Chapter 5

Discussion
Discussion

also influences in vitro morphogenesis by causing alteration and darkening of culture media, adsorption of vitamins, metal ions and by absorbing PGRs (Thomas 2008).

The effect of gamma rays on embryogenic cultures was evaluated in C. roseus. Physiological alterations in a large range of plants exposed to gamma rays have been described by many researchers (Kim et al. 2000a, 2004, 2005; Kovacs and Keresztes 2002; Wi et al. 2005). The changes frequently observed in the low- or high-dose-irradiated plants are enhancement or inhibition of germination, growth rate, and other biological responses (Kim et al. 2000b: Wi et al. 2005).

In the present investigation, the embryogenic callus biomass of C. roseus was improved at lower irradiation dosages (10 Gy and 20 Gy). Although no conclusive explanations for the stimulatory effects of low-dose gamma radiation are available until now, papers support a hypothesis that the low dose irradiation induces growth stimulation by changing the hormonal signaling network in plant cells or by increasing the antioxidative efficiency of cells to easily overcome stress routinely produced by fluctuations of light intensity and temperature (Kim et al. 2004). In contrast, the growth inhibition induced by the high-dose irradiation has been attributed to the cell cycle arrest at G2/M phase during somatic cell division and/or various damages in the entire genome (Preussa and Britta 2003).

In Arabidopsis, growth of seedlings exposed to low-dose gamma rays (1 or 2Gy) was slightly increased compared with that of the control, while the seedling growth was noticeably decreased by the high-dose irradiation of 50 Gy. The biological effect of gamma-rays is based on the interaction with atoms or molecules in the cell, particularly water, to produce free radicals, which can damage different important compounds of plant cell (Kovacs and Keresztes 2002).

In experiments of irradiation carried out with other species the effects of gamma radiation (on the basis of survival, growth, proliferation rate, etc.) were very variable, depending not only on the kind of explant and genotype utilized for each species (Shen...
et al. 1990; Pinet-Leblay et al. 1992; Fereol et al. 1996) but also on the developmental stage of the cultures (Simard et al. 1992).

Similar to the above findings, an increase and maximum fresh mass of EC was observed in *C. roseus* (20 Gy) after that fresh mass decreased. Palanivel (1998) subjected different explants of groundnut to different concentrations of mutagens including gamma rays and EMS also found that the lower dose/concentrations of mutagens increased growth rate of callus, multiple shoot induction, somatic embryo formation and germination of embryos. Lukey (1982) also observed that low doses of ionizing radiations have stimulatory effect on plant growth. Pawlicki et al. (2001) observed that the callogenesis was increased at lower concentration of mutagens (5, 10 Gy). In potato (Ahloowalia 1990) and *Setaria italica* (Reddy and Vaidyanath 1990) lower mutagen dose/concentration stimulated growth while higher doses inhibited their growth.

We observed that gamma irradiation induced changes in protein content of somatic embryos in *C. roseus*. The soluble total protein content was very similar to the reports of Watt and Breyer-Brandwijk (1962) and Hulse (1991), where they found decrease in protein content as the irradiation dose was increased. Aziz and Mahrous (2004), reported that there were no changes in protein, lipid or carbohydrate contents of gamma-irradiated wheat and bean seeds at a dose of 5 kGy. The present result suggests, dose-dependent depletion in total protein content, when embryos of *C. roseus* were treated with 10-100 Gy.

