 2015; 5: 74-82
3. **Gupta SK** and Mandal P. Assessment of the effect of nitric oxide and calcium ion on the therapeutic potential and oxidative stress status of fenugreek sprouts. *Asian Journal of Pharmaceutical and Clinical Research*. 2016; 9(2): 271-277
4. **Gupta SK** and Mandal P. Nitric oxide and calcium signalling in plants under salinity stress and their crosstalk . *NBU Journal of Plant Sciences*. 2016; 10:31-44
5. **Gupta SK** and Mandal P. Elicitation of trigonelline, a hypoglycemic agent in fenugreek sprouts by calcium and nitric oxide priming. *Asian Journal of Pharmaceutical and Clinical Research*. **In Press, 2017.**

## **APPENDIX-D**

## Research Article



## Improvement of Antioxidant Activity and Related Compounds in Fenugreek Sprouts through Nitric Oxide Priming

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### ABSTRACT

Nitric oxide (NO) is an important signalling molecule in plants. In the present work, the effect of some nitric oxide donors namely, sodium nitroprusside (SNP), sodium nitrite (NN) and potassium ferricyanide (FCN) on the antioxidant activity of *Trigonella foenum-graecum* L. was studied during germination. The treated seeds were germinated in dark for 72 h and the antioxidant activity were determined at interval of 24 h up to 3 days. The *in vitro* antioxidant activity was determined spectrophotometrically by the following methods, DPPH, ABTS, nitric oxide, anti-lipid peroxidation, beta-carotene bleaching, ferric reducing power and metal chelating as well as phytochemicals such as phenol, flavonol and carotene content were also evaluated. The results demonstrated that the seeds treated with nitric oxide donors showed gradual increase in their antioxidant potential from 24 h to 48 h and then declined at 72 h stages. Overall, the seeds treated with sodium nitroprusside and potassium ferricyanide showed higher antioxidant potential particularly at concentration 80 mM and 40 mM respectively, when compared with control. Our data supported the hypothesis that NO is a signalling molecule that plays an important role as an antioxidant component in plants.

**Keywords:** Antioxidant, Germination, Nitric oxide, Phenolics, *Trigonella foenum-graecum*.

### INTRODUCTION

*Trigonella foenum-graecum* commonly known as fenugreek, being rich in antioxidants and phytochemicals has been traditionally used as a food, forage and medicinal plant.<sup>1-2</sup> The pharmacological and folkloric uses of different plant parts of fenugreek have been reported by different researchers. Its seeds have been reported to have anti-diabetic,<sup>3-4</sup> anti-cancerous,<sup>5</sup> anti-inflammatory<sup>6</sup> and antioxidant activity.<sup>7</sup> Its leaves have been reported to possess potential anti-bacterial activity,<sup>8</sup> anti-diabetic<sup>9</sup> and antioxidant property.<sup>10</sup> Randhir *et al.*,<sup>11</sup> have also reported about the presence of potential antioxidant activity in the sprouts of fenugreek.

Nitric oxide is a short-lived bioactive molecule,<sup>12</sup> which is considered to function as prooxidant as well as antioxidant in plants.<sup>13</sup> Nitric oxide molecule is now recognized as an important signaling molecule and reported to be involved in various key physiological processes such as abiotic stress tolerance,<sup>14</sup> plant defense mechanism,<sup>15</sup> germination,<sup>16</sup> growth and development of plants<sup>17</sup> etc. In the cited study it was also shown that plant response to such stressors like drought, high or low temperature, salinity, heavy metals and oxidative stress, is regulated by NO.<sup>13, 18</sup>

Free radicals, such as reactive oxygen and nitrogen species, are an integral part of normal physiology. An over-production of these free radicals occurs, due to increase in oxidative stress brought by the imbalance of the bodily antioxidant defence system and free radical formation.<sup>19</sup> These highly reactive species on reaction with biomolecules can cause cellular injury and even death. This may lead to the development of several

disorders and chronic diseases such as cancers, Alzheimer's and Parkinson's diseases and those related to cardiac and cerebrovascular systems.<sup>20</sup>

Antioxidant compounds play vital role in protecting cell against destructive chemical compounds such as free radicals and reactive oxygen species (ROS) that are constantly produced by the cell metabolism and their concentration increases under stress conditions.<sup>21</sup> Phenolics and flavonoids are considered to be very important antioxidant components, which play very important roles in the prevention of human oxidative damages.<sup>22-23</sup>

