Plant tissue culture techniques have emerged as an inescapable tool with possibilities of complementing the conventional methods in plant breeding and have proved to be efficient and often economical for plant propagation (Murashige, 1974). It is an important technology being used for the conservation of important plants either through organogenesis, somatic embryogenesis or genetic transformation (Nasim et al., 2009a). Plant cell and tissue culture for secondary metabolite synthesis has attracted scientists and industrialists worldwide because of its various advantages.

*In vitro* cultures accelerate production of specific medicinal compounds at a rate similar or superior to that of intact plants by affecting the biosynthetic activity of cultured cells, which is done by regulating environmental factors, as well as artificial selection or induction of variant clones (Stafford et al., 1986). Thus, many industries use plant tissue culture techniques to develop cell cultures which yield secondary products, which is cheaper than extracting either the whole plant grown under natural conditions or synthesizing the product. Although the production of pharmaceuticals using plant cell cultures has been highlighted, other applications have also been suggested as a new route for the synthesis of products from plants that are difficult to grow, or in short supply, as a source of novel chemicals and as biotransformation systems. It is expected that the use, production, market price and structure would bring some of the other compounds to a commercial scale more rapidly and *in vitro* culture products may see further commercialization (Rao & Ravishankar, 2002; Nasim et al., 2009a; Nasim et al., 2010). Therefore, *in vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicines.

Garlic is widely cultivated plant with both culinary and medicinal uses owing to its biological activities related to organo-sulfur compounds, which include antibiotic, anti-cancer, anti-thrombotic and lipid-lowering cardiovascular effects (Nasim et al., 2009). Though such medicinal use of garlic existed for centuries, there was limited scientific support for its therapeutic and pharmacological properties. However, there has been a recent upsurge of research on garlic aiming to understand its exact mechanism(s) of action in each case so that garlic and its products may have more judicious future applications. Since garlic is vegetatively propagated, its improvement for desired traits through conventional means is difficult. Thus, the intervention of biotechnological methods such as tissue culture and tissue cultured nutritional enrichment based protocols hold great promise for improving this crop.
innovations in instrumentation and processing technologies coupled with more judicious experimental models could raise garlic to a reputable position in the medicinal world market (Bhagyalakshmi et al., 2005).

The aim of the present study was to understand the possible effect of plant growth regulators and/or their alteration or modification in medium composition with nutrients on various tissues and organs. An attempt has also been made to examine the effect of regulating factors such as solute (salt) and radiation (gamma) on regeneration and production of metabolite; and also to evaluate and compare production of metabolite in various in vitro grown tissues and organs. The observations during the course of present investigations have been discussed in terms of cause and effect relationships as far as possible and to derive some logically valid conclusions that are discussed under as follows:

5.1 Direct and Indirect In Vitro Regeneration

In vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines. This can be achieved through different methods including micro propagation. Micro propagation has many advantages over conventional methods of vegetative propagation which suffer from several limitations (Nasim et al., 2010a). With micro propagation, the multiplication rate is greatly increased. It also permits the production of pathogen-free material. Micro propagation of various plant species, including many medicinal plants has been reported (Nasim & Dhir, 2010). Propagation from existing meristems yields plants that are genetically identical with the donor plants (Chovelon et al., 1994). Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like Catharanthus roseus, Cinchona ledgeriana, Digitalis spp., Rehmannia glutinosa, Rauvolfia serpentina, Isoplexis canariensis etc. (Junaid et al., 2006; Perez et al., 2002). In this present investigation, different parts of the explant (basal, middle and tip of clove) were used in which regeneration was induced from middle and tip part of the clove while basal part of the clove showed remarkable response for direct and indirect shoot regeneration as in other similar studies (Nasim et al., 2010; Kapoor et al., 2012).

Numerous factors influence the success of in vitro propagation of different medicinal plants (Bhagyalakshmi & Singh, 1988). Of these, the effects of auxins and
cytokinins on shoot multiplication of various medicinal plants have been reported (Bhagyalakshmi & Singh, 1988). 6-Benzylaminopurine (BAP) at high concentration (1 to 5 ppm) is reported to stimulate the development of axillary meristems and shoot tips in *Atropa belladonna* (Benjamin et al., 1987). Similarly, it is also reported that cytokinin in optimal quantity is required for shoot proliferation in many genotypes but inclusion of low concentration of auxins (with cytokinins) triggers the rate of shoot proliferation (Tsay et al., 1989). According to Faria and Illg (1995), the addition of 10 μM BAP with 5 μM Indole-3-acetic acid (IAA) or 5 μM Naphthalene acetic acid (NAA) induced a high rate of shoot proliferation in *Zingiber spectabile* (Faria & Illg, 1995). It was also noted that the number of shoots/explants depends on concentration of the growth regulators and the particular genotype. The nature and condition of explants has also been shown to have significant influence on multiplication rate in *Clerodendrum colebrookianum* (Mao et al., 1995). Actively growing materials were found to be more responsive to shoot induction than dormant buds. Also, BAP was proved to be superior to 6-(γ-Dimethylallylamino) purine (2iP) and TDZ (Thidiazuron) for multiple shoot induction.

The plant growth regulators generally play a key role in different morphogenetic programme and therefore to get a particular response, explants are treated with specific plant growth regulators. These are able to produce direct organogenesis by inducing shoot and root formation (Nasim et al., 2009). In our study, higher percentage of shoot emergence (81.30%) was noted in medium with low concentration of BAP (0.5 mgL⁻¹) (Table 4.1.1). This result indicates that there is strong influence of BAP on the percentage of shoot emergence particularly at 0.5 mgL⁻¹ whereas other concentrations of BAP used in the study were shown to be less effective. These observations showed similarities to the earlier findings (Nasim et al., 2010). But for induction of shoot and root, BAP alone or with NAA was found to be most effective (Table 4.1.2). This result is consistent with previous reports (Nasim et al., 2010).

The induction of callus, its growth and subsequent differentiation and organogenesis is accomplished by differential application of growth regulators and by controlling conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. There are
many reports on regeneration of various medicinal plants via callus culture. Satheesh and Bhavanandan (1988) reported the regeneration of shoots from callus of *Plumbago rosea* using appropriate concentrations of auxins and cytokinins. Mantell and Hugo (1989) also reported a high frequency of shoot, root and microtuber production in *Dioscorea alata* depending on the culture medium used, the type of explant from which the calli originated, and the photoperiod. Plant regeneration has been achieved from leaf callus of *Cephaelis ipecacuanha* on MS medium supplemented with 4.5 mgL\(^{-1}\) Kn and 0.1 mgL\(^{-1}\) \(\alpha\)-Naphthaleneacetic acid (NAA) (Rout et al., 1992). The relative importance of genotype, explant and their interactions for *in vitro* plant regeneration via organogenesis in *Solanum melongena* has been investigated (Sharma & Rajam, 1995). Basu and Chand (1996) achieved shoot bud differentiation from root-derived callus in *Hyoscyamus muticus* in MS medium supplemented with 0.05 mgL\(^{-1}\) NAA and 0.5 mgL\(^{-1}\) BAP. Saxena et al. (1997) reported plant regeneration via organogenesis from callus cultures derived from mature leaves, stems, petioles and roots of young seedlings of *Psoralea corylifolia*. *In vitro* organogenesis in *Zingiber officinale* via callus culture has been described by Rout et al., 1992.

Although callus was regularly induced from nearly all explants, but there was marked difference in callus initiation, maintenance and yield from different explant sources. Among the various auxins, efficient callus induction and maintenance was seen in MS medium supplemented with 2, 4-D (Table 4.2.1). This may be due to the reason that 2,4-D is much more stable and less inactivated during culture processes compared to other auxins. Similar results were reported by Bhat et al. (2004). They proved superiority of 2, 4-D over other auxins for the callus induction and strongly antagonize any organized development.