Protein breakdown and recycling, which depend on the levels of proteolytic enzymes, are an essential part of the plant response to environmental stress (Hieng et al. 2004). In response to environmental abiotic and biotic stress cellular proteins need to be rebuilt. Degradation of damaged, misfolded and potentially harmful proteins provides free amino acids required for the synthesis of new proteins (Schaller 2004; Gudkowska and Zagdanska 2004). After gamma and neutron irradiation of pea seeds, an inverse correlation between growth and degrading enzyme activity has also been reported (Bagi et al. 1988).
After gamma ray irradiation soluble free amino acids were noted to increase in a dose-dependent manner. The observed increase in free amino acid content after ionising radiation is in agreement with findings of Satter et al. (1990), who documented increases in essential and non-essential amino acids of soybean when irradiated at a dose level of 0.10 kGy. Dose-dependent increase in amino acid content due to protein synthesis inhibition have also been cited in germinating wheat (Reuther 1969). However, Inoue et al. (1975), reported that release of amino acid in gamma-irradiated rice remained almost uninhibited when exposed at 0.10 to 0.40 kGy dose levels. Ananthaswamy et al. (1971), however, reported that endogenous amino acid pool sizes were significantly decreased in irradiated wheat seeds. The precise effect of ionising radiation on free amino acid content depends on various factors, such as sensitivity of the exposed system, the type of particular functional tissue and even with other applied conditions, such as aqueous soaking after irradiation, as has been indicated in the works of Sidduraju et al. (2002). Khattak and Klopfenstein (1989) reported that amino acids, e.g. phenylalanine, leucine and arginine, were increased with increasing gamma dose from 0.5 kGy to 5 kGy whereas methionine, threonine, valine, isoleucine were decreased at higher dose (5 kGy) in wheat, maize, mugbean and chickpea. Joseph et al. (2005) reported that, with the exception of tyrosine (which increased significantly), the amino acids in cowpea (acidic, basic, polar and non-polar amino acids) were decreased significantly with the increase of gamma radiation compared to their respective controls.

The present findings showed an increase in activity of all antioxidant enzymes SOD, CAT, APX and GR in a dose dependent manner. Gamma irradiation has been reported to induce oxidative stress with overproduction of ROS such as superoxide radicals (O$_2^-$), hydroxyl radicals (OH') and hydrogen peroxides (H$_2$O$_2$) (Xenia et al. 2000), which react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids causing disturbance of cellular metabolism (Salter and Hewitt 1992). To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of reactive oxygen species in cellular compartments (Bowler et al. 1992). One of the protective mechanisms was the enzymatic system, which operates with the sequential and simultaneous actions of a

Jamia Hamdard
number of enzymes including SOD, APX and CAT (Larson 1988). SOD which occurs in various cell compartments, dismutase $O_2$ to $H_2O_2$ and oxygen (Salin 1987). Catalases are synthesized in a tissue specific and age dependent manner, scavenge $H_2O_2$, generated during photorespiration and b-oxidation of fatty acids (Lin and Kao 2000). Peroxidases located in the cytosol, vacuole, cell walls as well as in extra-cellular spaces, use guaiacol as electron donors and utilize $H_2O_2$ in the oxidation of various inorganic and organic substrates (Shah et al. 2001). The role of GR in $H_2O_2$ scavenging mechanism in plant cells was well established in Haliwell-Asada enzyme pathways (Bowler et al. 1992).

There was a compelling evidence which showed that the activities of enzymes involved in ROS scavenging were altered by several environmental stresses, including gamma irradiation (Al-Rumaih and Al-Rumaih 2008). The expression patterns of GST, SOD, POX and CAT genes exhibited increased transcripts upon irradiation in *Nicotiana tabacum* (Cho et al. 2000).

The activity and isozyme patterns of peroxidase (POX) in *Nicotiana debneyi* and *Nicotiana tabacum*, SOD in *Nicotiana debneyi*, and CAT in *Nicotiana tabacum* increased in response to irradiation treatment (Wada et al. 1998), this observation was similar to our present study. Chaomei and Yanlin (1993) also reported an increase in activity of POX and CAT with a corresponding decline in growth of *Triticum aestivum* plants under higher irradiation doses (20, 40, 60, 80 Kr). Singh et al. (1993) reported induction of APX activity in two sugar cane varieties grown under gamma irradiation. The activities of POX, CAT and SOD in radish (*Raphanus sativus* leaves) were enhanced by irradiation (10 Gy) treatment (Lee et al. 2003). SOD activity showed an increase in the irradiation (2, 4, 8, 6 Gy) of red pepper (*Capsicum annum*) yeomyang variety and a decrease in joheung variety (Kim et al. 2004). Irradiation was reported to enhance POX activity of two *Phaseolus vulgaris* cultivars (Stoeva et al. 2001).

Several reports with other plants provided evidence of enhanced activities of APX (Singh et al. 1993), SOD (Zaka et al. 2002) and GR (Foyer et al. 1991) after gamma irradiation treatment. Generation of ROS, particularly $H_2O_2$ had been proposed to be part of the signaling cascades that lead to protection from stresses (Larson 1998).
Environmental stresses were shown to upset the balance between the production of ROS and quenching activity of antioxidants (Cakmak and Marschne 1992). Induction of antioxidant enzyme activities was reported to be a general strategy adopted by plants to overcome oxidative stresses (Foyer et al. 1994).

