DISCUSSION
*Medicago sativa* L. belongs to family *Fabaceae* (*Leguminaceae*) and is commonly known as alfalfa or lucerne. The genus *Medicago* has a wide distribution and comprises of more than 50 species exhibiting both annual and perennial habits. Lucerne is one of the most important and the only forage crop with a very long history, known to have been cultivated before the era of recorded history. Presently, it is grown on an estimated area of more than 33 million hectares in diverse environmental conditions throughout the world (Bolton *et al*., 1972). In India, it is cultivated in Deccan Plateau, humid belt of peninsular India and subtropical regions. In the country, it is the third most important forage crop after sorghum and berseem occupies one million hectare area of the cultivation and provides 60 to 130 tones of green fodder per hectare (Hazra, 1995). It is one of the most productive and high quality forage legume. Majority of lucerne grown worldwide is used to feed livestock and also dehydrated to produce protein supplement to be fed to the animals of all classes. It has also been recognized for its medicinal value to the sick animals. Its importance in cropping systems as a source of nitrogen and for soil improvement has been well recognized.

A number of adaptive features in lucerne, such as, its prostrate growth habit with multiple growing points close to the ground, vigorous regeneration capacity and persistence etc. have been considered quite favorable particularly for the survival under herbivore grazing conditions, as a legume.

Lucerne is a very complex species from the crop improvement point of view. It has some inherent problems for its genetic improvement, such as uncontrolled pollination, inbreeding depression, non-availability of desirable gene pool in the germplasm and lack of seed production etc. Because of these problems, the genetic improvement of this crop adopting conventional breeding methods has been quite difficult and the breeding activities have not resulted in any significant improvement in its yield potential in the last century (Rosellini and Veronesi.)
Biotechnology has gained momentum in almost all the aspects of life sciences during the recent past. It has greatly contributed to the developments in modern genetics and led to extensive use of biological systems for advancements in the improvement of crops leading towards agricultural production throughout the world in a significant manner. Most of these advances and their use in modernizing agriculture primarily stem from their successful use for plant tissue culture systems. The basic foundation of plant tissue and cell culture systems was laid down by Haberlandt (1902) by postulating the concept of “totipotency” in plant cells. However, the establishment of the phenomenon of totipotency could be realized only after the discovery of hormonal regulation of in vitro differentiation and the growth in the cells of higher plants by Skoog and Miller (1957). This was further strengthened by the development of experimental procedures for successful cultures of plant tissues (Reinert, 1959), cell suspension and eventually single cell cultures (Steward et al., 1958) and their differentiation leading to in vitro regeneration of plants.

The concept of totipotency has opened the vistas for several approaches aiming at inducing genetic modifications of plant cells and tissues, which would survive through tissue cultures and be expressed in the whole plant. Plant cell and tissue cultures have provided new options of obtaining increased genetic variability relatively rapidly. The variability generated by the use of tissue culture cycle has been referred as “Somaclonal Variation” and the somaclonal variation is now an established phenomenon (Larkin and Scowcroft, 1981). It has been established in a number of studies that genetic changes do occur in plant tissue cultures and these variations are transmitted to the regenerated plants and their progenies (Larkin and Scowcroft, 1981 and Shepard, 1981). The utilization of novel variations induced in tissue cultures has now become one of the major objectives of tissue culture based crop improvement. A series of reports on a wide range of genetic variability among plants regenerated from protoplasts, cell and callus cultures have stimulated the
interest in this type of variations for their use in genetic improvement of different crops (Shepard et al., 1980; Shepard, 1981, 1982 and Thomas et al., 1982). Majority of plant traits of agronomic value for which genetic variability is generated through tissue culture may provide valuable adjunct to the crop improvement. The heightened interest in somaclonal variation has emerged in part from the fact that modern genetically sophisticated cultivars could be improved by generating novel variations within co-opted gene complexes in a sequential manner by selection and for screening of plants regenerated through tissue culture and their progenies. Much of variation generated during tissue culture has been demonstrated to be heritable in many agronomically important plants together with its possible value in upgrading the genetic base in valuable varieties. The increase in genetic variability through tissue culture was first achieved in sugarcane followed by rice, wheat, lettuce and tomatoes (Larkin and Scowcroft, 1981).

