Micropropagation

Chicorium intybus L. is a widely used medicinal herb coffee substitute, therefore, we aimed at developing a reliable protocol for in vitro regeneration of this plant and thus examined the effects of several combinations and concentrations of plant growth regulators.

Micropropagation of C. intybus L. provides an opportunity to produce clonal populations, which can be multiplied on a commercial scale. Being the popular vegetable plant, it provides a variety of edible products from both leaves and storage roots. Nutritional aspects such as hypocaloric value, high fibre, vitamin and mineral salt content increase the importance of chicory for the diet of human beings. Besides the nutritional aspects it is being exploited for its secondary metabolites. The plant finds its name in modern pharmacopeia because of its vast medicinal uses. The species is self-incompatible and biannual; hence its propagation through seeds slows down the genetic improvement because of the difficulties of obtaining pure lines in a reasonable number of years. Technologies such as haploid production, in vitro vegetative multiplication, regeneration from vegetative tissues and genetic transformation are used as tools to speed up the improvement of traits in this species (Genga et al. 1994). These technologies can also be used to improve genetic traits of chicory.

The maintenance of aspect or sterile conditions is essential for the successful tissue culture procedures. Micro organisms must be completely removed from the explants which are to be cultured without damaging the cells and tissue. The choice of sterilizing
agent and time required for treatment depends on the sensitivity of the plant material to be disinfected. While perusing the literature it was noticed that various sterilants had been tried by different workers for achieving maximum seed sterilization.

In present studies, Seed sterilization of C. intybus L. was achieved using HgCl$_2$ (0.2%) for 10 min. A perusal of the literature showed that Caffar et al. (1982) sterilized roots of C. intybus with 20% commercial hypochlorite for 20 min and Rehman et al. (2003) reported complete sterilization of C. intybus after seeds were washed with cetrimide (0.5%) for 7 min and surface-sterilized by immersion in 0.1% (w/v) mercuric chloride for 7-10 min. Some other workers have also reported sterilization procedures for C.intybus (Nandagopal et al. 2006; Kumari et al. 2007; Nandagopal and Kumari, 2007).

Best seed germination as well as complete seedling formation was recorded on MS (1/2) basal medium, Similar results were achieved by Rehman et al. (2003); Abdin and Ilah (2006) while Buhara et al. (2007) reported seed germination of C. intybus L. on MS basal medium (Murashige and Skoog, 1962).

Regeneration of plantlets by cell, tissue and organ culture is a major goal of Plant tissue culture and great deal of work on regeneration has been devoted in finding equilibrium between the components of the medium. Numerous studies indicate that it is difficult to make extensive generalizations. Infact, an inductive treatment developed for a particular culture is not necessarily successful in other cultures. Consequently, there is no doubt that organogenesis in vitro depends on a complex system of endogenous and exogenous interacting factors (Alicchio et al. 1982). Although, it is known that regeneration ability depends on plant species and genotype, age of plant, physiological condition, type of explant the possibility of improving organogenesis efficiency by employing other phytohormone concentrations may also exist. A very efficient and rapid in vitro plant regeneration system through multiple shoot formation in C.intybus L. was developed. Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray, 2000). Different tissues may have different levels of endogenous
Discussion

hormones and, therefore, the type of explant source would have a critical impact on the regeneration success.

Keeping in view, the efficacy of different plant growth regulators used individually or in combinations, a number of concentration and combinations were tried for exploiting maximum potential for organogenesis/regeneration. Since a number of media formulations are available attempts were made to see the effect of two different media with an array of similar phytohormones.

In our study, shoot tip was much more productive for shoot formation than nodal explants and MS medium was more effective than B5. Ray and Jha (2001) in W. somnifera; Bini-Zachariah et al. (2001) and Varghese et al. (2003) in H. muticus have also reported MS medium the most suitable medium for morphogenesis. MS has relatively high total nitrogen and ammonium content and has been used in all successful studies on morphogenesis in explants of S. melongena (Yamada et al. 1967; Kamat and Rao, 1978; Matsuoka and Hinata, 1979). The difference in response lies in the concentrations and constituents of the media. It contains only nitrate whereas other media contain nitrate and ammonium. Bhojwani and Razdan, (2005) held the view that chief differences in the composition of various commonly used tissue culture media lies in the quantity of various salts and ions. Qualitatively, the inorganic nutrients required for various plant tissues appear to be fairly constant. When mineral salts are dissolved in water they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt. For example in MS (1962) medium NO\textsubscript{3} ions are contributed by NH\textsubscript{4}NO\textsubscript{3} as well as KNO\textsubscript{3} and K\textsuperscript{+} ions are contributed by KNO\textsubscript{3} and KH\textsubscript{2}PO\textsubscript{4}. In present studies, it seems that difference in responses in terms of growth and morphogenesis appears due to the quantity of various salts and ions present in MS and B5 media. Therefore, MS media, a high salt medium, was found most suitable medium for overall growth and morphogenesis of the plant. It has been reported that fragments of green or etiolated leaves or other parts of witloof chicory shoots cultivated in vitro are capable of forming whitish calli on which newly formed roots and shoots can be observed (Toponi, 1963; Bouriquet and Vasseur, 1973; Liebert and Tran Thanh Van, 1972; Grazia et al.
1984; Vasseur et al. 1995). Successful attempts to initiate callusing in C. intybus L. were also made by Vasil et al. (1964) who grew mature embryos on nutrient medium supplemented with coconut milk, 2,4-Dichlorophenoxy acetic acid (2,4-D) and NAA. This callus tissue was regularly sub-cultured on a medium without 2,4-D. Differentiation was obtained on MS medium supplemented with thiamine HCl (0.1 mg l$^{-1}$), nicotinic acid (0.5 mg l$^{-1}$), pyridoxine HCl (0.5 mg l$^{-1}$) glycine (2 mg l$^{-1}$), meso-inositol (100 mg l$^{-1}$) and kinetin (0.04 mg l$^{-1}$). Gwozdz (1973) analysed the effect of IAA on the growth, organogenesis and RNA content of root callus. They observed that IAA induced intensive callus growth accompanied by an increase in RNA content and a simultaneous decrease in RNAase activity.