Involvement of APX (Singh et al. 1993), CAT (Sah et al. 1996), SOD (Zaka et al. 2002) and GR (Kim et al. 2004) enzymes in maintaining overall defense against the effect of irradiation was earlier mentioned. Blokhina et al. (2003) attributed the induction of POD, SOD and GR activities to enhanced production of toxic ROS levels in living organisms under stress. CAT, in cooperation with APX and other enzymes were shown to destroy the \( H_2O_2 \), produced by SOD and other reactions (Foyer et al. 1994).

It was established that the peroxidase was involved in the compensatory mechanisms of inhibition of free radicals formed upon gamma irradiation, so is the increase in activity when embryogenic calli were treated with gamma rays (Al-Rumaih and Al-Rumaih 2008).

The effect of gamma rays at lower irradiation induced enhanced alkaloid content in \( C. roseus \). In fact, several mutagenic agents and specially ionizing radiations have been reported to induce considerable improvement in secondary metabolite production of different medicinal and aromatic plants (Kak et al. 1982). To test this possibility, we determined the terpenoid alkaloid contents (vincristine and vinblastine) after irradiated cultures, establishing a comparison with those produced by unirradiated cultures (control plants).

An improvement in secondary metabolite production have been recently found in different studies using gamma radiation as compared with controlled samples (Klu et al. 1997, Sharma et al. 1997). Gavidia and Bermúdez (1997), in \( Digitalis obscura \) found considerable variability in plantlets developed from irradiated shoot tips in their cardenolide production (878 to 3291 \( \mu g/g \) d.w.), including variants with similar or even higher productivities than the native T4 plant. Gamma irradiation has been widely applied in medicine and biology in terms of biological effects induced by a counter-
intuitive switch-over from low dose stimulation to high-dose inhibition (Chunga et al. 2006). Previous studies have shown that relatively low-dose ionizing radiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance, and crop yields (Charbaji and Nabulsi 1999; Kim et al. 1999/2000; Chakravarty and Sen 2001; Kim et al. 2005; Baek et al. 2006). There have been a number of reports on the use of UV, low-energy ultrasound, hormone, and feeding of precursors to regulate the production of secondary metabolites such as shikonin, anthraquinone, saponins, silymarin, anthocyanin, etc. in suspension culture system (Mohil et al. 1985; Yoshikawa et al. 1986; Lin and Wu 2002; Edahiro et al. 2005).

The effect of salinity stress (NaCl) on embryogenic cultures was evaluated in *C. roseus*. Plant cell culture is a useful technique for the study of responses to environmental stress at cell level. In the present study we observed that the NaCl level inhibited cell growth, so the embryogenic callus biomass was low with increased NaCl concentrations. This may be due to nutritional imbalance, osmotic and metabolic disturbances etc (Niknam et al. 2006). Elkahoui et al. (2005) in *C. roseus*, Cherian and Reddy (2003) in *Suaeda nudiflora*, Niknam et al. (2006) in *Trigonella* species, and Shibli et al. (2001, 2007) in *Lycopersicon* species also observed such responses. The soluble salts decrease the availability of water to plants by decreasing free energy of water. Salts may exert detrimental effects on plant growth through the toxicity of one or more specific ions present in high concentration (Alam 1999; Arshi et al. 2002). Generally, plant biomass is inhibited by an excess of the solute taken up by plants from the saline growth media. Na accumulation in tissues under salinity stress is generally considered as a major factor behind the adverse effect of salinity on nutrient uptake and growth (Shibli et al. 2001). Higher salinity levels in external medium are known to effect various physiological and metabolic processes, leading to cell growth reduction (Ashraf and Harris 2004). Growth reduction have been described also in many other NaCl-treated cell lines, for instance, in *P. sativum* calli adapted to 85.5 mM NaCl, a 65% reduction of dry weight was shown, compared with sensitive calli (Olmos et al. 1994). Also in NaCl tolerant *citrus* lines, 170 mM NaCl reduced growth by 5 fold when compared to control (Piqueras et al. 1996).
Discussion

Osmotic stresses have been shown to be important for induction of embryogenic cultures in wheat (Benkirane et al. 2000). In the present study, there was an improvement in embryo proliferation at lower concentration of NaCl (25 mM). The addition of NaCl enhanced the rate of multiplication as well as yielded hardy somatic embryos in Ipomea batatas (Mukharjee 2002). This observation is in accordance with results obtained in a number of previous reports dealing with salt-stress in tissue cultures of rice and other cereal species. Different groups of scientists [Reddy and Vaidyanath (1986), and Galiba and Yamada (1988)] observed that salt pre treatments have positive effects on plant regeneration of selected callus lines that developed on a salt-free medium. These results suggest a beneficial effect of salt stress on the embryogenic process (D'onofrio and Morini 2002).