Although the tissue culture of most of the species of agriculture interest has been possible, yet relatively few such species of one of the most important group, Leguminaceae have exhibited reproducible and efficient regeneration systems. Modern biotechnological methods, such as somaclonal variation, somatic hybridization and genetic transformation are becoming gradually very important in genetic improvement of lucerne. Optimization of tissue culture and regeneration protocols is an essential component for the successful application of tissue culture cycle in crop improvement. M. sativa has been found amenable for in vitro plant regeneration in a number of studies (Saunders and Bingham, 1972; Parrot and Bailey, 1993 and Moursy et al., 1999). Nevertheless, since each lucerne plant behaves genetically different due to high heterogeneity, much genotypic variation exists for in vitro response in this crop. Lack of regeneration potential among the target germplasm sources might have been an early limitation to exploit somatic cell genetic improvement in lucerne. Genotypes and explants have been identified as important factors that influence in vitro regenerating potential of lucerne (Walker et al., 1978 and Meijer and Brown, 1987). The existing technology is not yet adequate for regenerating full range of lucerne germplasm. Application of biotechnology for the improvement of popular Indian cultivars has not been attempted so far. Thus.
In view of this background the present study was conducted in eight genotypes of *M. sativa*. The first task was to optimize *in vitro* callus production, regeneration of plants and their establishment in the field. The second task to was evaluate the *in vitro* regenerated plants (somaclones) for variations expressed in terms of important morphological or agronomic traits, isozyme patterns, RAPD polymorphism and forage quality traits.

### 5.1. Optimization of callus induction and morphogenetic responses of callus differentiation and plant regeneration:

A number of factors, such as, nutrient media, adjuvants and growth regulators, genotypes and explants affect callus induction and morphogenetic responses of callus for plant regeneration. Basic parameters for measurement of production of good callus are callus induction frequency and regenerability of callus. In the present study, these parameters were measured as per cent explants responding for callus induction and the quality of the callus was determined in terms of colour and texture of the calli. Callus induction and plantlet regeneration have been dependent on genotype, explant, medium and culture conditions (Vasil, 1986).

#### 5.1.1. Callus Induction:

Excised hypocotyl, epicotyl and cotyledon form *in vitro* germinated seeds were used as explants and they were put aseptically on the culture medium. The observations on callus induction were recorded 20 days after inoculation. Differences with respect to callus induction frequency with respect to genotypes, growth regulators and media were studied.
1.1.1. Effect of media:

Effect of different tissue culture media on callus induction frequency was determined. The callus induction frequency in the present study revealed non significant differences among SH, MS and Blaydes media for all the three explants, namely hypocotyl, epicotyl and cotyledon.

The mean callus induction frequency was recorded maximum on MS medium followed by SH medium while Blaydes medium showed minimum callus induction response. The over all maximum callus induction responses was observed on MS medium from hypocotyls explant followed by callus induction from cotyledon explant on MS and SH medium, both. The minimum callus induction response was observed from cotyledon explant on Blaydes medium. On the basis of callus induction frequency from all the explants MS medium was found to be the best followed by SH medium.

In case of callus quality, there was no over all differences in the texture of calli raised on the three different mediums. However, the best quality of callus, in terms of its colour, was produced on MS medium followed by SH medium. The cotyledon explant exhibited the best quality of callus production followed by epicotyl.

Mariotti et al. (1984) found that MS medium supplemented with NAA and BA was most suitable for callus induction and growth in lucerne out of the four tissue culture media tested. Moursey et al. (1999) suggested that solidified MS medium with 2.0 mg/l 2,4-D and 1.5 mg/l kinetin was the best for callus induction in M. sativa. Arcioni et al. (1990) found MS medium more suitable than B5 medium in maintaining the regenerative capability of the calli in cultures of alfalfa cv. Roboto. However, Walker et al. (1978) observed that callus induction and growth of the calli in lucerne was substantially improved on MS medium as compared to that on Blaydes medium. According to Kim et al. (1999), among SH, MS and N6 medium, the SH medium gave the highest efficiencies in callus formation and plant regeneration. Verga and Badea (1992) also found SH basal medium to be most effective among SH, MS and B5 media for callus induction in lucerne.
1.1.2. Effect of genotype and explant:

