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 β-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 *Gingko 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 α-amylase and α-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-hyroxy 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^{-1} and 8ds m^{-1} 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^{-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. 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

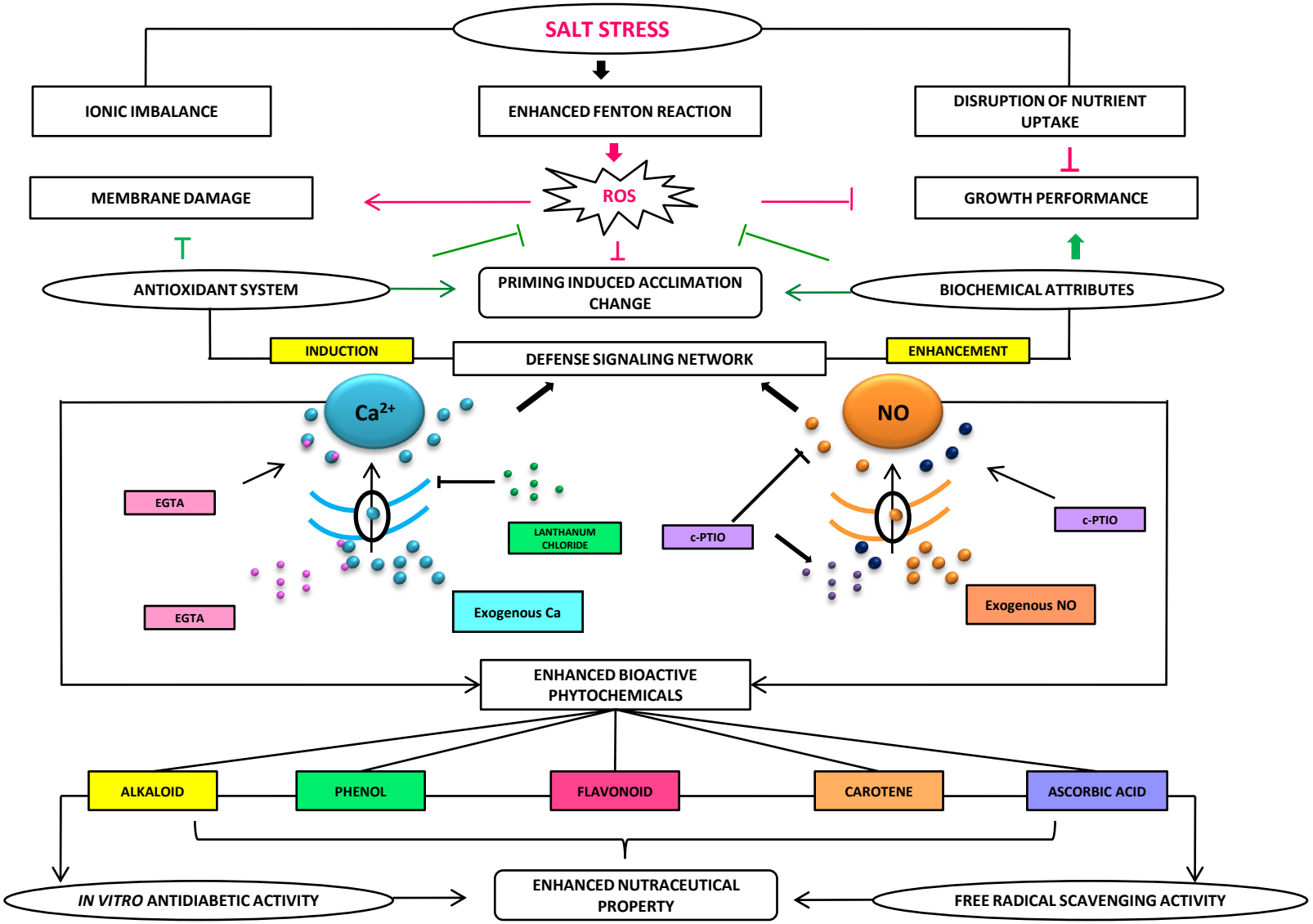

Figure 8.1: Schematic representation of involvement of nitric oxide and calcium in enhancement of nutraceutical property and oxidative stress management in fenugreek

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.