Micropropagation from leaf explants has been achieved previously on BA (4.4µM) (Genga et al. 1994); IAA, NAA or IBA in the concentrations ranging from 0.0 to 2.0 µM with or without BAP 1.0µM (Vermeulen et al. 1992) and 2, 4-D (1.3µM) and Kn (1.3µM) (Yasseen et al. 1995). Micopropagation from the leaf explants has also been achieved previously using IAA/Kn combination (Topon, 1963; Liebert and Tran Thann Van, 1972). However, Vasseur and Sene (1984) obtained regenerated buds from callus using IAA and Kn in combination. Dabin (1985) achieved regeneration from the leaf explants using IAA and BAP in combination. Rehman et al. (2002) reported callus formation from leaf explants on MS medium supplemented with IAA, BA, and Kn, with or without CH. The calluses obtained on MS + IAA (0.5-2.0 µM) + Kn, BA (5.0 µM) + CH (1000 mg l$^{-1}$) were compact and pale yellow but were friable on MS + IAA (0.5-2.0 µM) + Kn, BA (5.0 µM). A method for totipotent callus formation has been recently developed in C. intybus by Hamid et al. (2010) from basal portion of shoot tip explants on MS medium supplemented with different concentrations of plant growth regulators (PGR) like 6-benzyl amino purine (BAP); Kinetin (Kn); BAP with Indole 3-butyric acid (IBA). It was observed that cultures growing under the influence of BAP+IBA produced considerably more callus than cytokinins used alone. Re-differentiation of such callus leading to multiple shoot formation was obtained on same medium after 3 weeks.

Velayutham (2006) studied different types of explants (cotyledon, leaf, hypocotyls and root) for callus induction. The explants were cultured on MS medium supplemented
with various concentrations and combinations of plant growth regulators including 0.5, 2.5, 5, 7.5, and 10 \( \mu \text{M} \) of IAA, IBA, NAA or 2, 4-D in combination with 2 \( \mu \text{M} \) callus induction and reported order of effectiveness of plant growth regulators for callus induction was NAA>IBA>IAA>2,4-D. Malarz et al. (2005) also obtained callus from leaf explants of C. intybus on to solidified MS medium containing (2,4-D; 1 mg/l), Kn (0.3 mg/l) and 3% sucrose.

However in our study, IAA/TDZ combination was found to be the best treatment of all the treatments for callus induction as well for callus biomass among all the combination of auxins and cytokinins used and less callus biomass was obtained on 2,4-D/IAA, 2,4-D/NAA, IBA/Kn and IBA/Kn combinations while BAP/IAA, BAP/NAA, BAP/IBA, Kn/IAA and Kn/NAA produced moderate amount of callus biomass.

Organogenesis

C. intybus L. has been regenerated earlier from the primary explants, such as storage roots (Margara et al. 1966), green leaves (Toponi, 1963; Nitsch and Nitsch, 1964; Wagner and Eneva, 1996), etiolated leaves (Bouriquet and Vasseur, 1966), floral stems (Bouriquet and Vasseur, 1973), suspension cultures (Yasseen and Splittstoesseç, 1995), leaf and petiole (Eung et al. 1999), leaf midrib (Wagner and Eneva, 1998), pollen (Castano and De Proft, 2000) and protoplasts (Varotto et al. 1997; Saski et al. 1986). Plants have also been regenerated from tissue cultures of mature tap-roots (Heirwiegh et al. 1985). Shoot regeneration from callus obtained from root segments was achieved on modified Heller medium supplemented with 2,4-D (Caffaro et al. 1982; Profuma et al. 1985). Hamid et al. 2010 observed dedifferentiation of callus from shoot tips leading to multiple shoot formation under the influence of BAP/IAA on MS medium.

The major factor influencing the proliferation rate is the interaction of the physiological state of the plant material with the culture medium and its additives. Effect of growth regulators is not specific in most cases. Even different growth regulators belonging to the same class may elicit different morphogenetic response in a given tissue (Bhan, 1998) so is the case with present studies where BAP, Kn belonging to the same class i.e. cytokinins differs in their responses. In our study both types of cytokinins (Kn & BAP) when used alone, were able to produce shoots although BAP was nearly twice more
effective than Kn in terms of both the frequency of explants producing shoots and the
mean number of shoots produced per explants similarly, Velayutham, (2006) found BAP
more effective than Kn in terms of shoot induction. Our results are in line with Buhara et
al. 2007 as they reported BAP was more successful than Kn. Also the similar results
were well documented in Niger (Nikam and Shitole, 1993) and Chlorophytum (Purahit
et al. 1994).

In our study, indirect shoot regeneration was obtained when shoot tips and nodal
explants were cultured on different concentrations of BAP. A maximum of 19.1±1.28
number of shoots on MS+BAP (9 µM) were produced. Earlier similar type of work was
carried out by Kamili et al (2003), who achieved reproducible protocol for
micropropagation of C. intybus through in vitro culture of mature nodal explants and
reported maximum number of shoots(13.1±0.8) on MS+ BAP (15µM).Combined
interaction of cytokinins and auxins resulted in maximum multiple shoot regeneration
suggesting that interaction of auxins and cytokinins perhaps resulted in shifts in
endogenous synthesis of auxins as well as cytokinins thus making it either suboptimal or
supra-optimal hence resulting in varying needs in exogenous supply of both the
phytohormones [Ying-Hua, 2011]. Rehman et al. (2003) established study on in vitro
regeneration of C. intybus L. They found that mean number of shoots per leaf explants
was 5.92 and percentage of the shoot formation was 84.4% on MS semi solid basal
medium containing (0.5µM) IAA+(5µM) Kn in the presence of 1000mg/l casein
hydrolyzate (CH). In addition, they also examined shoot formation using 1.127mg/l
BAP + 0.086 mg/l IAA in the presence of 1000mg/l CH and obtained a mean value of
3.9 shoots per leaf explant and 61% of shoot formation. The most successful treatment
of Yucesan et al. (2007) was the combination of MSMO + 0.5 mg l⁻¹ Kn and 0.3 mg l⁻¹
IAA, producing a mean number of shoots (19.7) per explant from Lamina explants.
However, they reported combinations of either Kn or BAP with NAA were effective for
shoot production. The maximum shoot number 54±0.71 (100% response) were recorded
on MS+ Kn (7.5µM) +IAA (5µM) and 23.1±1.91 on Kn (3.5µM ) + NAA (5µM) and
also the maximum multiple shoots 28.8±0.78 on BA (7.5µM ) + IAA (5µM) and
19.8±1.31on MS + BAP (4.5µM) + NAA (2.5µM) were achieved.
In our study, the mean shoot number/explants was very high as compared with earlier studies (Rehman et al. 2003; Buhara et al. 2007) and it has been also observed that 2, 4-D alone produced non regenerative callus and was ineffective in in vitro regeneration of shoots however 2,4-D in combination with BAP/Kn induces callusing as well as in vitro regeneration of shoots, however regeneration potential, average mean number of shoots and percentage of callusing was found very low and our results are in agreement with the findings of Velayutham (2006) who observed 2,4-D alone to be ineffective in producing regenerative callus in C. intybus. There are some reports where 2, 4-D alone was not found effective in inducing callusing i.e. Arnica genus (Kalynyak et al. 1995) and Stevia rebaudiana (Gupta et al. 2010) Petrova et al. (2011) has also reported that high level of BA (1 mg/l) and lower concentration of 2, 4-D (0.1 mg/l) in MS medium were required for shoot induction Arnica Montana.