In recent times seed sprouting is gaining more significance commercially because it enhances the nutritional value of the seed. A large number of chemical changes occur to mobilize the stored carbohydrates and protein reserve into the germinating sprout.<sup>11, 24</sup> Sprouting also removes some anti-nutritive factors such as enzyme inhibitors from the seed that make sprouts safe for consumption. Sprouting in fenugreek is known to improve its soluble protein and fibre content and reduce the phytic, tannic acid and trypsin inhibitors.<sup>24</sup>

The purpose of the present study was to evaluate the effect of nitric oxide donor namely, sodium nitroprusside, potassium ferricyanide and sodium nitrite on the antioxidant activity of *Trigonella foenum-graecum* sprouts germinated under dark condition. To our knowledge, this is the first report on the effect of nitric oxide donors in counteracting oxidative stress in fenugreek sprouts.



# Involvement of Calcium ion in Enhancement of antioxidant and antidiabetic Potential of Fenugreek Sprouts

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## ABSTRACT

**Introduction:** Calcium ion ( $\text{Ca}^{2+}$ ) is considered as a key signal transducer in plants which is involved in various physiological processes. The aim of the present study was to evaluate the influence of  $\text{Ca}^{2+}$  in enhancement of antioxidant as well as anti-diabetic activity of the fenugreek seedlings during developmental phase. **Methods:** The fenugreek seeds primed with Calcium chloride ( $\text{CaCl}_2$ ), Calcium chelator EGTA [Ethylene glycol-bis(2-aminoethylether)-N,N,N', N, tetra acetic acid] and calcium channel blocker  $\text{LaCl}_3$  (lanthanum chloride) and germinated for 3 days. The sprout extracts were investigated for their antioxidant potential by DPPH, ABTS<sup>+</sup>, metal chelating, reducing power, nitric oxide scavenging capacity and anti-lipid peroxidation as well beta-carotene bleaching assays along with *in vitro* antidiabetic activity by  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition. Along with this, phytochemicals such as phenol, flavonol and carotene were also estimated. **Results:** The results demonstrated that the seeds treated with calcium chloride showed enhanced antioxidant as well as antidiabetic potential over control; on the other hand the action was reversed by EGTA and  $\text{LaCl}_3$ . Similar trend was observed in the phytochemical contents of the sprouts. **Conclusion:** Our data suggested that the improvement in nutraceutical value of fenugreek sprouts by calcium chloride could be due to the involvement of  $\text{Ca}^{2+}$  in signaling pathways associated with related phenolic compounds.

**Key words:** Antioxidant, Antidiabetic, Calcium ion, Germination, Phenolics.

## INTRODUCTION

Several pharmacological as well as experimental studies have suggested that consumption of foods rich in antioxidant is significantly associated with reduced risk of various disorders and human diseases, including diabetes.<sup>1</sup> Some of the food types such as fruits, vegetables and sprouts and herbal drugs have been found to be very rich in bioactive compounds such as polyphenols, vitamins C and E,  $\beta$ -carotene etc, which possess potential antioxidant activity. Therefore, in recent times the regular consumption of sprout or germinated seeds, fruits and vegetables, is highly recommended as they are considered to provide long term health benefits.<sup>2</sup>

Reactive oxygen species (ROS) or free radicals leaked during the process of metabolism have been the major source for the oxidative stress in the living system. These free radicals are found to be responsible for several chronic diseases and disorders in human body system.<sup>3</sup> The increased level of ROS has been associated with the degradation of pancreatic beta-cells leading to type 1 diabetes and the onset of type 2 diabetes by insulin resistance.<sup>4</sup> The inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are responsible for post-prandial hyperglycemic conditions have gain more attention by the medical practitioner for the diabetic treatment. The antioxidant compounds present in these food sources and herbal drugs could be attributed to their properties such as hydrogen donors, reducing agents and metal ion-chelators.<sup>5</sup> It has also been reported that the natural sources of antioxidants can play vital role in controlling the post-prandial hyperglycemic conditions via inhibition of the key enzymes  $\alpha$ -amylase,  $\alpha$ -glucosidase which is a potential approach towards diabetic treatment.<sup>6</sup> *Trigonella foenum-graecum* commonly known as fenugreek, the different plant

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## ASSESSMENT OF THE EFFECT OF NITRIC OXIDE AND CALCIUM ION ON THE THERAPEUTIC POTENTIAL AND OXIDATIVE STRESS STATUS OF FENUGREEK SPROUTS

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### ABSTRACT

**Objective:** The aim of the present study was to investigate the effect of priming with exogenous sources of calcium ion and nitric oxide on the antioxidant activity, antidiabetic activity, and related phenolic contents along with the histochemical status of fenugreek sprouts.