Callus induction response from hypocotyl, epicotyl and cotyledon explants in the eight genotypes were observed in the present study. Highly significant differences were observed among the media, genotype and their interactions with regard to callus induction frequency from hypocotyl and epicotyl explant where as in case of cotyledon explant highly significant differences were found among different media compositions and their interaction with genotypes only and no significant differences were detected with genotypes. The maximum callus induction frequency was recorded in the genotype IG-1212 followed by IL-75 from hypocotyl and cotyledon explants where as in case of epicotyl explant the maximum callus induction response was observed in the genotype LLC-9 followed by Anand-2. The minimum callus induction frequency from hypocotyl explant was recorded in the genotype LLC-3 and Anand-2 and from epicotyl explant, it was observed in the genotype A-3 and IG-1212. The cotyledon explant exhibited lowest callus induction frequency in the genotype C-10. Among the different media compositions the highest callus induction response from hypocotyl and cotyledon explants was recorded on MS medium containing 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP where as the maximum callus induction response from epicotyl explant was observed on SH medium supplemented with the same combination of growth regulators. SH medium supplemented with 4.0 mg/l IAA and 1.0 mg/l kinetin showed minimum callus induction response from hypocotyl and cotyledon explants where as the epicotyl explant showed minimum callus induction response on SH medium containing 1.0 mg/l NAA and 1.0 mg/l kinetin. However, no callus induction response was observed in any of the eight genotypes under study on SH medium containing 1.0 mg/l kinetin and 2.0 or 4.0 mg/l IAA from epicotyl explant only where as hypocotyl and cotyledon explants responded for callus induction in all the genotypes, though poorly.

Significant differences were observed for both the colour and texture of the callus from hypocotyl explant in different media and genotype where as in case of epicotyl and cotyledon explant the genotypic and media differences were significant with
respect to the callus colour only and for the callus texture in both these explants, the
genotypic differences were found significant but the media differences were
observed to be non-significant.

The best callus quality in terms of callus colour was observed from both hypocotyl
and epicotyl explants in genotype IG-1212 where as the genotype AL-95-12
exhibited best callus colour quality from cotyledon explant. The best callus texture
was observed in genotype Anand-2 and AL-95-12 from hypocotyl explant. in
genotype C-10, IL-75 from epicotyl explant and in all the genotypes except LLC-3
in cotyledon explant. MS and SH medium supplemented with 2.0 mg/l 2,4-D, 1.0
mg/l NAA and 0.2 mg/l BAP in general showed the best callus quality from
different explants.

In corroboration to the present study, the genotype specificity of the in vitro
response of M. sativa has been described by several authors. (Mitten et al., 1984;
Brown and Atanassov, 1985 and Meijer and Brown, 1985). It is well documented
that alfalfa (M. sativa) exhibited both intervarietal (Bingham et al., 1975; Atanassov
and Brown, 1984; Mitten et al., 1984 and Brown and Atanassov, 1985) and
culture. Brown et al. (1984) reported wide variation among cultivars in the ability to
form callus. They tested 76 lucerne cultivars for callus formation from hypocotyl
and cotyledon explants. Bianchi et al. (1988) observed that the ability to produce
callus varied widely both between and within the lines when petiole and hypocotyl
explants of 20 genotypes of tetraploid and diploid populations of M. sativa and
tetraploid populations of M. falcata were cultured in vitro. The best results from the
experiment were obtained with hypocotyl and petiole explants of the tetraploid
genotypes. Saunders and Bingham (1972) for the first time regenerated plants in
alfalfa from the callus tissue initiated from hypocotyl, internode and ovary explants.
In lucerne, various explants for callus induction and regeneration from different
genotypes have been tried by different workers. Many of them have reported the
superiority of petiole and hypocotyl explants over the others with respect to callus
Mariotti et al. (1984) found that root and hypocotyl explants were better than those from the leaves or cotyledon for producing embryogenic callus. Nam and Heszky (1987) found that callus from hypocotyl, generally, had a higher regenerative ability than that from the other explants. Scarpa et al. (1991) reported that hypocotyl provided the best explants for callus production. Chen et al. (1987) suggested that cotyledons were a good explant source to screen for embryogenic genotypes based upon callus induction response. Hammad et al. (1993) found that leaves as explants always gave the best callus for subsequent regeneration. Okumura et al. (1993) reported that shoot tip followed by hypocotyle explant gave better callus for further regeneration.

Denchev and Atanassov (1988) found a stringent correlation between the stage of development of the initial explant and the process of dedifferentiation. They observed that interaction between explant and media or between explant and cultivars were significant. They also suggested that genotype background was most critical factor than either the medium or the nature of explant for callus induction and further regeneration. Scarpa et al. (1993) also found that the callus induction and regeneration responses were affected by explant source.