It is pertinent to mention here the role of TDZ (Thiadizuron) in the cultures of our study showed effect of suppression of elongation as we tested the effects of TDZ/IAA combination for shoot formation and low concentrations of TDZ was found effective for shoot formation. Also among all the combinations of auxins and cytokinins used for shoot multiplications, the TDZ was found to be effective for shoot multiplication with maximum shoot number of 144.3±5.86 were reported on Ms + TDZ (1µM) + IAA (5.5µM) and at higher doses of TDZ/IAA a sharp decrease in shoot length was noticed. According to Heutteman and Preece (1993), the low concentration of TDZ induces the shoot proliferation but may inhibit the shoot elongation. They also reported that higher concentration of TDZ in woody plants increased the formation of calluses. Although C. intybus is a perennial herb or shrub, those of reported findings on some woody plants are consistent with our data. Nevertheless, application of TDZ seems to be good promoter of shoot formation (Chen et al. 2002; Leddbetter and Preece, 2004; Thomas and Philip, 2005) and somatic embryogenesis in plant tissue culture (Singh et al. 2003).

The difference in mean number of shoots could arise from basal medium used in present studies. In terms of vitamin contents of MS and Murashige and skoog medium with Minimal Organics (MSMO), myo-inositol and thiamine are considered essential in plant tissue culture media. Myo-inositol content of the MS and MSMO is same however
thiamine contents are different i.e. MSMO and MS contain 0.4mg/l and 0.1mg/l thiamine respectively. Thiamine is a B vitamin and is essential for carbohydrate metabolism and biosynthesis and has also been found to influence organogenesis in some plant cultures and myo-inositol takes a role as intra cellular messenger and enzyme activation (Ranys and Fowler, 1993). In addition to myo-inositol and thiamine, the nicotinic acid and pyridoxine–HCl are present in only MS basal medium.

Growth regulators are able to direct organogenesis in the direction of the root and shoot formation (Liebert and Tran Thanh Van, 1972). High concentrations of auxins alone tend to induce rhizogenesis, while cytokinins alone enhance caulogenesis. Auxins and cytokinins do not enhance cell division in the callus when applied separately. Exogenously applied growth regulators have a significant influence on callus development and differentiation. It has been reported that auxin alone at high concentrations inhibit shoot development in chicory, but stimulate root formation and callussing which gradually dominate organogenesis (Profuma et al. 1985). Gwozdz and Szweykowska (1969) observed that high concentration of IAA alone render the callus loose and soft, not only by the increase of water uptake but also by an increase of protein synthesis. Here also in our case no effect on shoot induction was noticed when IAA, NAA and IBA were used alone except elongation of shoot tips and nodal segments.

It has been found in our study that the frequency of in vitro regeneration was remarkably higher than those reported for C. intybus so far (Profumo et al. 1985; Pieron et al. 1993; Vesseur et al. 1995; Belletre et al. 1999; Rehman et al. 2003; Bennici et al. 2006). Some of these studies also employed different approaches in order to increase the frequency of shoot or somatic embryo production from cultured explants of different types, such as pretreating leaf explants with 330 mM glycerol (Belletre et al. 1999), the inclusion of low concentrations (1.3–2.6 mg l−1) of the antibiotic cefotaxime in the culture medium (MixWagner and Eneva, 1996) or the anti-mitotic agent colchicines (0.001%) used alone or combined with kinetin (Bennici et al. 2006).

To our knowledge, very less work has been carried on the effect of TDZ on in vitro regeneration of C. intybus but we were able to regenerate shoots that too with very high frequency of about 144.3±0.86 shoots from a single shoot tip explant within a period of
Discussion

2 months. Our system, therefore, provides not only a rapid clonal propagation but also an excellent platform for gene transformation studies. It is known that rapid shoot bud development helps to ensure more successful and stable genetic transformation (Dandekar and Fisk, 2005).

Effects of Plant Growth Regulators on Root Formation

Auxin is generally considered necessary for the acquisition of the meristematic competence of the responsive cells. Once this competence has been established, excessive auxin concentrations were often found to be inhibitory for further embryonic (Charriere et al. 1999) or adventitious root (Gurel and Wren, 1995) development. It was reported that activated charcoal enhance rooting by reducing illumination to the submerged parts of the culture (Rayns, 1993). Velayutham et al. (2006) reported the rooting of elongated shoots on MS medium supplemented with (2.5–12.5 μM) IAA, IBA or NAA and found that IBA was found to be the best rooting hormone than IAA or NAA. Similar results are well documented (Tiwari et al. 2000; Yu et al. 2000; Rehman et al. 2003).

In our experiment, elongated multiple shoots were obtained in various trials were isolated and sub-cultured on MS and B5 medium and augmented with different concentrations of IBA, IAA and NAA. IAA as an auxin had the best response for root formation after 4 weeks initiation. Indirect multiple root regeneration was recorded at all concentrations of NAA and yielding thick and long root formation. Direct rooting was noticed at lower concentrations of IAA and IBA however an increase in the auxin concentrations (IAA and IBA) resulted in indirect root induction. It is obvious that the auxin (IAA) was more effective auxin. Our result are similar to Buhara et al. (2007) who reported rooting of the shoots was readily achieved on medium containing different concentrations of IAA or IBA and reported IAA was more effective than IBA in terms of the rooting percentages and percentage response.