It was evident from the present investigation that combinations of Kn and 2,4-D exhibited profound influence, where a combination of 0.50 mgL\(^{-1}\) Kn and 0.50 mgL\(^{-1}\) 2,4-D was more effective while higher concentrations has shown poor performance (Table 4.2.2). This result collaborated with the findings of Robledo et al. (2000) who reported that good callus induction was observed using root tip in MS medium supplemented with a combination of Kn and 2,4-D. In our study, different concentrations of 2,4-D with various concentrations of BAP were used in MS medium (Table 4.2.3). It was observed that MS medium supplemented with 2.0 mgL\(^{-1}\) 2,4-D and 0.50 mgL\(^{-1}\) BAP exhibited highest percentage of callusing (75.40%). Similar
results were obtained by Nasim et al. (2010). Several studies also reported that callus formation was higher on the medium containing BAP, NAA and low concentration of 2,4-D (Barandiarian et al., 1998; Nasim et al., 2009, 2010). These results coincide with our finding that the combination of high concentration of NAA and BAP and a low concentration of 2,4-D produced excellent results (84.17 %) in terms of percentage of callus formation (Table 4.2.4).

In our study, higher frequency of shoot regeneration via caulogenic pathways was observed in MS medium supplemented with BAP and NAA. Similar result pattern was also reported by several other workers in garlic (Barandiarian et al., 1998; Nasim et al., 2010). These results are also in agreement with other plants including *Dalbergia lanceolera* (Dwari & Chand, 1996). However, our finding is contradictory to Pandey et al. (1996) where best response for regeneration via caulogenic pathway was noted by using BAP alone.

### 5.2 Regeneration through Somatic Embryogenesis

Somatic embryogenesis is a process where groups of somatic cells/tissues lead to the formation of somatic embryos which resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium. Plant regeneration via somatic embryogenesis from single cells that can be induced to produce an embryo and then a complete plant, has been demonstrated in many medicinal plant species. Efficient development and germination of somatic embryos are pre-requisites for commercial plantlet production. In *Allium sativum* L. cv. Yamuna Safed 3, the embryogenic culture was initiated and the various stages of embryo development were studied in detail. Our results indicated that the rate of embryogenesis was quite high in *Allium* as was reported in many other plant groups (Pullman et al., 2003). The initiated cultures continued to proliferate for a period of two years or more making the culture a useful stable embryogenic source for future research purposes.

Application of exogenous PGRs was found to be essential for the induction of callus, embryogenic culture establishment, proliferation, maturation and germination of embryos into plantlets. Although calli production was noted to be maximum on medium amended with BAP + NAA + 2,4-D (Table 4.2.4), individual application of 2,4-D was also very effective in inducing callus from basal clove explants (Table
4.2.1). Generally, 2,4-D is considered to be one of the most important PGRs that regulate somatic embryogenesis *in vitro* (Zhang et al., 2007).

During induction into the medium, 2,4-D increases explants’ endogenous auxin level, one of the crucial signals that determine cultured cells’ fate to become embryogenic (Victor, 2005). Becwar et al. (1988) earlier reported that compared to higher concentrations, low levels of 2,4-D were more effective when combined with BAP for inducing embryogenic tissue. In auxin amended medium, cultured cell or tissue produce more ethylene than the auxin free cultures, which suppresses embryo development as the tissue multiplication continues to proceed without much check, the embryonic clumps develop into mature embryos only on medium amended with a very low level of 2,4-D (Razdan, 1993). These observations support our present study that the auxin (2,4-D) has no significant effect on induction of embryos rather it has a considerable positive effect on callus production (Table 4.4.1). Similar observation was noted in other plants like *Melia* where embryos were formed from pre-embryogenic determined cells, and did not depend on 2,4-D requirement (Evans et al., 1981; Litz & Schaffer, 1987). In this present study, the application of BAP and NAA at high concentration (1.0-2.0 mgL⁻¹) with a low level of 2,4-D (0.50 mgL⁻¹) showed maximum influence in producing embryogenic callus (Table 4.4.1, 4.5.1). Embryo maturation and simultaneous germination to obtain plantlets is one of the important steps in *in vitro* embryogenesis, which has been partially depending on embryo quality. Recently, a number of compounds like abscisic acid, sugar, sugar-alcohol, polyethylene glycol, activated charcoal, low temperature etc. have been added during embryo maturation (Lipavska & Konradova, 2004; Robichaud et al., 2004).

In our preliminary experiment, the presence of ABA appeared to be ineffective for embryo maturation and germination as it prevented embryo maturation and reduced plant regeneration (Table 4.6.1). This result is consistent with hybrid chestnut where addition of ABA was found to have no effect on embryo maturation (Vieitez, 1995). In contrast, ABA-induced improved embryo maturation with quality embryo was reported in several studies of somatic embryogenesis particularly in conifers (Lelu-Walter et al., 1999; Maruyama et al., 2007). In this present cultivar of *Allium*, we observed that the introduction of GA₃ had significant influence on embryo maturation and germination (Table 4.6.1). On GA₃ (0.5 mgL⁻¹) added medium, somatic embryos grew in size and synthesized photosynthetic pigment, an early
indicator of being autotrophic identity. These pigments may help to improve photosynthetic ability, extra storage reserves like lipid, triglycerides, protein and other important carbohydrates which favour fast \textit{in vitro} germination.

The germination of somatic embryos has been determined by the quality of embryos. The morphology and biochemical reserves which are very similar to zygotic embryos generally produce normal plantlets. In our studied material, some of the induced embryos had ‘nearly normal’ morphology with stunted hypocotyls and developed shooty end, but these types of embryos showed poor rate of embryo germination. The quality of embryos, was however, improved on addition of various carbohydrates, like sucrose, maltose, glucose and fructose.

In this variety of \textit{Allium sativum}, the maturation and germination of somatic embryos were repressed by 3-6% glucose, 6% each of sucrose, maltose, and fructose; but application of fructose, maltose and sucrose at 3% individually were very effective (Table 4.6.2). Similar observations were also observed in other plants where rapid somatic embryo maturation with 3% sucrose or maltose was noted (Corredoria et al., 2003). In several woody species, addition of maltose in culture induced fast embryo maturation (Druart, 1990; Alemanno et al., 1997; Li et al., 1998). Although the relationship of maltose-enriched medium with early embryo maturation has not been established yet, it somehow caused low hydrolysis (Norgaard, 1997) or it may induce a nutritional stress, which eventually improved embryo germination (Blanc et al., 1999). In \textit{Catharanthus} L. G. Don (Apocynaceae), 3% fructose also showed a positive influence on maturation and germination of the cultured somatic embryos (Junaid et al., 2006). A similar mechanism might have played a role during maturation and subsequent germination of somatic embryos in the case of \textit{A. sativum} L. It seems, therefore, that in the present investigated variety, PGRs and carbon sources (3% each of fructose, maltose and sucrose) influenced embryogenesis by improving embryo size through cellular expansion and by extra accumulation of storage products as was earlier reported during embryo maturation (Merkle, 1995). Variation in embryo size (length) could therefore, be considered a good marker for monitoring embryo maturation process. Maturation treatments that produced larger embryos not always yielded maximum plant recovery. The high and consistent embryogenic ability may, however, suggest a stable embryogenic cell line, which could effectively be used in
transgenic programme. Similarly, the regeneration methods established here may be used for fast \textit{in vitro} propagation and cryopreservation purposes.

Lowering of growth regulator concentrations in culture media has improved embryo development and germination in many medicinal plants (Wakhlu et al., 1990). Germination of the somatic embryos is achievable on MS medium without growth regulator (Wakhlu et al., 1990). However, Arumugam and Bhojwani (1990) noted that inclusion of BAP (2 \( \mu \)M) and gibberellic acid (GA\(_3\), 2.8 \( \mu \)M) in the medium stimulated embryo development in \textit{Podophyllum hexandrum}, although 75\% of the embryos germinated on basal MS medium devoid of growth regulator. Wakhlu et al. (1990) reported that the somatic embryos of \textit{Bunium persicum} matured and germinated on the basal MS medium supplemented with 1.0 mgL\(^{-1}\) Kn. Further, Kunitake and Mii (1997) reported that 30 to 40\% of somatic embryos of \textit{A. officinalis} germinated after being treated with distilled water for a week; they were subsequently transferred to half-strength MS medium supplemented with 1.0 mgL\(^{-1}\) IAA, 1.0 mgL\(^{-1}\) GA\(_3\) and 1\% sucrose.