The effect of NaCl stress on various biochemical parameters was evaluated in C. roseus. Increase in soluble protein content was recorded in almost all embryogenic stages as the salt concentration was increased in the medium. The increase in soluble protein content has already been described in different plant species in response to NaCl stress (Cusido et al. 1987). Although the possible mechanisms of salt tolerance are not completely uncovered, the increase or decrease of proteins in response to salt stress could be important in adaptation of plants to saline substrates (Ashraf and O'leary 1999).

In all embryogenic stages, decrease in amino acid content was noticed with increase in NaCl concentrations in the medium. Similar to our present observation, in wheat variety cv. Giza 155, the free amino acids accumulation showed marked decrease as the concentration of NaCl was increased (Hamada and Khulaef 1995). This may be due to the fact that salinity may promote conversion of the other amino acids into proline (Shaddad et al. 1990; Hamada and Khulaef 1995).

Accumulation of proline as a compatible solute has been reported in the present study when embryogenic cultures were subjected to salt stress. Mattioni et al. (1997) reported that proline accumulates in the cytosol in response to salt stress and acting as osmoregulator. Other soluble N-containing compounds such as other amino acids,
polyamines and soluble proteins also could protect plant tissues against osmotic stress (Rai 2002b). In addition, proline's role as a protector of enzyme denaturation, a reservoir of nitrogen and carbon (Fukutaku and Yamada 1984), or as a stabilizer of the machinery for protein synthesis (Jacobsen et al. 2005) were also reported. Olmos and Hellin (1996) viewed that the increase in proline content under stress conditions can protect proteins against salt ions and inhibit breakdown of protein in leaf tissues. This might be true for our plant system too, however the significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Meloni et al. 2004).

Salinity also induced a gradual increase in soluble carbohydrates levels in cultures and tissues, as the NaCl concentration increased. Soluble carbohydrates and soluble proteins were also increased in wheat cv. Giza 155 (Hamada and Khulaef 1995). These results are in agreement with the results obtained by many other authors (Venekamp et al. 1989; Shaddad et al. 1990). The increase in soluble sugar may play an important role in osmotic adjustment; a conclusion which is in accordance with the results obtained by Shaddad et al (1990) while working with glycophytic plants. Soluble sugar accumulation under salt stress was reported in many species such as tomato (Bourgeais-Chaillou and Guerrier 1992), Populus euphratica (Watanabe et al. 2000) where they seemed to be implied in osmotic adjustment. In our results, it was observed that salt-tolerant embryogenic calli accumulated more soluble sugars than the salt free cultures.

NaCl induced a dose dependent increase in SOD activity in C. roseus callus which could represent a defense mechanism against NaCl induced generation of superoxide anion (O₂⁻), hydroxyl radical (·OH), singlet oxygen (O₂). Increased activity of SOD under saline conditions was earlier reported in many other plants (Elkahoui et al. 2005; Cherian and Reddy 2003). The CAT and APX activity also showed a progressive increase with increasing salinity in the medium. NaCl increased APX and CAT activity in embryogenic callus in C. roseus, that indicated that these cells have a higher efficiency to scavenge H₂O₂ generated by SOD. Which may be required for preventing the peroxidation of membrane lipids, generated by salt stress (Hernandez et al. 2000; Cherian and Reddy 2003; Niknam et al. 2006).
Salt treatments have had a great impact on vinblastine and vincristine yield at all the embryogenic and morphogenetic stages. In the present study, lower salt concentration increased vinblastine and vincristine content. In leaves maximum vinblastine yield was recorded in NT-1 and NT-2 (25 mM and 50 mM NaCl), while a maximum vincristine yield was recorded in 25 mM NaCl. Higher salinity resulted in loss of alkaloids. Similarly, increased solasodine synthesis in response to salt stress was also observed in case of solasodine obtained from in vitro cultures of Solanum nigrum (Bhat et al. 2008). The increase of terpenoid alkaloids at lower NaCl concentration may be due to the fact that perception of stress signals created by NaCl (called elicitors) activates a number of signal transduction pathways in plants, promote transcriptional activation of cascade of genes and which eventually produce de novo synthesis of a variety of defense proteins and protective secondary metabolites (Nimchuk et al. 2003). Coordinated transcriptional control of biosynthetic genes, often regulated by various external signals emerges as a major mechanism dictating the accumulation of secondary metabolites in plant cells (Dutta et al. 2007). However, information about stress regulation of secondary metabolites and the molecular mechanisms regulating these specialized pathways are poorly understood.