Effect of carbohydrate sources

In the present study, the in vitro response including regeneration was affected by the supplemented carbon sources. Sugar added in culture medium does not only act as a
carbon source but also plays a role in osmotic regulation of water stress. Sugars like glucose, sucrose, fructose and maltose were used at different concentrations (3%, 4%, 6%) for various morphogenetic responses. Glucose (3%) and sucrose (4%) showed their influence in developing micro shoots and maximum number of shoots was found to be 16.4±0.96 and 18.7±1.33 respectively after 8 weeks of culture period. However, fructose and maltose at all concentrations had little effect on regenerations. Among all sugars tested, glucose (3%) followed by sucrose (3%) was found to be more responsive in root induction process, both in terms of number and growth, however fructose at all concentrations had more effect on root length as compared with other carbon sources. Sucrose, by virtue of being the most abundant sugar (Strickland et al.1987) is often assumed to be the best carbon source for in vitro cultures. However, several experimental evidences indicate that callus growth and morphogenesis can be significantly influenced by other carbohydrate supplements as well (Swedlund and Locy, 1993; Jeanin et al. 1995; Jain et al. 1997; Lemos and Baker, 1998). This behavior and the difference of response if any could not be linked to the carbohydrate nutritional status but to the osmotic condition induced by carbohydrates. Carbohydrates control morphogenesis by acting as energy source and also by altering osmotic potential of the culture medium (Pritchard et al.1991). Buter et al. (1993) reported a toxic compound 5-hydroxymethyl)-2-furaldehyde (HMF), a derivative of fructose, which was generated by high temperature during media sterilization.

**In vitro flowering**

Chicory, which is a cross-pollinated crop, provides an extra advantage for the study of various clones through in vitro seed setting and germination (Badila, 1991). In the present study, auxins such as IBA, IAA and NAA showed role in promoting floral bud formation and flowering without undergoing vernalization but IAA and IBA were more effective than NAA. The supply of exogenous growth regulators in the medium was essential for flower bud induction without vernalisation. It is known that the plant growth regulator requirement of plants for in vitro flowering varies. Empirical guides show that exogenous auxin could act as a principal floral inhibitor (Scorza, 1982) but in the present study IBA, IAA and NAA in the medium did not inhibit in vitro flower bud
formation (except NAA 0.03µM/0.1µM/0.25µM/0.5µM;IAA,0.5 µM) i.e. the formation of flower buds depended on the concentration of the applied plant growth regulators. The essentiality of auxins for flower bud induction and development has been reported for a few plants such as Pisum sativum (Franklin et al. 2000), Vigna radiate (Avenido and Havlea, 1990) and Vigna mungo (Ignacimuthu et al. 1977). Our results were also in line with Erdag and Emek, (2009) who reported IBA was found to be more efficient than IAA in the induction of in vitro flowering of Anthemis xyllopoda. Similar findings were obtained for another Asteraceae member Pentanema indicum (Duan and Yazawa, 1994). Sheeja and Mandal (2003) have also reported in vitro flowering and fruit formation in tomato at high levels of endogenous auxins.

**Transfer to non-sterile conditions**

Shoots grown in vitro with or without roots are not adapted physiologically to survive in soil; therefore, they have to be gently weaned to accept such conditions. Rayns and Fowler, (1993) indicated two major dangers for plantlets to get might become desiccated and might be subjected to fungal rot. This fact can be explained in the following way; relative humidity in culture containers is usually close to 100%. As a result, the plantlets do not necessitate regulating their transpiration rates. Consequently their stomata remain open all the time and they recover the ability to close stomata over a rather extended period. As such we kept the plantlets in growth chamber for two weeks.

In addition, as reported transfer of such plantlets to soil in a relatively low humidity atmosphere resulted in desiccation (Rayns, 1993). Therefore, we avoided this, providing high humidity to the room by humidifier, besides, the plantlets were watered every two days and more so large plantlets with healthy appearance were selected.

**Induced mutations**

Induced mutation techniques have been successfully used to improve yield, quality, disease amid pest resistance in crops, or to increase the attractiveness of flowers and ornamental plants (Saleem et al. 2005). Variation has been promoted by the use of radiation (UV, Gamma, X-rays, thermal and fast neutrons etc.) or by chemical mutagens i.e. alkylating agents, Nitrous acid, hydroxyl amine, azides of Sodium and Potassium etc.
(Micke et al. 1987) and by colchicine, which has been used to change ploidy (Heinz and Mee, 1970).

A combination of in vitro technology and radiation/chemical induced mutation has been recommended to improve cultivars of vegetatively propagated crops (Novak, 1994; Maluszynski et al. 1995). The use of in vitro cultures in mutation breeding offers several advantages over the in vivo techniques including obtaining explants from preexisting cultures and recovering mutants and rapidly micropropagating them under controlled environmental conditions.

As plant tissues are composed of heterogeneous array of cells of various ages, different physiological states and degree of differentiation and cells with different ploidy levels exists, by placing cells in the tissue culture, the genome at different molecular states is suddenly placed under stress to cope up with in vitro conditions, resulting in the genome undergoing restructuring as a response to the new condition (McClintock, 1984). The changes in tissue culture conditions could influence the frequency of variation that seems to be an array of genetic/epigenetic changes (Phillips et al. 1994). 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 (Predieri, 2000).