**Methods:** The fenugreek seeds were primed with calcium chloride (CC), calcium chelator ethylene glycol-bis (2-aminoethylether) -N, N, N', N tetra acetic acid (EG), sodium nitroprusside (SNP) and 2-(4-carboxyphenyl) -4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CP) and germinated for 72 hrs. The sprout extracts were investigated for their antioxidant potential by 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, reducing power as well as beta-carotene bleaching assays along with *in vitro* antidiabetic activity by  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition. Along with this, phytochemicals such as phenolics, flavonoids, and carotene content were also estimated, and the histochemical detection of reactive oxygen species in roots was performed.

**Results:** The results demonstrated that the seeds pre-treated with CC and SNP showed enhanced antioxidant as well as antidiabetic potential over control; on the other hand, their action was reversed by their antagonists, EG, and CP. A similar trend was observed in the phytochemical contents of the sprouts. Furthermore, it was evident from the histochemical detection of  $H_2O_2$  and superoxide localization as well as lipid peroxidation and plasma membrane integrity that the exogenous supply of calcium ion and nitric oxide exhibited protective role in the germinating seedlings.

**Conclusions:** The study suggests the active involvement of the signal molecules,  $Ca^{2+}$ , and nitric oxide in signaling pathways associated with related phenolic compounds and oxidative stress management.

**Keywords:** Antidiabetic, Antioxidant, Calcium ion, Germination, Histochemical localization, Phenolics, Nitric oxide, *Trigonella foenum-graecum*.

### INTRODUCTION

The fact that the consumption of antioxidant-rich food is extensively associated with reduced risk of various disorders and diseases has been suggested by several researchers [1]. Natural resources such as fruits, vegetables, and also sprouts have been found to be a potential source of bioactive compounds such as polyphenols, ascorbic acid,  $\beta$ -carotene, etc., which possess high antioxidant activity. Therefore, the habitual intake of sprouts, fruits, and vegetables is highly recommended by the nutritionists, as they provide long-term health benefits [2].

Free radicals, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), are considered to be a fundamental part of normal physiology. Oxidative stress occurs as a consequence of the imbalance in the antioxidant defense system and free radical production [3,4]. These free radicals are mainly responsible for several oxidative stresses mediated chronic diseases and disorders in the human body system, including diabetes [5]. Excessive production of ROS leads to degradation of the pancreatic  $\beta$ -cells, thus causing Type 1 diabetes and Type 2 diabetes with insulin resistance. The inhibiting agents of  $\alpha$ -amylase and  $\alpha$ -glucosidase (AG), which are responsible for post-prandial hyperglycemia, have gained more attention for diabetic treatment [6]. Antioxidant compounds play a vital role in preventing the cellular damages, against the highly unstable chemical components such as free radicals and ROS, which are constantly produced by the cell metabolism and their concentration increases under stress conditions [7]. It has also been reported that the natural sources of antioxidants can efficiently control the post-prandial hyperglycemia via inhibition of  $\alpha$ -amylase and AG without any negative effects [8].

*Trigonella foenum-graecum* commonly known as fenugreek has been reported to possess several pharmacological and folkloric applications.

Its leaves have been reported to show potential antioxidant property, antimicrobial as well as antidiabetic activity. The *in vivo* hypoglycemic activity of fenugreek seeds has been established in various animal model systems. In addition, fenugreek seeds possess potential hypocholesterolemic effect, antioxidant property and are also very effective in the treatment of diabetic disorders [9].

Nitric oxide is a bioactive molecule, which functions both as a pro-oxidant as well as antioxidants in plant system [10]. The chemical properties of nitric oxide make it a versatile signaling molecule that functions via interactions with several cellular components [11]. It is also considered as an RNS and its concentration-dependent impacts on different systems were reported to be either protective or toxic [12].