In this study, successful cryopreservation of embryogenic cell suspensions of C. roseus has been achieved. The protocol developed herein provided fast proliferation of uniform embryogenic cultures that in turn produced abundant somatic embryos.

In this protocol, a vitrification-based method has been evaluated using sucrose, sorbitol, DMSO, glycerol and PEG as cryoprotective agents. These types of agents have been widely used in a large number of plant groups (Winkelmann et al. 2004). These compounds contribute to cellular dehydration, prevent cell damage, and restore regrowth of cultured cells (Winkelmann et al. 2004). The level and simultaneous influx/efflux of water determine the degree of cell/tissue survival under low-temperature preservation conditions as these prevent freezing injury and improve post-thawing viability (Mazur 2004). Thus, cellular water content must be reduced either by use of cryoprotectants, such as DMSO (Vendrame et al. 2001), Polyethylene glycol (PEG)
al. (1990) have preserved *C. roseus* LD50/h13 cell lines for over 6 months in the presence of sorbitol and 5% DMSO, and using a two-step freezing process that produced secondary metabolites, even following cryopreservation. Several reports have indicated that the use of a vitrification solution, a mix of protective agents, enhanced success of cryopreservation (Langis et al. 1990; Sakai 1990). It has been reported that a mixture of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v), and 0.4 M sucrose is recommended. However, the mechanism of action of DMSO is somewhat different from those of sugars as it penetrates deep into the cell membrane while protecting cells from freezing damage (Merymann 1971). Although higher levels of DMSO and glycerol have reduced viability of *C. roseus* in this study, viable cells are capable of dividing and regrowing upon culture.

Suspended embryogenic cells of *C. roseus* subcultured on solid medium showed growth rates similar to those of non-cryopreserved embryogenic cells. Embryogenic cultures of *Hevea brasiliensis* showed a higher regeneration ability following freezing than did unfrozen cultures, and therefore cryopreservation has been suggested to be an important method in enhancing regeneration efficiency in older cultures (Engelmann et al. 1997). Similarly, in *Vitis vinifera* cryopreserved cells showed improved regeneration capacity as compared to unfrozen control cells, and regenerated plantlets were similar to those of control and no visible morphological differences were observed (Wang et al. 1994, 2002).

In the present study, dehydration treatment that included preculture and pretreatment and air drying, was found to enhance embryo regrowth when compared to control. Similar positive effects of dehydration were reported in many other plants (Wang et al. 2004; Danso and Ford-Lloyd 2004)

Wang et al. (2002) have reported that cryopreservation may influence regrowth of embryogenic cultures as follows: (i) the morphogenetic ability of cryopreserved cultures remains the same as that of control, as noted in the present study; (ii) it may decrease regrowth following cryopreservation as reported in *Gossypium hirsutum* (Rajeskaran et al. 1996); and (iii) regrowth may improve following cryopreservation as
reported in *Festuca* and *Lolium* species (Wang et al. 1994), and in *Pinus sylvestris* (Haggmann et al. 1998). In this study, the observed morphology of cryopreserved and non-cryopreserved embryogenic cultures and regenerated plantlets is similar. Earlier, Norgaard et al. (1993) have reported that the cultural morphology of *Picea abies* have not changed even after cryopreservation. No significant morphological alterations have been noted in cryopreserved and non-preserved somatic embryos and derived plantlets *Pseudosuga menzeisii* (Gupta et al. 1995).

In conclusion, our results demonstrate a simple and an effective method for storage of *C. roseus* via cryopreservation. As *C. roseus* is an important medicinal plant, those promising embryogenic cell lines that have been selected with enriched alkaloids can be cryopreserved for a short to longterm basis.