**Chemical mutagenesis**

Chemical mutagens are being used to induce mutations and generate genetic variations in plants from which desired mutants may be selected. The most commonly used chemical mutagens are alkylating agents such as Ethyl methane sulphonate (EMS), Ethyl ethane sulphonate (EES), Diethyl sulphate (DS) etc. In the present study, ethyl methane sulphonate (EMS) was used to generate mutation for the selection of plants with desired characters. The alkylating agents induce mutation by adding either methyl or ethyl group to one of the four DNA bases, leading to the altered base formation. Among these bases, beside the mutagenic bases, toxic bases (3-alkyl adenine) are also produced. When such toxic bases are inserted in template DNA, DNA replication is blocked (Britt, 1996) and therefore, suppress cell division and differentiation.
EMS is a chemical mutagen of the alkylating group and has been commonly used in plant breeding because it can cause high frequency of gene mutations and low frequency of chromosome aberrations (Van Harten, 1988). It has been used to treat in vitro explants of many species (Duron 1992) and efficient mutagenesis of plant cell culture is possible, as the cells can be uniformly treated. Compared with physical agents, chemical mutagens are perhaps more capable of leading to specific and predictable mutations. EMS is one of many stress elements, and its stimulative effect on plant regeneration in the culture of somatic tissue has been reported (Pius et al. 1994). Stress treatment stimulates initiation of embryogenesis in microspore cultures (Touraev et al. 1997). EMS is a potent and efficient mutagen to cause GC-AT transitions, although it does produce some small deletions and other chromosomal rearrangements (Haughn and Somerville, 1987; Anderson, 1995; Greene et al. 2003). EMS mutagenesis is a standard technique for induction of point mutations (Greene et al. 2003). TILLING (Targeting Induced Local Lesions in Genomics) is now a popular technology to screen point mutations with EMS mutagenized plants (McCallum et al. 2000). EMS has been also used to induce mutations in mature seed and cell suspension cultures of soybean (Wilcox et al. 1984; Fujii and Tano, 1986). Further, Van et al. (2005) describe the generation of many super-hypernodulating soybean mutants by EMS mutagenesis with mature seeds from three different soybean genotypes. The use of DMSO increases absorption and penetration into tissues of dissolved drugs and other chemicals (Leake, 1967; Leonard, 1967; Siddiq and Majid, 1969) and also enhances the germination of certain tree seeds (Smale, 1969). Because of its phenomenal ability as a biological tissue penetrant, study were undertaken to determine the effect of DMSO on the mutagenicity of EMS in C.intybus. Our results indicated that the presence of dimethylsulfoxide (DMSO) greatly enhanced uptake of EMS into the apical meristematic dome, leaf primordial and tissue which is supported by the findings of Omar and Novak (1990) who studied the effect of DMSO on in vitro plant regeneration and EMS uptake in somatic embryos of date palm.
Effect of EMS on seed germination

The results of the study indicated highly significant differences (P<0.05) for seed germination and the percentage response for seed germination was found to be maximum (85%) on Msx1/2 medium followed by MS basal medium. The seeds treated with different EMS concentrations were cultured on MSx1/2 medium and % response for seed germination decreased with increase in concentration of EMS, however the no. of leaves per seedling and length of seedlings were found to be maximum on MS x1/2 and our results are in agreement with Cheng and Gao (1988) who reported a significant decrease in the germination percentage in barley seeds when treated with sodium azide. Turkan et al. (2006) also reported the effect of NaN$_3$ on seed generation of four pea cultivars and found that seed germination decreases with increase in mutagenic concentration baring from few exceptions in which authors have reported no significant decrease in seed germination resulting from application of EMS (Dhamayanthi and Reddy, 2002). Kumar and Mishra (2004) reported that in okra (Abelmoschus esculentus) germination percentage generally decreased with increasing doses/concentrations of gamma rays and EMS. Reduced germination percentage with increasing doses of gamma radiation has also been reported in Pinus (Thapa, 2004); Rye (Akgun and Tosum, 2004); Chickpea (Khan et al. 2005; Toker et al. 2005). Gradual reduction in germination percentage was also observed with an increase in concentration of mutagen, reaching more than 50% lethality at 0.5% EMS in two genotypes of tobacco (Amernath and Prasad, 1998). Reduction in seed germination with EMS might be due to the increase in production of active radicals responsible for seed lethality. The total DNA content and bonds intension decreased with increasing EMS concentrations which is anticipated main effect of EMS on viability of barley seeds (Jyoti et al. 2009). Higher exposures of mutagens may also cause injury in seeds (Mehetre et al. 1994) and usually shows inhibitory effects on seeds of angiosperms and gymnosperms (Akhaury and Singh, 1993; Thapa, 2004) or damage to chromosomes and mitotic retardation (Al Safadi and Simon, 1996).
Effect of EMS on callusing

The percentage callusing was found to be maximum on lower EMS concentration and it decreased with increasing EMS concentration followed by browning of callus. A marked difference in morphological appearance of callus was noticed with increasing EMS concentrations. Our findings are in line with Svetleva et al. (2005) who reported that after treatment of leaf petiole explants by EMS and N-nitroso-N’-ethyl urea (ENU) influenced callus growth of common bean by increase in callus weight at each subculture on a fresh medium. Vu Duc Quang et al. (1988) also studied the effect of mutagenic treatment of rice (Oryza sativa) panicles at the uninucleate pollen stage and found that the lowest concentrations of the mutagens stimulated callus induction and its growth. Junaid et al. (2008) reported low EMS concentrations on in vitro developed nodal stem of Dracena sanderiana significantly increased callus induction and biomass production. Gahukar and Jambhule (2000) also found similar type of decrease in callus obtained with increased dose of gamma rays and EMS in sugarcane. Similar decrease in callus fresh weight was observed in castor bean and sugarcane (Reddy et al. 1987; Singh and Singh, 1993). Higher doses of mutagens caused considerable tissue damage, which perhaps in turn leads to reduction in callus fresh weight. This reduction in fresh weight may be caused by the reduced amount of endogenous growth regulators, especially the cytokinins, as a result of break down due to irradiation. The chemical mutagens mediated reduced survival and regeneration of the calli could be linked to the production of toxic products (Sedgewick and Vaughan, 1991). Vu Duc Quang et al. (1988) studied the effect of mutagenic treatment of rice (Oryza sativa) panicles at the uninucleate pollen stage and found that the lowest concentrations of the mutagens stimulated callus induction and its growth. Moustafa et al. (1989) also obtained dependence between applied doses of gamma irradiation and ENU on cultured maize callus growth and plant regeneration.