Calcium ( $Ca^{2+}$ ) is another important secondary messenger and signaling molecule which is actively involved in various physiological and developmental processes. In the cited literature,  $Ca^{2+}$  has also shown a protective effect against stress by mitigation of oxidative damages and membrane stabilization [13].

Seed priming is a pre-sowing technique in which seeds are subjected to the low external water potential that limits hydration which does not allow the protrusion of radicle through the seed coat. This technique is known to enhance the primary development of seeds under unfavorable environment [14,15]. The priming of seeds with various substances such as water, inorganic salts, osmolytes, and hormones has been successful and reported as a cost-effective strategy to enhance tolerance under saline conditions [16].

Sprouting has been considered as the effective means by which the nutritional quality of the seeds is enhanced. During germination process, mobilization of complex macromolecules such as stored

## **Review Article**

# **Nitric oxide and calcium signalling in plants under salinity stress and their crosstalk**

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### **Abstract**

Salinity is considered as one of the major factor affecting the crop production throughout the world. The oxidative stress induced by salinity can retard plant growth and yield as major part of energy is wasted on conserving water and improving ionic balance. The free radicals produced during stress are considered to be a major factor for most of the damages as these free radicals attack vital biomolecules such as lipids, protein and carbohydrates which are the basic requirement of almost all physiological and developmental processes. Understanding the mechanism of stress tolerance along with the involvement of important signalling molecules in stress signalling network is essential for crop improvement. Likewise, the two signalling molecules nitric oxide and calcium ion have been reported to be actively involved in upregulation of various stress response mechanisms thus indicating the existence of a possible cross talk among these molecules and other associated pathways. In this review, emphasis was given on the impact of salinity and oxidative stress mediated damages on plant system. Additionally, the role of nitric oxide and calcium ion as signalling molecules in response to stress signals and their implication in mitigation of salinity stress has also been discussed.

**Keywords:** Calcium ion, Free radicals, Nitric oxide, Salinity, Signalling.

### **Introduction**

Salinity is considered as one of the major factor affecting the crop production throughout the world. Salinity either in water or soil represents one of the major abiotic stresses especially in arid and semi-arid regions, which can severely limit the agricultural production (Shanon, 1998). High concentration of salt creates ionic imbalance and hyper osmotic stress in plant system which consequently leads to oxidative damages. Such drastic changes in plant system cause retardation of growth, molecular damages, membrane disruption and even death. For the plant to be tolerant to salinity stress: their homeostasis must be re-established along with detoxification mechanism must be boosted (Zhu, 2001). Most of the cellular damages caused by salinity are usually associated with ROS mediated oxidative stress (Parida and Das, 2005).

Nitric oxide and calcium both are considered as highly versatile signalling molecules. Various literatures have reported

the significant involvement of both of these molecules in wide range of physiological and developmental processes in plants. Additionally, these molecules have found to mitigate the adverse effect of varied environmental stresses including salinity (Wilson *et al.*, 2008; Sirova *et al.*, 2011; Lecourieux *et al.*, 2006).

### **Effect of salinity on plant system**

The two major consequences of salinity on plant system are osmotic stress and ionic toxicity; these physical conditions affect all other physiological, biochemical and developmental processes in plants (Yadav *et al.*, 2011). High salt content in the substratum creates rise in osmotic pressure of the substratum thus, affecting the water uptake capacity of plants. Furthermore, decrease in the turgor pressure of the plant cells cause closing of stomata which leads to reduced carbon fixation but increase in ROS production. These highly reactive and unstable free radicals disrupt various cellular processes by damaging the major biomolecules like lipids, proteins, and nucleic acids (Parida and Das, 2005). Ionic toxicity is the physiological state

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## ELICITATION OF TRIGONELLINE, A HYPOGLYCEMIC AGENT IN FENUGREEK SPROUTS BY CALCIUM AND NITRIC OXIDE PRIMING

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### ABSTRACT

**Objective:** This work was performed to evaluate the effect of priming with exogenous sources of calcium ion and nitric oxide on the antidiabetic activity and the alkaloid contents of fenugreek sprouts along with isolation and identification of trigonelline, a bioactive alkaloid responsible for hypoglycemic property of fenugreek.