In the present study, up to 83\% of the embryogenic callus formed globular somatic embryos (Table 4.5.1) after two months on embryogenic callus medium. The best combinations of growth regulators were (0.25 mgL\(^{-1}\) BAP + 1.00 mgL\(^{-1}\) 2,4-D) for indirect embryogenesis in terms of both embryogenic events and embryo numbers. Embryogenesis could happen in the absence or at low doses of 2,4-D and BAP. However, any combination of both (2,4-D and BAP) enhanced the regeneration process and this is called cumulative effect of growth hormones. Callus derived from basal part of clove explant produced a significantly higher number of globular embryos compared with callus from other segments.

The percentage of conversion of somatic embryos into plants, when added with BAP, is given in Table 4.7.1. The globular embryos subsequently developed a shoot and root apex, and formed a complete plantlet within five weeks (Plate 4.9). In the absence of BAP, the frequency of conversion was rather low (8.14\%). The addition of BAP (0.5 mgL\(^{-1}\)) increased conversion by up to 30.63\%. With 0 to 0.5 mgL\(^{-1}\) BAP, the plants seemed to show a normal development, but higher concentrations (2 mgL\(^{-1}\)) promoted abnormalities such as curled and dark green leaves.
or multiple shooting. Normal plants were transferred to plant growth medium and sub-cultured at regular interval of five-weeks.

5.3 SELENIUM TREATMENT

Selenium taken up by plants can be incorporated into their biological systems to substitute sulphur, although it is not essential for plant growth and development (Kapoor et al., 2012). The toxicity of Se can be attributed to its pro-oxidative effects, as well as to metabolic disturbances (Hartikainen et al., 2000). A higher Se dosage (1.0 mg kg⁻¹ soil) proves toxic to plants thereby inducing reduction in yield, affecting primary branching and seed production. On the other hand, its activity as an antioxidant, inhibiting lipid peroxidation in ryegrass at concentrations 0.1 and 1.0 mg Se (Hartikainen et al., 2000) and increased yield in pumpkin (Cucurbita pepo) at a concentration 1.5 mg L⁻¹ have also been reported. It can also delay senescence and promote the growth of ageing seedlings by preventing the reduction of tocopherol concentration and by enhancing superoxide dismutase (SOD) activity (Xue et al., 2001).

The medicinal properties of garlic have been attributed primarily to the presence of alliin [alk(en)yl cysteine sulphoxide (CSO)] and allicin (Jones et al., 2007; Nasim et al. 2010). The sulphur supply in growing medium enhanced the production of metabolite in garlic (Nasim et al., 2010). Since garlic has the ability to take Se from its immediate surrounding medium and incorporate high concentrations of Se into amino acids and phytochemicals (Arnault et al., 2003), so attempt has been made to explore the effects of Se on metabolite production and other associated aspects (like amino acid, protein, proline and antioxidant enzymes) in various tissues and organs of garlic. These observations in present investigations have been discussed in terms of cause and effect relationships under major headings as follows.

Effect of Se supplementation on callusing

Selenium treatment showed significantly increased callusing with increasing selenium concentration except at concentration of 4 mgL⁻¹ (Table 4.8.1). The induction of callusing was significantly higher at Se supply up to 2 mgL⁻¹ (88.59 %). Garlic has the ability to readily uptake Se from immediate medium and incorporates it into amino acids. Se uptake by garlic produces various seleno-derivatives, enzymes
that accelerate callus formation (Arnault et al., 2003; Nasim et al., 2010). It is supposed that like S, Se input improves quality of organoseleno-enzymes or increase production of seleno intermediates acting as precursor for callus production. This is in accordance with earlier observations where addition of low concentrations of Se to medium induced callusing when compared to control. However, high concentrations of selenate are toxic and cause declination of glutathione peroxidase activity which is responsible for callus formation in ryegrass (Hartikainen et al., 2000). Further, Hartikainen et al. (2000) showed that selenite was more efficient than selenate for causing increase in glutathione peroxidase activity in ryegrass. This is in accordance with our observation where at higher concentrations, selenium significantly reduced callus formation (58.27 %) (Table 4.8.1).

**Effect of Se supplementation on induction of somatic embryogenesis**

Plant growth regulators play a key role in inducing somatic embryogenesis. There are many other factors that have been found to affect the disposition of a particular tissue to undergo somatic embryogenesis. The range of possible induction treatments suggests that it is unlikely that a single inducing molecule is responsible. Other factors that can direct the transition from somatic cells to cells able to form embryo-like structures are: in *Citrus* suspension cultures a change in carbon source from sucrose to glycerol (Jiménez & Guevara, 1996); in carrot the NH$_4^+$ concentration (Smith & Krikorian, 1989) and pH changes (Smith & Krikorian, 1992) in the culture medium; in *Brassica* microspores a temperature shock (Pechan & Keller, 1988); also pre-treatment of donor plants and subculture duration (Morocz et al., 1990), to name only a few factors. A more detailed description of some of these factors was given by Harada (1999). In our study, somatic embryogenesis increased significantly with increasing selenium concentration in medium except at concentration of 4 mgL$^{-1}$ (Table 4.8.2). The reason may be enhancement of selenocysteine methyltransferase enzyme which accelerates embryo formation on embryogenic callus by Se uptake at low concentrations in garlic.

**Effect of selenium on dry weight of tissues and organs**

In our study, the selenium treated garlic showed higher biomass in terms of dry weight than untreated ones in *in vitro* conditions (Graphs-4.1-4.4). The enhancement was significant at Se treatment of 2 mg L$^{-1}$. Earlier studies demonstrated
that the application of 0.5 mg kg\(^{-1}\) selenium stimulated growth and dry matter yield of Indian mustard (Singh et al., 1980). Selenium applied at low concentrations enhanced growth and antioxidative capacity of both mono- and dicotyledonous plants. The growth-promoting response to Se was demonstrated in lettuce and ryegrass (*Lolium perenne* L.) (Hartikainen et al., 2000). Pennanen et al. (2002) also observed Se induced starch accumulation in chloroplasts of young leaves. A higher Se dosage (1.0 mg kg\(^{-1}\) soil) was toxic to lettuce and reduced the yield of young plants. In senescing plants, it diminished the dry weight yield but not the fresh weight yield (Xue et al., 2001). These findings coincide with our present work in which higher concentrations of Se reduced dry weight in various *in vitro* raised tissues and explants (Graphs-4.1, 4.2, 4.3, 4.4).

**Effect of Se supplementation on biochemical parameters**

**Protein and Amino acid content**

Effect of different concentrations of Se on amino acid and protein contents in the various *in vitro* raised tissues and explants is shown in Table 4.8.3 and Table 4.8.4 respectively. Selenium treatment significantly enhanced amino acid and protein contents in tissues grown under *in vitro* conditions. The amino acid and protein contents in all tissues and explants increased significantly with Se supply up to 2 mgL\(^{-1}\) and slightly decreased thereafter. Selenium enters plants and is assimilated through the sulfur (S) pathway and there it gets incorporated into the amino acids. These amino acids are then utilized by the plants as normal components of proteins or acting as precursor for protein production. So Se at optimum level increased the total protein and amino acid contents (Kapoor et al., 2012). Earlier studies demonstrated that addition of low concentrations of Se to the soil induced increased levels of all detectable amino acids in broccoli, tea and *Eruca sativa* when compared to the control (Lee et al., 2005).