Effect of EMS on shoot regeneration:

At lower concentration of EMS, regeneration potential of in vitro raised nodal explants was improved as compared to control. The maximum average numbers of shoots were observed to be 68 at 0.8% v/v EMS as compared to the control however shoot length
showed a sharp decrease with increase in EMS concentrations. Our findings were in line with Moustafa et al. 1989 who reported low doses of $\gamma$-radiation and N-nitrose-N’-ethylurea (ENU) on cultured maize favored plant regeneration. Junaid et al. (2008) also reported that callusing which was achieved from nodal stem segment explants of D. sanderiana treated with various concentrations of EMS (0.2-1.6 cm$^3$ m$^{-3}$) on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D 1.5 gm$^{-3}$) showed a high regeneration potential and an increase in number of shoots and significant difference in shoot length was noticed when compared to control and sometimes started to be uncountable due to rosette of microshoots with stunted shoot growth similarly Moustafa et al. 1989 reported low doses of $\gamma$-radiation and N-nitrose-N-ethylurea (ENU) on cultured maize favored callus growth and plant regeneration. Kumari et al. 2007 reported decreased regeneration of Citrus jambhiri Lush with increase in EMS conc. The reduction in length of root and shoot was attributed to the effects of mutagens on the physiological system (Gaul, 1977) such a reduction in length of root and shoot arising out of mutagenic treatments was previously reported in crop plants (Reddy and Gupta, 1989; Amarnath and Prasad, 1998; Uma and Salimath, 2001). The stimulatory effect was observed in lower doses/concentrations of gamma rays and EMS on the length of root, shoot and seedling. The hypothetic origin of these stimulations by irradiation and EMS treatments was due to in cell division rates as well as an activation of growth hormone e.g., auxin (Gunckel and Sparrow, 1961; Zaka et al. 2004).

**Effect of EMS on rooting:**

The inhibitory effect of mutagens on the length of seedling was evident from the decrease in length of roots with increasing concentration of EMS however the stimulatory effect was observed in lower concentrations of EMS on the root development and length of roots. The maximum average numbers of roots 20.1 were observed at 0.4% v/v EMS concentration as compared to the control. Cemalettin et al. 2006 also reported in four cultivars of pea after irradiated with Gama rays the decrease in root development and root length with increase in mutagen concentration. Seeds of three varieties of tomato (Lycopersicon esculentum) Mill were treated with
Diethylsulphate (DES) and both seed germination and root length decreased with increase in mutagen concentration (Aliyu and Adamu, 2007). Gamma rays and EMS was drastically reduced the length of root, shoot, seedling and vigour index in J. curcas at higher doses / concentrations. Similar observations were made by several workers in sunflower (Giriraj et al. 1990 and Jayakumar and Selvaraj, 2003). The hypothetic origin of these stimulations by irradiation and EMS treatments was due to cell division rates as well as an activation of growth hormone e.g. auxin (Gunckel and Sparrow 1961; Zaka et al. 2004). The reduction in length of root and shoot was attributed to the effects of mutagens on the physiological system (Gaul, 1977) such a reduction in length of root and shoot arising out of mutagenic treatments was previously reported in crop plants (Reddy and Gupta, 1989; Amarnath and Prasad 1998; Uma and Salimath, 2001).

**Effect of EMS on pigment content**

The results obtained showed that the overall pigment contents were found in higher concentrations in field grown plants as compared with in vitro raised plants. Further it was incurred that among the EMS treated in vitro raised plantlets, the pigment concentrations i.e. chl. a, chl. b, Total chl. and Carotenoids were found in higher amounts at lower doses of EMS. Asmahan, (2006) studied the mutagenic treatments of tomato with 2,4 K/rad gamma rays and 0.001 ml/l NaN$_3$ and reported an increase in chlorophyll a and b of about 80.28% and 40.93% respectively and carotenes 85.71% with respect to control. However, in present study, the chlorophyll content showed irregular distribution among the EMS treated plantlets. This result is supported by Kim et al. 2004 whereby chlorophyll was found insensitive to low doses of gamma irradiation. The concentration of chlorophyll a was relatively higher than chlorophyll b in both EMS treated and untreated plantlets. Strid et al. 1990 has reported that gamma irradiation resulted in greater reduction in the amount of chlorophyll b as opposed to chlorophyll a. The reduction in chlorophyll b may be due to more selective destruction of biosynthesis or degradation of chlorophyll b precursors (Marwood and Greenberg, 1996). Abu et al. 2005 stated that an increase in chlorophyll a, b and total chlorophyll levels was observed in Paulownia tomentosa plants that were exposed to gamma irradiation. However, their contents decreased with increasing EMS concentration. The
incidence of chlorophyll mutation induced by EMS was mentioned already by Natrajan and Upadhya (1964). The effect of mutation on chlorophyll and starch was also studied by Joseph et al. 2004 and they found less chlorophyll in Cassava. These results were almost in agreement with those of Asmahan, (2000); Osama, (2002), who reported that the improvement of yield components and chlorophyll parameters in various plants such as tomato, maize, rice and wheat was induced after various mutagenic treatments such as EMS, NaN₃ and gamma rays.

**Effect of EMS on sugar content**

In present investigation, the soluble sugar content showed maximum value of 35.72±0.79 mg/g in EMS treated in vitro raised shoots at concentrations of 0.8%. The total sugar content was found in lower concentration in callus as well as in shoots of field grown plants which were supported with the findings of Junaid et al. (2008) who reported marginal increase in sugars at lower EMS treated lines (ET1 and ET2) of Dracaena sanderiana. This increase might suggest delay of senescence. In addition to essential roles as substrates in carbon and energy metabolism and in polymer biosynthesis, sugars have important hormone like functions as primary messengers in signal transduction. Biochemical, molecular, and genetic experiments have supported a central role of sugars in the control of plant metabolism, growth, and development and have revealed interactions that integrate light, stress, and hormone signalling (Roitsch, 1999; Smeekens, 2000; Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002) and coordinate carbon and nitrogen metabolism (Stiff and Krapp, 1999; Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). Variations in the environment can decrease photosynthetic efficiency and result in sugar limited conditions in parts of the plant, which down-regulate biosynthetic activity to conserve energy and protect cells against nutrient stress while up regulating starch degradation and protein and lipid catabolism to sustain respiration and metabolic activity (Yu, 1999; Fujiki et al. 2001). Soluble sugar has been postulated to act as regulatory molecules that help to control seed and embryo development (Borisjuk et al. 2004). A statistically significant increase in reducing sugars following low doses of EMS may be due to some changes in starch distribution.
The increase in sugar content supports some changes in the grain starch granules which have been reported earlier (Blaszczak et al. 2002; Gralik and Warchalewski, 2006).