**Methods:** The fenugreek seeds were pre-treated with calcium chloride (CC), lanthanum chloride (LC) a calcium channel blocker; ethylene glycol-bis (2-aminoethylether) -N, N, N', N tetra acetic acid (EG) a calcium chelator; sodium nitroprusside (SNP) and 2-(4-carboxyphenyl) -4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CP) a nitric oxide scavenger and germinated for 72 hrs. The sprout extracts were evaluated for their *in vitro* antidiabetic potential by  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition along with their trigonelline content. Trigonelline was isolated from fenugreek sprouts and identified by Infrared analysis and nuclear magnetic resonance (NMR) spectroscopy.

**Results:** The results revealed that sprouts pre-treated with CC and SNP exhibited enhanced antidiabetic potential as well as alkaloid content over control; on the other hand, their action was reversed by their antagonists, EG, LC, and CP. The sprouts pre-treated with 2mM CC showed the best elicitation of alkaloid content and antidiabetic activity followed by SNP-20 mM.

**Conclusions:** The study suggests probable involvement of the signaling molecules, calcium ion, and nitric oxide in pathways associated with biosynthesis of bioactive compounds responsible for hypoglycemic activity of fenugreek sprouts one of which being trigonelline.

**Keywords:** Antidiabetic, Calcium, Fenugreek sprouts, Nitric oxide, Priming, Trigonelline, Nuclear magnetic resonance.

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### INTRODUCTION

Plants are the basic source of knowledge of modern medicine. Almost all the parts of a plant, namely leaves, flowers, fruits, bark, roots, stem, and seeds are known to have various medicinal properties [1]. The trend of using natural products has increased, and the active plant extracts are frequently screened for new drug discoveries and for the presence of potential bioactive components [2,3].

India with the highest number of people suffering from diabetic disorders has been considered as the diabetic capital of the world by the "International Journal of Diabetes in Developing Countries." There is an alarming rise in diabetes patients in India; approximately 3.4 million deaths occur due to complication related to high blood sugar [4].

Diabetes mellitus is a serious metabolic disorder that leads to hyperglycemic condition due to decreased insulin production or inefficient insulin utilization. It is usually characterized by hyperglycemia, lipoprotein abnormalities, high basal metabolic rate, impairment in the activity of important enzymes, and oxidative stress which damages the pancreatic beta cells. It is the most common endocrine disorder which disrupts glucose homeostasis causing severe diabetic associated complications in major organs such as eye, blood vessels, and brain [5,6]. Multiple risk factors responsible for the disease to occur include persistent stress and depression, obesity, environmental pollutants, and sedentary lifestyle [7].

Some of the synthetic antidiabetic components such as metformin, acarbose, biguanides, and voglibose are found to be used clinically in combination with another diet to control diabetes, but moreover, they

exhibit adverse side effects after long term use [8-10]. To prevent or overcome the side effects of these synthetic drugs and also to create other safer alternative drug choices, it has become essential to seek other inhibitors for further drug development. Thus, in recent years, several efforts have been made for increasing the availability of glucosidase inhibitors from natural sources [11,12].

Likewise, fenugreek besides having several pharmacological properties it is also reported to possess potent antidiabetic property both *in vitro* and *in vivo* system. Fenugreek is known to be a rich source of various bioactive components having a different therapeutical property such as sapogenins, fenugreekine, nicotinic acid, phytic acid, and trigonelline [13,14]. Trigonelline is a pyridine alkaloid known to be mostly found in Fabaceae members and is reported to be metabolically active as a hypocholesterolemic agent along with potential hypoglycemic effect [15-18]. Fenugreek has been successfully implemented as antidiabetic remedy for both types I and II diabetes [19]. Moreover, fenugreek has been reported to be enriched with wide spectrum of pharmacological and folkloric significance [20].

This study deals with the isolation and identification of trigonelline by 1-D and 2-D nuclear magnetic resonance (NMR) from fenugreek sprouts. Further, the elicitors of calcium and nitric oxide were applied for the enhancement in the alkaloid content of fenugreek sprouts along with the *in vitro* antidiabetic property. This study may provide an insight in the role of these signaling molecules in modulating the biosynthesis of alkaloids, a potential hypoglycemic agent; in addition, the utility of 2-D NMR spectroscopy in identification of bioactive compounds.