**Soluble sugar content**

Effect of different concentrations of Se on total sugar content in various *in vitro* raised tissues and explants of garlic is shown in Table 4.8.6. Selenium treatment significantly enhanced the sugar content in tissues grown under *in vitro* conditions. The sugar content in all tissues and explants increased significantly with Se supply up
to 2 mg L\(^{-1}\) and significantly decreased thereafter. The mechanism through which Se acts on carbohydrate metabolism of plants is not fully understood. Pennanen et al. (2002) indicated that plant growth promoted by Se is the result of increased starch accumulation in chloroplasts. It was shown that Se also has positive effects on potato carbohydrate accumulation (Turakainen et al., 2004). The positive effects of Se on the recovery of potato from photooxidative and paraquat-generated oxidative stress point to mechanisms that, although unknown, protect chloroplasts during stress (Seppänen et al., 2003). It was shown that Se has the ability to regulate the water status of plants under conditions of drought (Kuznetsov et al., 2003) and that the protective effect of Se under drought stress conditions was achieved by increasing the water uptake capacity of the root system (Kuznetsov et al., 2003). The authors concluded that Se-induced increase in growth was related to the inducible role of Se in chloroplast enzymes and carbohydrate metabolism.

**Proline content**

Comparison of proline contents in various *in vitro* raised tissue and explants is shown in Table 4.8.5. Proline content noted increase in all Se treated garlic tissues and plantlets but results were significantly higher at 4 mg L\(^{-1}\) Se treatment (Table 4.8.5). Proline has been shown to play an important role in recovering from environmental stresses in plants and its accumulation might be induced as a result of reactive oxygen species (ROS) formation. The mechanisms by which proline reduce oxidative damage include physical quenching of singlet oxygen and chemical reaction with hydroxyl radicals (Salter & Hewitt, 1992). Due to the chelating ability, proline also functions as a defense mechanism for survival of stressed plants by binding with metal ions. Increase in proline contents with increasing Se concentration is indicative of free radical scavenging (Wi et al., 2007).

**Antioxidative enzyme activity**

Enzymatic antioxidant system is one of the ways to resist the oxidative damage caused by abiotic stresses (Mittler, 2002). Enzymes such as SOD, CAT, GR, APX have a physiological function under non-stressed conditions, but their activity or quantity is increased under oxidative damage. However, the role of antioxidant systems in detoxification of Se is still not clear. Activity of CAT noted an increase with increasing Se treatment but the highest activity was noted in *in vitro* raised tissue.
and explants exposed to 4 mgL\(^{-1}\) Se (Table 4.8.9). Our results demonstrated that Se toxicity mediated changes in the activities of antioxidant enzymes are dose dependent. Increasing the activities of CAT, which are scavengers of H\(_2\)O\(_2\), might result in reduced formation of superoxide anion radicals (Wi et al., 2007). Higher Se levels were also reported to induce the activity of CAT in *Spirulina platensis* (Mittler, 2002). In contrast, no significant alteration was observed in SOD activity at all Se levels (Table 4.8.7), except at a treatment of 4 mgL\(^{-1}\) Se. Leaves of barley seedlings treated with Se have also been reported to show no significant change in total SOD activity (Wi et al., 2007). The enhanced activities of SOD have been recorded in selenate subjected plants.

The GR activity increased in a dose dependent manner (Table 4.8.8) with a slight increase in all tissues up to a treatment of 2 mgL\(^{-1}\) Se and a significant increase thereafter. Leaves of barley seedlings treated with Se also showed significantly higher activities of GR (Wi et al., 2007). Stimulation of GSH synthesis by oxidative stress is reported by Yamane et al., 2009. High concentrations of Se show interference in GSH synthesis in plants, thus diminishing plant defense against hydroxyl radicals and oxidative stress.

Increase in APX activity when exposed to sodium selenite supports our observations in which APX activity increased in a dose-dependent trend (Table 4.8.10). In all *in vitro* raised tissues and plantlets, the APX activity increased slightly up to a Se treatment of 2 mgL\(^{-1}\) beyond which a significant increase was noted.

**Alliin content**

Selenium treatment increased alliin production in tissues and explants grown under *in vitro* conditions except at concentration of 4 mgL\(^{-1}\) (Table 4.8.11). The concentrations of alliin were significantly higher in all tissues and explants at Se supply up to 2 mgL\(^{-1}\). The highest content of 3.68 \(\mu\)g g\(^{-1}\) dry wt. was recorded in leaf. Earlier studies showed that leaves of plants treated with S showed higher alliin content (Nasim et al., 2009a). Garlic has ability to readily uptake Se from the soil and incorporates it into amino acids. Se uptake by garlic produces various seleno-containing compounds and selenoamino acid derivatives, including Se-methyl-L-selenocysteine (MeSeCys) and \(\gamma\)-glutamylmethylselenocysteine (GluMeSeCys). These selenoamino acids and seleno proteins are involved in cancer prevention.
especially against mammary and prostate tumors, as well as leukemia (Nasim et al., 2009a). It is supposed that like S, Se input improves quality of organo-seleno compounds or increase production of seleno intermediates acting as precursor for metabolite production. These Se compounds have superior anticancer properties than their corresponding S analogues (Kapoor et al., 2012). The production of alliin depends on availability of cysteine as it plays a vital role in activating enzymes such as cysteine synthase or glutathione transferase that are involved in alliin synthesis (Jones et al., 2007). It has been reported that Se incorporation into garlic increases the bioactivities of garlic (Kapoor et al., 2012).

5.4 GAMMA IRRADIATION

Effect of gamma radiations on garlic

In the last decade, gamma-irradiation has drawn attention as a new and rapid method to improve the qualitative and quantitative characters of many crops. Gamma irradiation has been widely applied in medicine and biology in terms of biological effects induced by a counter intuitive switch-over from low doses stimulation to high-doses inhibition (Wi et al., 2005). Previous studies have shown that relatively low-doses of ionizing radiations on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Wi et al., 2005; Jan et al., 2012). In vitro mutagenesis is a combination of in vitro culture and mutation induction which provides the opportunity to increase variability in an economically important cultivar or used on plants in developing varieties that are agriculturally important and have high productivity potential (Jain et al., 1998). Traits induced by mutagenesis include plant size, blooming time and fruit ripening, fruit colors, self-compatibility, self-thinning, resistance to pathogens and it is also known to increase nutritional values of food sources and enhance and accelerate growth of certain vegetables (Jan et al., 2012). Induced mutation technique is a valuable tool but not yet fully exploited in fruit breeding (Maity et al., 2004). Tissue culture makes it more efficient by allowing the handling of large populations and by increasing mutation induction efficiency, possibility of mutant recovery and speediness of cloning selected variants (Barros et al., 2002).
However, the low dose irradiation did not cause changes in the ultra structure of chloroplasts. Irradiation of plants with high doses of gamma rays disturbed the synthesis of protein, hormone balance, leaf gas-exchange, water exchange and enzyme activity (Esfandiari et al., 2008). The salient findings obtained during the present investigation are discussed in the following paragraphs in relation to available literature. Following variable doses of the gamma rays, the overall effects experienced by the plant has been assessed in terms of growth, development, synergistic interaction between metabolites and physiological, morphological and biochemical analysis of the model plant. Attempts have been made to examine, evaluate and demonstrate the findings of the present investigation recorded in the course of time in dose and response relationship and to draw valid conclusion.