**Effect of EMS on proline**

The proline content increased significantly with increasing EMS concentrations. The higher proline content 63.43 mg/g was found in EMS treated in vitro raised tissues at 1.6% v/v EMS concentration as compared with control used. Proline has been earlier shown to act as a compatible osmolyte and its increased production confirms osmotolerance in plants (Nanjo et al. 1999). Carletti et al. (2003) reported that proline may play a regulatory part in plant metabolism and even small increase in proline biosynthesis might have a large impact on the extent to which the cellular NADP pool was reduced. Proline accumulation may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy needed for recovery. High levels of proline synthesis during stress may maintain NAD (P) + NAD(P)H ratios at values compatible with metabolism under normal conditions. The increased NADP+/NADPH ratio mediated by proline biosynthesis is likely to enhance activity of the oxidative pentose phosphate pathway. This would provide precursors to support the demand for increased secondary metabolite production during stress as well as nucleotide synthesis accompanying the accelerated rate of cell division upon relief from stress, when oxidation of proline is likely to provide an important energy source for ADP phosphorylation (Hare and Cress, 1996).

**Effect of EMS on protein content**

There was quite significant difference among the plantlets treated with varying concentrations of EMS compared with control at P<0.05. The protein content at lowest EMS doses showed increment comparatively with the control used in both the invitro raised shoots and callus formed. Biochemical differentiation based on total soluble protein content revealed that plantlet treated at 0.8% v/v EMS contain the highest amount of total soluble protein, 5.46±0.70 mg/g fw, whereas only 3.91±0.30 mg/g fw of total soluble protein was detected at 1.6% EMS. The control plantlets exhibited significantly lesser total protein content than those of EMS treated plantlets at lower concentrations. However, the total soluble protein content in shoots of the field grown...
plants was lesser than in in vitro raised shoots (with and without EMS treatment) which significantly differed from one to another. In vitro raised aerial shoots of the C. intybus were analyzed by 10% SDS-PAGE against the aerial shoots of the field plant as control. 5.0 ug/ml of total protein was loaded in each well. The expression of proteins was quite distinct and nearly same in all treatments groups as well in the control group. However, in vitro raised shoots with EMS treatment 0.4% v/v & 0.8% v/v showed higher expression of ~80KDa protein as compared to other treatment groups as well as control. The in vitro raised shoots also showed higher expression of lower molecular weight proteins <25KDa at all treatments compared to the control. The medium range marker (18.4-116KDa) was used as reference.

Besides being an integral factor for plant growth, proteins are involved in signal transduction, anti-oxidative defence system, anti-freezing and heat shock, anti pathogenesis or osmolyte synthesis which is essential for plant development and physiology (Gygi et al. 1991). In all the EMS treated mutagenic lines, a significant increase in protein was noticed with low concentration which started to decrease at higher doses that may have been due to activation of enzymes and release of energy (in form of packets ‘photon’) throughout the growth period, especially because they are all involved in the biosynthesis of amino acids, the building blocks of proteins. It may be also due to a decreased stomatal conductance, intercellular CO₂ concentration, contents of photosynthetic pigments and inactivation of photosynthetic enzymes including Rubisco (Ribulose-I, 5-biphosphate carboxylase/oxygenase), the key enzyme in photosynthetic carbon reduction cycle (Seemann and Sharkey, 1986; Miteva et al. 1992). The inhibition in photosynthetic mechanism produces a lower pool of RuBP that may lower ATP formation and reduction in the rate of electron transport. Rubisco enzyme is thought to be involved in the process of plant growth development and productivity (Miteva et al. 1992). Phillips et al. (1994) reported that several proteins are synthesized and accumulated in plant tissues under a range or stress conditions. Such proteins referred to as stress proteins have been noted to be induced in response to several stress factors. Humera and Javed (2010) stated that the stress reaction of plants often results in the alteration of protein metabolism. The most crucial function of plant cell is to respond to chemical mutagen stress by developing defenses mechanisms. This
defense was brought about by alteration in the pattern of gene expression (Corthals et al. 2000). This led to modulation of certain metabolic and defensive pathways (Zolla et al. 2008). Owing to gene expression altered under mutagens, qualitative and quantitative changes in total soluble protein content was obvious (Corthals et al. 2000). In our study, high concentrations of EMS reduced protein content and this reduction may be due to the stress proteins or over production of proline. The stress reaction of plants often results in the alteration of protein metabolism.

**Effect of EMS on enzyme activities**

In present study, the EMS treatment significantly enhanced the enzyme activity in the shoots of C.intybus. The maximum activity of SOD (0.77 EU mg\(^{-1}\) protein h\(^{-1}\)), GR (0.25 EU mg\(^{-1}\) protein) and APX (4.67 EU mg\(^{-1}\) protein) was recorded in in vitro raised shoots at 0.8% v/v EMS concentration as compared to control and with further increase in EMS concentration there was fall in all enzyme activities. However, the enzyme activities in shoots of field grown plants were low as compared to EMS treated in vitro raised shoots.

In order to counteract the oxidative stress, the plants have developed intracellular defense strategies. These strategies are represented by an enzymatic and a non enzymatic antioxidant system. The non enzymatic system includes ascorbic acid, \(\alpha\)-tocopherol, carotenes, polyphenols, flavones and the enzymatic system includes superoxide dismutase, catalase, peroxidase, ascorbate oxidase, glutathione reductase and polyphenol oxidase. The function of these antioxidant systems relies in the prevention of formation or in the destruction of toxic radicals formed during the oxidative stress, thus ensuring the survival of plants in improper conditions (Omar, 1988).

In plants, antioxidant enzymes such as superoxide-dismutase, peroxidase and catalase are seen as the defensive team, playing the role of protecting the cells from the injuries caused by oxidative stress (Mittler, 2002). The increase of the activity of these enzymes represents the most common pathway that leads to the elimination of the reactive oxygen species.

In the present study, an attempt has therefore been made to study oxidative stress-induced adaptive response against EMS. Oxidative stress is also known to invoke genomic responses that include inducible global adaptive responses involving changes in
gene expression that provide cellular protection and adapt cells to a variety of hostile environments, including genotoxins (Garrison, 1987). Inducible adaptive responses have now been observed at every level from the repair of DNA damage (Predieri, 2001) to the induction of antioxidant enzymes such as superoxide dismutase (SOD), Catalase, Peroxidase as well as small molecules, such as glutathione, a-tocopherol and ascorbate that scavenge reactive oxygen species before they cause damage (Pollard, 1964; Filali, 1997). In the present study, EMS induces Peroxidase, SOD and GR activities in in vitro raised shoots of C. intybus which were supported by the findings of Mierlici et al. (2011) who reported that EMS treatments produced an obvious increase in the superoxide-dismutase, catalase and peroxidase activities in Barley plants, as a consequence of the oxidative stress caused by the EMS. These enhancements of these activities represent some of the biochemical markers of oxidative stress (Garrison, 1987). Activation of the Ascorbate glutathione cycle has been found to be essential in stressed plants to combat oxidative damage (Alscher et al. 1997). The increase in SOD activity in all EMS treatments may be attributed to the increased production of ROS (Somashekariah et al. 1992) or increased expression of gene encoding SOD (Bowler et al. 1992). However a reduction in SOD activity at higher concentrations may be attributed to the inactivation of enzyme by H$_2$O$_2$ which is produced in different cellular environments and also from a number of enzymatic and non enzymatic processes in cells (Dixit et al. 2001). The decrease may be also associated with degradation caused by induced peroxisomal proteases or may be due to photo inactivation of enzyme (Sandalo et al. 2001). The increased APX activity under EMS stress depicts its role in the detoxification of H$_2$O$_2$ into water using ascorbate as the electron donor, resulting in the formation of dehydroascorbate. It is recycled back to ascorbate using reduced GSH as an electron donor and the oxidized glutathione (GSSG) is converted back to GSH by NADPH dependent enzyme glutathione reductase (Asada and Takahashi, 1987). The participation of GR in this pathway which is activated upon EMS stress as has also been observed in our study, is the best documented role for this enzyme (Chaoui et al. 1997). These enzymes provide antioxidant protection and preserve membrane integrity. The removal of H$_2$O$_2$ produced in chloroplasts is essential to avoid inhibition of the Calvin cycle enzymes (Tanaka et al. 1982).
Effect of EMS on Esulin content

Taking into consideration the fact that esulin compounds are important bioactive secondary metabolites with a narrow margin of safety between therapeutic and toxic dose, quality control of esulin drugs/extracts is very necessary. In this direction we have carried out the estimation of esulin in C.intybus using a simple and reliable HPLC method. The HPLC method used by Ohta et al. (1988) was followed in the present studies. A gradient reversed-phase high-performance liquid chromatography (HPLC) method using a C30 column was also developed for the simultaneous determination of astaxanthin, astaxanthin monoesters and astaxanthin diesters in the green algae Chlorococcum sp., Chlorella zofingiensis, Haematococcus plu-vialis and the mutant E1, which was obtained from the mutagenesis of H. pluvialis by exposure to UV-irradiation and EMS and they have reported higher amounts of esters in lower treated lines (Juan et al. 2008). However, the mobile phase composition has been changed to meet the requirements for the development of completely resolved chromatogram. In the present study, estimation of esulin content was carried out in treated in vitro raised tissues as well as in field grown plant parts (aerial part as well as root). The highest amount of esulin was found in roots of field grown plants (0.032 µg) followed by roots of in vitro raised plants (0.029 µg) however, among the treated extracts, the esulin content was found maximum at lower EMS concentrations, but its content decreased at higher concentrations. The callus was reported to contain the least amount of esulin. Similarly, Kumari et al. (2007) has reported higher amount of esulin content in the in vitro roots of C.intybus. Rehman et al. 2003 has also reported the esulin accumulation was higher in in vitro plants of C.intybus. Esulin and cichoriin have earlier been detected in Taraxacum and Cichorium species (Williams et al. 1996). The rapid multiplication of medicinal plants and aromatic plants can help to solve some problems connected with the pathway of biosynthesis of chemical compounds in plants, and the relationship between organogenesis and the production of secondary metabolites (Bajaj et al. 1988) e.g. flavor compounds in celery tissues were absent in undifferentiated callus but were produced by embryoids (Collin and Watts, 1983). C.intybus L. contains large number of compounds, which are pharmaceutically very important. The groups of compounds include sesquiterpene lactones, coumarins, flavonoids, anthocyanins, organic acids,
cytokinins etc. George et al. (1999) has shown that esculin is formed from Cichoriin in cell free extracts of C.intybus L. Coumarins are also reported to accumulate in various desired plants (Murray et al. 1983) and this protocol has been employed successfully for inducing coumarin synthesis in parsley and intracellular signalling system has been proposed for rapid induction of coumarin accumulation (Tietjen et al. 1983). Our results were further supported by the findings of Chauhan and Kumar, (2007) who reported that alkaloid content was quite high in plant parts of Phyllanthus niruri raised from EMS treated seeds when compared with that of control.

Understanding the basic network of metabolic intermediates and enzymes is a first step in unraveling secondary metabolism. Subsequent steps should aim at dissecting the regulatory mechanisms that control the enzyme-encoding genes, the mechanisms that control the activities of transcriptional regulators, and the mechanisms of intra- and inter-cellular transport of metabolites and enzymes. Although some progress has been achieved in understanding the transcriptional regulation of the phenylpropanoid and flavonoid pathways. Transcriptional regulators have also been identified in glucosinolate and TIA biosynthesis. The picture is far from complete, however, and how the expression and the activities of these transcription factors are regulated remains largely mysterious (Vom et al 2002). The mechanisms of intra- and inter-cellular transport of metabolic intermediates and enzymes remain poorly understood aspects of secondary metabolism, but Arabidopsis genetics and genetic tools clearly offer promising opportunities for studying this neglected part of plant secondary metabolism (Memelink, 2005).

In general, EMS was efficient in inducing morphogenetic potential regeneration of C.intybus. This might be due to the fact that at small doses, the mutagen enhanced the action of auxin because the cells divided more actively; instead, at higher doses it inhibited cell multiplication (Martin 1945). This phenomenon of synergism may be involved in the response of C. intybus. However, in regard to this particular position of mutagens upon morphogenetic processes in plant materials, only few reports exist (Barnabas and Kovacs 1990; Zaki and Dickinson, 1995; Barnabas et al. 1999). Moreover, a close correlation between EMS concentration and morphogenetic and
biochemical responses have been observed. Moreover, it is possible to hypothesize a relationship between these morphogenic effects of EMS and a stress condition resulting by the poisonous action of the EMS itself, as suggested by Gmitter et al. (1991) in a work on sweet orange cultures and Zaki and Dickinson (1995) that mention the induction of stress proteins.