**Radiation sensitivity test**

Radiation sensitivity test is a prerequisite step before the mutagenic treatment is started. The main purpose of this test is to investigate the most effective dosage of irradiation to be used and also to estimate the frequency and mutation spectrum using gamma irradiation. The results in the present study for radiation sensitivity test based on germination percentage of irradiated and non-irradiated garlic demonstrated that significant reduction in germination percentage was observed with increasing gamma dosage under both *in-vivo* & *in-vitro* conditions. These results are in accordance with the radiation sensitivity test done by Kim et al. (2000) whereby increasing gamma dosages decreased the germination percentage of tomato and okra. The inhibition of seed germination at higher doses of radiation may have resulted from damage to chromosomes and subsequent mitotic retardation (Inoue et al., 1975; Wi et al., 2006; Jan et al., 2012) or the reduction in garlic germination with gamma irradiation might be due to the increase in production of active radicals responsible for seed lethality. These findings are also in accordance with those reported by many researchers such as Al-Jassir, 1992 in *Allium sativum* L.; Zeerak et al., 1994; Graham & Stevensen, 1997 in potatoes; Osama, 2002 in tomato, Kovacs & Keresztes, 2002 in wheat. Based on the sensitivity to radiations, a gamma dosage range between 1-4 Gy was selected for the present study.
Effect of low doses on callusing

The effect of low doses of gamma radiations on callusing is presented in Table 4.9.1. There was a significant ($p<0.05$) increase in callus induction with increase in doses of gamma radiation at optimum PGR treatments (Table 4.9.1). Under *in vitro* conditions, induction frequency in our study showed a direct co-relation with increase in doses of gamma radiation. The maximum enhancement in callus induction was observed with an absorbed dose of 2 Gy (86.65%). However, a dose dependent reduction was observed with an absorbed dose of 4 Gy (59.63%) (Table 4.9.1). The change in $\gamma$-irradiated tissues may be due to acquisition of the capacity to synthesize hormones. Studies also showed that callus exposed to $\gamma$-irradiation acquired new capacities for the synthesis of cell division factors (Wi et al., 2005). The gamma rays application at low doses has been reported to result in biosynthesis of endogenous phytohormones (e.g. indole acetic acid, gibberellic acid, cytokinins) and nitrogenous compounds (Jan et al., 2012). Another study (Elliot et al., 1987) has also demonstrated that a critical endogenous level of growth regulators has to be attained before cell division and organogenesis could occur. It is reasonably conjectured that gamma ray could stimulate callus regeneration by some mechanism such as activation of retro-transposon (Hirochika et al., 2002). These observations demonstrate that mutations have probably taken place in garlic tissues due to gamma irradiation. Apart from the dose effects, the responses are controlled by a number of parameters, including the genotype, the type of explant, the orientation of the explant on the culture medium and the origin of the explant from the mother plant (Douglas, 1985).

Effect of low doses on somatic embryo induction

Somatic embryos are formed by embryogenic cells, which arise from somatic cells of an explant, callus or suspension cells (Robichaud, 2004). It has become widely recognized that somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli, generally called stress factors. Osmotic pressure applied in an induction medium was reported to stimulate somatic embryogenesis in explants of *P. ginseng* (Choi et al., 1998). Stress factors that can stimulate morphogenic process under *in vitro* conditions are also induced by mutagens. There is increase in somatic embryogenesis with increase in doses of gamma radiation at optimum PGR treatments as observed in our study (Table 4.9.2).
Maximum embryogenesis was observed at a dose treatment of 2 Gy (85.80%) which declined thereafter. This may be the stimulatory effect of \( \gamma \)-irradiation on embryogenesis as was reported earlier in anther or microspore culture (Maluszynski et al., 1996) and in somatic tissue cultures. Similarly, \( \gamma \)-radiation stimulated somatic embryogenesis in culture of *H. annuus* immature zygote embryos (Wi et al., 2005). All stress factors induce a common reaction of somatic cells manifested by their de-and re-differentiation to somatic embryos. However, the stimulatory effect of stress treatment on cell differentiation and morphogenesis remain elusive. Whatever the detail of mechanisms is, stress treatment triggers expression of factor(s), which effect gene expression and cell cycle regulation and thus induce somatic embryogenesis. Identified factors controlling stress specific response genes (Aarts & Fiers, 2003) can help to elucidate the stress response in plants and its relation to embryogenesis. The increasing \( \gamma \)-radiation doses induce decrease in callus fresh weight and regeneration percentage. These results are in accordance with those obtained in tomato (Locy, 1995) and sweet potato (El-Fiki, 2004).

**Effect of low doses on dry weight of tissues and organs**

The effect of low doses of radiation on dry weight of various tissues and organs is shown in Figure 4.5, 4.6, 4.7, 4.8. *In vitro* grown garlic leaves and plantlets showed highest dry weights in comparison to other plant tissues and explants tested (Figure 4.6). The metabolites are synthesized primarily in leaves and translocated to clove during bulb development (Nasim et al., 2010). The mechanism by which *in vitro* grown tissues or plantlets overproduce metabolites and other compounds is unknown (Nasim et al., 2010). It has been reported that synthesis of vital compounds is controlled by several factors (Moreno et al., 1993, Nasim et al., 2010). When irradiated with different doses of gamma radiation (0-4 Gy), it was observed that in all *in vitro* grown tissues and organs, weight increased up to a dose of 2 Gy, and on further increase to 4 Gy, a decline in the dry weight of tissues and plant organs was observed (Figures 4.5, 4.6, 4.7, 4.8). The increase in weight of tissues/organs at low doses of gamma rays has also been reported by workers in bean, orange and carrot (Al-Safadi & Simon, 1990; 1995). A reduction in weight at higher doses as a result of gamma irradiation has also been reported by Bajaj (1973). This reduction in fresh weight may be caused by the reduced amount of endogenous growth regulators.
especially the cytokinins, as a result of breakdown, or lack of synthesis due to irradiation.

**Effect of low doses on biochemical parameters**

**Total soluble protein**

In this study, it was found that there was an irregular distribution of total soluble protein content in irradiated tissues, organs and plantlets under *in vitro* grown conditions (Table 4.9.3). According to the results obtained in the present study, it was observed that absorbed doses (1 Gy and 2 Gy) displayed increased total soluble protein content (Table 4.9.3), which is in accordance with Pradeep et al. (1993) who observed that gamma irradiation did not induce significant loss in water soluble components such as total soluble proteins. Highest protein content of 7.18 mg g\(^{-1}\) f.wt. in leaf and 10.12 mg g\(^{-1}\) f.wt. in complete plantlet at gamma irradiation with 2 Gy was noted in the present study. Humera and Iqbal, 2010 stated that the stress reaction of plants often results in the alteration of protein metabolism. Several proteins are synthesised and accumulated in plant tissues under a range of stress conditions. Such proteins, referred to as stress proteins, have been noted to be induced in response to several stress factors. The most crucial function of plant cell is to respond to gamma stress by developing defense mechanisms. This defense was brought about by alteration in the pattern of gene expression (Salter & Hewitt, 1992). This led to modulation of certain metabolic and defensive pathways (Riley, 1994). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein contents were obvious (Salter & Hewitt, 1992). These proteins might play a role in signal transduction, antioxidative defense, antifreezing, heat shock, metal binding, antipathogenesis or osmolyte synthesis which were essential to a plant’s function and growth (Wi et al., 2005). Gamma irradiation significantly influences the cell metabolism and protein synthesis in plant meristem (Stajner et al., 2007). Inoue et al. (1975) reported that irradiation caused a slight increase in total soluble protein content in soyabean as compared to the non-irradiated seeds. Also, quantity of carbonyl groups in oxidatively modified proteins significantly increased. Introduction of carbonyl groups into amino acid residues of proteins was a hallmark for oxidative modification due to gamma rays exposure (Variyar et al., 1998; Stajner et al., 2007).
Total amino acid content

In vitro grown garlic leaves and plantlets showed highest total amino acid content in comparison to other plant tissues and explants tested (Table 4.9.4). The amino acid content in samples irradiated with 2 Gy was significantly higher than that of control samples (Table 4.9.4). The highest amino acid content was observed in in vitro grown leaves (6.92 mg g⁻¹ f.wt.) and plantlets (7.80 mg g⁻¹ f.wt.) at 2 Gy gamma irradiation. The results of this research show that different doses of gamma radiation have different effects on plants’ biochemical characteristics, such as increase in total soluble protein and total soluble amino acids and proline content. It is clear that this technique can be used for production of mutants with tolerance to environmental stress. The effect of ionizing radiation on total amino acid content depends on various factors, such as sensitivity of the exposed system, the type of particular functional tissue and even other conditions, such as aqueous soaking after irradiation, as reported by Siddhuraju et al. (2002). The presence of amino acids especially proline may play a role in protection from desiccation and from the harmful effects of solute accumulation. However, proline can act as an osmo-protector of cytosolic enzymes and cellular structures (Barros et al., 2002). Proline accumulation may help the plant to survive short periods of drought and recover from stress. Besides, high proline concentration measured in sludge-treated nodules could also contribute to a protective role as scavenger of ROS (Tubiana, 2008; Lee et al., 2009). All these factors could result in improved adaptation ability and growth of plants under ionizing radiation conditions.

Total soluble sugar

Soluble sugar can improve stability of soluble proteins. As shown in Table 4.9.6, the increase in gamma irradiation treatments reflected a high increase in total soluble sugars. Soluble sugar content significantly increased on irradiation with doses up to 2 Gy. Our results indicate that total amino acids and soluble sugar are significant contributors to metabolism under gamma irradiation stress. This is in agreement with those of Nassar et al. (2004) and Moussa (2011) who reported an increase in carbohydrates and soluble sugars in response to plant irradiation. The increase in total soluble sugars might be attributed to degradation of starch fractions (Salter & Hewitt, 1992). There is no doubt that total soluble sugars exert a positive role in the
alleviation of the imposed stress via osmotic adjustment in plants (Lee et al., 2009). Some of the soluble sugars accumulate in stressed cells to maintain membrane phospholipids in the liquid crystalline phase and to prevent structural changes in soluble proteins, while others play a key role in stress induced metabolic processes (Kerepesi & Galiba, 2000). Substantial osmotic adjustment was observed in adapted soybean cell suspension cultures exposed to water stress, mainly due to increased glucose, fructose and sucrose (Wi et al., 2005). Although the content of total soluble sugars changed insignificantly by the application of gamma-irradiation alone, it increased in much pronounced levels in response to a combined stress of irradiation and drought than in response to drought stress alone. This behavior confirms that increasing osmolyte/osmoprotectant contents may come as another defensive mechanism via the activation of genes responsible for the expression of the enzymes involved in the accumulation of these osmolytes, which is caused by pre-exposing to gamma rays, leading to more protection for the upregulating enzymes involved in the anabolism of these contents (Wi et al., 2005).

**Total proline content**

In the present study, proline content in *in vitro* tissues and organs showed an unwavering significant increase with increasing doses of gamma radiations (Table 4.9.5) which is in accordance with the findings of Esfandiari et al., 2008. Gamma radiations have been reported to induce oxidative stress with overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, which react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids causing disturbance of cellular metabolism (Al-Rumaih & Al-Rumaih, 2008; Ashraf, 2009; Noreen & Ashraf, 2009). To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of reactive oxygen species in cellular compartments (Kiong et al., 2008). This defense was brought about by alteration in the pattern of gene expression. This led to modulation of certain metabolic and defensive pathways. One of the protective mechanisms is the synthesis of osmolytes which is essential to plant growth - proline (Esfandiari et al., 2008). The results of this study show an increase in proline content in all irradiated tissues and plantlets. It has been reported that osmolyte synthesis such as proline involved in protective mechanisms is altered by several environmental stresses, including gamma irradiation.
(Al-Rumaih & Al-Rumaih, 2008). Proline is a compatible osmolyte and it may interact with enzymes to preserve enzyme structure and activities. Indeed, proline has been shown \textit{in vitro} to reduce enzyme denaturation caused due to heat, NaCl stress, gamma stress etc. (Kavi Kishor et al., 2005). In the present study, a significant increase in proline content was observed at a gamma dose of 4 Gy in all tissues and organs (highest content of 2.87 mg g\textsuperscript{-1} f.wt. in leaf and 3.95 mg g\textsuperscript{-1} f.wt. in plantlet). The increase in proline content has been reported to cope with the problem of oxidative reagents. However, Falahati et al., 2007 contradicted this statement by proposing that the radiations may have promoted the level of antioxidants and consequently there would be no need for extra amount of proline to cope with the same problem of oxidative reagents. The results of this research showed that different doses of gamma radiation has different effects on biochemical plant characteristics, such as increase in proline content, stimulation of germination and seedling growth. It is clear that this technique can be used for production of a mutant with ability for environmental stress tolerance.

\textbf{Antioxidative enzyme activity}

Activities of the antioxidant enzymes SOD, GR, CAT and APX were significantly (p<0.05) stimulated by irradiation treatments in garlic tissues and organs and this stimulation reached its maximum at dose level of 4 Gy (Table 4.9.7, 4.9.8, 4.9.9, 4.9.10). The irradiated tissues (callus, embryos, leaf, root and plantlets) exhibited significant increase in APX activities at the highest irradiation dose of 4 Gy. CAT activity in the irradiated tissues also showed significant increase at a dose of 4 Gy as compared to the control tissues. The SOD activity also showed a positive correlation with the increasing dose levels of gamma-radiation. The highest specific activity of SOD was recorded at a gamma dose of 4 Gy. Similarly, the gamma-irradiation treatment at 4 Gy doubled the GR activity in all tissues as compared to the untreated cultures. This change in the enzyme activity is a strong hint that irradiation treatments actually led to oxidative stress. Several reports with other plants provided evidence of enhanced activities of APX, SOD, CAT and GR by gamma irradiation treatments (Foyer, 1994).

Gamma irradiation was shown to induce oxidative stress with overproduction of reactive oxygen species (Kim et al., 2004). Generation of ROS, particularly H\textsubscript{2}O\textsubscript{2}
had been proposed to be part of the signaling cascades that lead to protection from stresses. Induction of antioxidant enzyme activities was reported to be a general strategy adopted by plants to overcome oxidative stresses (Foyer et al., 1994).

The antioxidant enzymes APX, CAT, SOD and GR function as effective quenchers for ROS and their level may also determine the sensitivity of plants to lipid peroxidation (Pramanik, 1997). Catalases (CAT) and peroxidases (POD) play an essential role in scavenging \( \text{H}_2\text{O}_2 \) toxicity. The combined action of CAT and SOD converts the toxic superoxide radical (\( \text{O}_2^- \)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) to water and molecular oxygen (\( \text{O}_2 \)), thus averting the cellular damage under unfavorable conditions (Tubiana, 2008). Superoxide dismutase is an essential component (coenzyme) in plants and represents an antioxidative defense system since it transforms hydrogen peroxide to water and oxygen (Wada et al., 1998).

Riley (1994) associated the oxidative bursts as well as the dramatic changes in the activities of various antioxidant defenses with the alteration in gene expression in a variety of tissues from phylogenetically diverse organisms. Moreover, Tubiana (2008) found that the expression patterns of GR, SOD, POD and CAT genes exhibited increased transcripts with irradiation of \textit{Nicotiana tabacum}. This over expression probably occurs by an efficient regulatory mechanism, while adjusting necessary enzyme expression by positive regulation of the corresponding genes to provide cells with resistance (Zaka et al., 2002). Wada et al. (1998) reported that the stimulation in SOD activity in response to stresses is possibly attributed to the \textit{de novo} synthesis of the enzymatic proteins. The results obtained in the present study also demonstrate clearly that CAT and SOD protect plant cells from the destructive effects of reactive oxygen species and constitute key components of the cellular antioxidant defense systems. Thus the results of this study suggest that APX, CAT and GR activities along with SOD activity play an important protective role in the \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) scavenging process.

\textbf{Alliin content}

Radiation treatment increased alliin production in all tissues and explants grown under \textit{in vitro} conditions in comparison to the control (Table 4.9.11). The increase in alliin content was more prominent at radiation dose of 2 and 4 Gy in all tissues and explants (Table 4.9.11). Our results corroborate with the findings of Lee et
al. (2009). They reported increase in metabolite contents such as total phenols, total flavonoids by increasing radiation in plants. Such increase in total phenols and total flavonoids is due to the release of phenolic compounds from glycosidic components and the degradation of larger phenolic compounds into smaller ones by gamma irradiation as suggested by Wi et al. (2005). Irradiation exerts its effects as direct and indirect mechanisms. In case of indirect mechanism, radiolysis of water results in the production of free radicals such as hydroxyl radicals, hydroperoxide radicals and hydrated electrons. These radicals may break the glycosidic bonds of procyanidin trimer, tetramer and hexamer that are present in plants, leading to the formation of procyanidin monomers, which increase the total phenolic and total flavonoids content in irradiated plants (Lee et al., 2009). In plant tissues, many phenolic compounds are potential antioxidants: flavonoids, tannins and lignin precursors may act as ROS (reactive oxygen species) scavenging compounds. The observed increase in total phenolic and tannin content was beneficial as antioxidant properties of irradiated soybean seeds due to polymerization of phenolic constituents and cross-linking and fragmentation, which were the key reactions controlling the properties of macromolecules such as proteins (Tagawa et al., 2004; Stajner et al., 2007).

5.5 SALT TREATMENT

Salinity is one of the most limiting factors directly responsible for decrease in crop yields. Thus, the increasing demand for agricultural crops requires the development of salt-tolerant plants. In recent years, attempts have been made to develop salt-tolerant plants through tissue culture (Purohit et al., 1998; Singh et al., 2003). This has included using a number of systems (i.e. callus, suspension culture, somatic embryos or shoot culture etc.) to screen for cells and tissues that show variation in their ability to tolerate relatively high levels of NaCl in media (Woodward & Bennett, 2005). In vitro selection of salt tolerant cell lines has been reported for several species (Tal, 1994). Plant tissue culture techniques provide a promising and feasible approach to develop salt tolerant plants in several more species such as wheat (Barakat & Abdel-Latif, 1996).

Reports on the effect of salinity on the metabolic activities and alkaloid contents in medicinal plants are scanty. There is also less information pertaining to garlic grown under saline conditions. Hence attempts were made to develop a reliable in vitro protocol for callus induction, embryogenesis and subsequent plantlet
regeneration, under different plant growth regulator treatments. Each morphogenetic stage was subjected to NaCl treatment in order to assess stress impact on morphogenetic, biochemical and phytochemical processes. The observations in present investigations have been discussed in terms of cause and effect relationships under major headings as follows.

**Effect of salt treatment on callusing**

Salt treatment increased the induction of callusing significantly under *in vitro* conditions except at concentration of 50 mM (Table 4.10.1). The callusing was significantly higher at NaCl supply up to 25 mM (88.93%). Increasing growth of calli in NaCl medium indicated that the tissues were tolerant as reported in rice (Basu et al., 2002). It was reported that rice calli selected for salt tolerance maintained high viability and regrowth capacity in the presence of NaCl in comparison with the non-selected calli. Our results are in agreement with those reported in *Catharanthus roseus* suspension cells adapted to 50 mM NaCl (Junaid et al., 2006). A long-term culture in 68 mM NaCl medium enabled the tissue to accumulate more Cl⁻ in comparison to the non-selected tissue while both selected and non-selected tissues accumulated same quantity of Na⁺ in NaCl free medium. This suggests that gradual adaptation of tissue in response to repeated culture in saline medium is directly related to Cl⁻ uptake. Selected calli accumulated more proline and soluble sugars than the non-selected calli in the absence of stress. Perhaps Cl⁻ ions were sequestrated in vacuoles and organic solutes accumulated in cytoplasm as osmolytes. However, our result indicated that induction of callus declined significantly at 50 mM (63.35%). A possible explanation for the decrease in callusing at increasing treatments of salt could be the loss of organogenic potential which may be due to increased osmotic potential of the saline medium. A higher osmotic potential affects water and nutrient uptake, which may in turn inhibit the metabolic activities necessary for bud initiation and growth (Mukhtar et al., 2008).

**Effect of salt treatment on embryo induction**

In the present study, the percentage induction of somatic embryos increased significantly up to NaCl supply of 25 mM NaCl at optimum PGR in medium (85.37%) (Table 4.10.2). However, on further increasing the concentration of NaCl to 50 mM, intensity of embryogenesis reduced significantly (69.71%) (Table 4.10.2).
Almost similar observation was reported in rice (Binh & Heszky, 1990), *Cymbopogon martinii* (Patnaik & Debata, 1997) and *Dendrocalamus strictus* (Singh et al., 2003). Torpedo stage somatic embryos developed on the medium devoid of NaCl showed worse germination in comparison to those developed in the presence of NaCl. It suggests that the salt tolerance could be retained by somatic embryos due to the development on salt conditions. Similar observations have also been made by Patnaik and Debata (1997) in *C. martini* and Singh et al. (2003) in *Dendrocalamus strictus*.

**Effect of salt treatment on dry weight of tissues and organs**

The effect of salt stress on biomass of garlic tissues and plantlets was found to be directly related to its concentration and exposure time (Figure 4.9, 4.10, 4.11, 4.12). In the present investigation, biomass increased significantly with increased salt concentration in the medium. Our results are in agreement with Amor et al. (2005) and Mukhtar et al. (2008). It has been reported that the fresh weight of explants increased up to the moderate salt concentration which probably increased water uptake from the medium, diluting the concentration of toxic ions within the tissues (Mukhtar et al., 2008). In our study, the lower weight of explants at highest salinity level of 50 mM could probably be attributed to the reduced capacity of dilution, when the toxic ion levels reached a point where plant metabolism was being severely affected by toxicity. The decrease in growth characters mentioned before may be due to the inhibitory effect of salt stress on protein accumulation as reported by Abd-El-Baki et al. (2000) in *Zea mays*. The obtained results of growth characters coincided with the results obtained by many investigators (Javed et al., 2003; Tester & Davenport, 2003; Aziz & Taalab, 2004). Such stimulation in weight was also recorded in the vegetative parts of flowering plants in the halophytes and succulent plants after being subjected to salinity treatments (Gale et al., 1970; Winter, 1974).

**Effect of salt treatment on biochemical parameters**

**Total soluble protein**

There was a significant linear increase in soluble protein content with NaCl supply up to 25 mM at optimum PGR concentrations in all tissues, organs and plantlets (Table 4.10.3). These findings are in agreement with earlier reports on salt
tolerant barley cultivars by Hurkman et al. (1989) and rice (Pareek et al. (1997)). The increased protein content has been reported by El-Baze et al. (2003), who suggested that newly synthesized proteins might be due to expression of salt stress related genes to produce salt induced proteins. Reports also suggest that initial response to salt stress involves increased protein synthesis that is prevented when stress becomes too severe. In our investigation, a similar trend was observed and there was a significant decline beyond NaCl supply of 25mM (Table 4.10.3). Protein degradation in higher saline environment has been attributed to the decrease in protein synthesis, accelerated proteolysis, decrease in the availability of amino acid and denaturation of enzymes involved in protein synthesis (Mukhtar et al., 2008).

**Amino acid content**

Soluble amino acid content in tissue culture raised samples gradually increased with increased salt concentrations in the medium (Table 4.10.4). Parida et al. (2004) also reported similar results of increased amino acid pool by salinity in Aegiceras corniculatum. Pattern of changes in proline has also been reported to be opposite to that of amino acid, indicating that the increase in proline content is at the expense of other amino acids through an effect of salinity in promoting their conversion. But our results are contradictory to other works where information on accumulation of amino acids in response to salt stress was reported (Ashraf, 1994). In the present study, amino acid content increased only up to a moderate concentration of NaCl (25 mM) and a significant reduction in amino acid content was noted on further increasing the NaCl concentration to 50 mM in all tissues and organs. However, free amino acids are reported to increase with an increase in NaCl salinity in Catharanthus roseus (Jaleel et al., 2007).

**Total soluble sugar**

Soluble sugar content increased significantly with increase in salt concentration in the medium in all in vitro raised tissues and organs (Table 4.10.6). Maximum increase in sugar content was recorded at a NaCl treatment of 25 mM in in vitro raised leaf (28.25 mg g\(^{-1}\) f. wt.) and plantlets (34.16 mg g\(^{-1}\) f. wt.). Similar findings were reported by Tester and Davenport (2003) in Populus enphratica in vitro.
cultures where maximum enhancement was found in leaves at 25 mM NaCl. An increased trend in total carbohydrate content in the plant cells was observed with increase in NaCl treatment (Mukhtar et al., 2008). Increase in sugar content in in vitro raised leaf and plantlets of garlic could be due to increase in glucose and fructose concentrations when high concentration of NaCl was present in the medium (Hasegawa et al., 2000). Accumulation of organic compounds like sugar in the cytoplasm plays an important role in osmotic adjustment in plants. Accumulation of sugars might make a greater contribution to osmotic adjustment than proline. Francois (1994) reported that the accumulation of sugars in *Populus deltoids* clones might lower the leaf osmotic potential, which would help to maintain turgidity under water stress. Furthermore, it is suggested that the accumulation of proline and sugar may be related to osmotic and saline stress tolerance.

**Total proline content**

Comparison of proline content in various *in vitro* raised tissues and explants under influence of salinity is shown in Table 4.10.5. Proline content noted gradual increase in various tissues of garlic up to NaCl treatment of 25 mM. Results were significantly higher at 50 mM with the maximum proline content recorded in leaf (3.64 mg g⁻¹ f.wt.) and plantlet (5.28 mg g⁻¹ f.wt.). Proline concentration in the shoots of *T. ammi* increased significantly, particularly at the highest external salt level, thus showing the positive role of proline in the salt tolerance of this crop. Proline is known to contribute to membrane stability (Gadallah, 1999), and mitigates the effect of NaCl on cell membrane disruption (Munns, 2002). In addition, proline may act as a signalling/regulatory molecule able to activate multiple responses that are component of the adaptation to abiotic stresses including salt stress. In contrast, Hasegawa et al. (2000) suggested that proline accumulation does not lead to salinity adaptation, but it may be synthesized due to initiation of other responses to salt stress. The results reported here for proline can be explained in view of some earlier reports that proline accumulation is one of the common characteristics in many plants under saline conditions (Dow et al., 1981; Francois, 1994). Increase in proline due to salinity has also been reported in some medicinal plants.
Antioxidative enzyme activity

Activities of the antioxidant enzymes SOD, GR, CAT and APX increased non-significantly with increase in NaCl concentration up to 25 mM and thereafter a significant increase was noted (Table 4.10.7, 4.10.8, 4.10.9, 4.10.10). At high SOD activity, ROS, especially superoxide radical scavenging was done properly and thus, damage to membranes due to oxidative stress decreased, leading to increased tolerance to oxidative stress. Salt stress increases the superoxide level in the cells. If this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler, 2000). Moreover, it inactivates antioxidant enzymes which are very important for H$_2$O$_2$ scavenging such as catalases (Ashraf et al., 2010) and peroxidases (Wang et al., 1999). On the other hand, in the wheat cultivar Alvand, superoxide radical production increases with the increase of salt stress, however, SOD activity remains constant at all salt stress levels. Due to this reason, scavenging of this dangerous radical in this cultivar is not done perfectly. Consequently, this radical attacks vital biomolecules and damage the membranes in this cultivar. Esfandiari et al. (2007) had similar findings and expressed that the increase in SOD activity and decrease in oxidative damage were closely related. In our study also, SOD activity increased significantly at high NaCl treatment of 50 mM in all tissues and organs suggesting plant tolerance to stress.

Our results show that CAT activity increased slightly with increasing salt concentrations in the medium up to 25 mM beyond which a significant increase was noted. Leaves exhibited maximum activity (2.45 EU mg$^{-1}$ protein) at 50 mM NaCl treatment (Table 4.10.9). Similar results were reported by Ashraf et al. (2010) in Calendula officinalis, who found that catalase activity increased at higher concentrations of NaCl in leaves (25%) than roots. Kovtun et al. (2000) found significantly higher constitutive concentrations of catalase in salt tolerant cotton than salt sensitive lines. CAT activity has been crucial for cellular defense against salt induced photorespiration in peroxisomes of leaves (Wi et al., 2005). CAT is involved in scavenging hydrogen peroxide formed by the dismutation of superoxide anions catalyzed by SOD (Amor et al., 2005). Our observation on CAT activity was very similar as earlier reported by Sudhakar et al. (2001) who reported increase in CAT activity in mulberry cultivar at higher concentrations of NaCl stress.
APX activity followed the same trend as catalase (Table 4.10.10) and this corroborates with the findings of Wang et al. (1999) who concluded that high salt tolerance was due to maintenance of higher SOD and APX activity. Furthermore, Wang et al. (1999) found that compared to cultivated tomato (*Lycopersicon esculentum*), better protection of wild salt tolerant tomato (*Lycopersicon pennelli*) root plastids from salt induced oxidative stress was correlated with increased activities of SOD and APX. Zhu et al. (2001) also reported increased APX activity in the leaves after 10 days treatment with salt stress in *Cucumis sativus* L. Moreover, under salt stress H$_2$O$_2$ concentration enhanced in different parts of cells and H$_2$O$_2$ has been shown to induce cytosolic APX. Therefore, accumulation of APX at higher salinity conditions may be a good signal for adaptive responses to stress.

Our results of increasing GR activity corroborate to the findings of Zhu et al. (2001) who showed increased GR activity in the leaves under salt stress. Like SOD, CAT and APX, GR activity also exhibited a slight increase up to a NaCl treatment of 25 mM (Table 4.10.8). Beyond this concentration, there was a significant increase in GR activity in all tissues and organs at 50 mM. The maximum activity was recorded in leaf (4.81 EU mg g$^{-1}$ protein). Roxas et al. (2000) also reported that NaCl treatment resulted in stress-induced increase in glutathione reductase in cotton cultivar. Maas and Poss (1989) demonstrated that the foliar level of GR activity was significantly increased in the leaves of cucumber (*Cucumis sativus* L.) by chilling stress. The increased GR activity has also been reported in *Triticum aestivum* and *Triticum durum* because of high temperature stress (Mukhtar et al., 2008). The role of glutathione and GR in the H$_2$O$_2$ scavenging in plant cells has been well established in Halliwell-Asada enzyme pathway (Fadzilla et al., 1997). GR catalyses the rate limiting last step of ascorbate-glutathione pathway. The elevated levels of GR activity perhaps could increase the ratio of NADP$^+$ / NADPH, thereby ensuring the availability of NADP to accept electrons from the photosynthetic electron transport chain. Under such a situation, the flow of electrons to O$_2$ and therefore the formation of O$_2$ can be minimized.
Alliin content

Salt treatment (NaCl) increased alliin production in tissues and explants grown under *in vitro* conditions except at concentration of 50 mM (Table 4.10.11). The concentration of alliin was significantly high in all tissues and explants at NaCl supply up to 25 mM. *In vitro* raised leaf (2.81 µg g\(^{-1}\) dry wt.) and plantlet (3.19 µg g\(^{-1}\) dry wt.) recorded highest alliin content at 25 mM NaCl treatment. This result corroborates with the earlier reports where enhanced metabolite production was noted with increased NaCl level. Various biotic and abiotic factors, used as elicitors, have been reported to increase secondary metabolite yield. Application of NaCl also enhances metabolite production. In tomato hairy root cultures, increased concentration of jasmonic acid was noted on 100 mM NaCl amended media. The biosynthesis of solasodine starts from acetyl coenzyme (A) which later converts to mevalonic acid, *via* mevalonic acid pathway. In this pathway, cholesterol, a key intermediate of solasodine, is synthesized. High salinity seems to enhance *in vitro* cholesterol production, which in turn increases solasodine in tissues, or the enhanced yield may also be due to over expression of genes.