5.1.1.3. Effect of growth regulators:

2,4-D, NAA, IAA and cytokinins were the growth regulators for the choice of callus induction from different explant in *M. sativa*. According to Stuart and Strickland (1984), 2,4-D played important role in explant dedifferentiation and induction of callus formation. In the present study, it was observed that different concentrations of these growth regulators in different combinations influenced callus induction from hypocotyl, epicotyl and cotyledon explants differently in the eight genotypes of lucerne under study. The lower concentration of NAA with 1.0 mg/l kinetin was generally found better for callus induction from hypocotyl explant in the genotypes LLC-3, AL-95-12, IL-75, Anand-2 and LLC-9. Application of 2.0 mg/l 2,4-D in
combination with lower concentrations of NAA and BAP both in MS and SH medium showed maximum callus induction response in the genotypes LLC-9, LLC-3, C-10, IG-1212, A-3, Anand-2 and AL-95-12 from hypocotyl explant. This combination of growth regulators also increased callus induction significantly from epicotyl explant in all the genotype except LLC-9. The callus induction response from epicotyl explant also improved considerably with the increase of NAA concentration in the media in the genotypes C-10, IL-75, A-3, LLC-3 and Anand-2. In the cotyledon explant, the extent of callus induction increased with the increasing concentrations of NAA in the genotype IL-75 and A-3, while it decreased in other genotypes such as C-10 and IG-1212. In general, the growth regulator combination of 2.0 mg/l 2,4-D+ 1.0 mg/l NAA + 0.2 mg/l BAP in MS or SH medium was found most suitable for callus induction frequency and the quality of callus from all the three, hypocotyl, epicotyl and cotyledon explant in all the eight genotypes used in the present study.

Kraic et al. (1994) also induced callus from leaf, petiole and stem segments of lucerne on a B5 based medium supplemented with 2,4-D, kinetin and NAA. Musiyaka et al. (1998) observed that the tissues of lucerne cultured on B5 medium with 2,4-D, kinetin and NAA initially lost its morphogenetic potential. induced dedifferentiation leading to callus formation. Wenzel and Brown (1998) obtained good callus form petiole explant of a clone of M. sativa var. Rangelander when exposed for 10 days to 22.6μM 2,4-D and 4.7μM kinetin on MS medium. Kim et al. (1999) found that in four cultivars of alfalfa, among the four media containing the same combination of growth regulators, the SH medium gave the highest efficiency in callus formation when supplemented with 3 mg/l 2,4-D, 5 mg/l NAA and 2 mg/l kinetin. According to Takamizo et al. (1991), BAP was more effective than any other cytokinin in terms of callus growth.

5.1.1.4. Callus growth:

In the present study, the calli were initially raised on MS medium from the three explants of the eight genotype of lucerne and luxuriantly growing calli from these explants were individually selected for callus growth rate experiment. A measured
amount of callus in each case was taken for successive subcultures at a regular interval of 20-21 days on MS medium supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP and maintained up to eighty days. Callus growth rate in terms of fresh weight and dry weight of callus was measured in each case up to eighty days of callus growth. The rate of the callus growth from all the three explants from all the eight genotypes of lucerne exhibited genotype dependant growth behavior.

In case of hypocotyl explant, all the genotypes showed increase in growth of callus with the increasing duration of culture. However, in the genotype Anand-2 alone on the fresh callus weight basis and in Anand-2 and AL-95-12, on the basis of dry weight of the callus, the growth stabilized after 60 days. The maximum growth was observed after 40 days in LLC-9. Regular increase in growth rate was observed in calli of the genotypes LLC-3, C-10, IL-75, IG-1212 and A-3 even up to 80 days on both fresh and dry weight basis. The genotype C-10 had maximum dry matter content in the calli up to 40 days of subculture followed by Anand-2 and IL-75. Maximum dry matter content at 60 days of culture was recorded in IL-75 and maximum dry matter content after 80 days of culture was recorded in genotype LLC-3.

In case of epicotyl explant, all the genotypes exhibited steady and regular growth rate of callus in all the passages of subcultures. The maximum callus growth rate at 40 and 60 days in culture was exhibited by IL-75, whereas the maximum growth rate at 80 days in culture was found in genotype C-10. The maximum dry weight of callus at 40 days was observed in IL-75 and genotype AL-95-12 performed best in terms of dry callus weight at 60 and 80 days in culture. The genotype AL-95-12 had maximum dry matter content accumulated at 40 days of culture while genotype Anand-2 and AL-95-12 showed maximum dry matter content in their calli at 60 days of culture. The genotype AL-95-12 also showed maximum dry matter content in the calli up to 80 days of culture.
In case of cotyledon explants also, callus growth rate in all the eight genotypes increased with increase in days in subcultures on fresh callus weight basis but on the basis of dry callus weight, the callus growth rate increased upto 60 days only. The genotype IL-75 and LLC-3 had maximum dry matter content upto 80 days of culture and the genotype IG-1212 had maximum per cent dry matter content at 60 days and the 80 days of culture.

The results of callus growth rate in terms of fresh and dry callus weights and the per cent dry matter content in the calli in all the eight genotypes during various passages of subculture indicated that there was a marked variation in the response of these parameters as effected by different genotypes. In general, all the genotypes showed steady growth rate upto 40-60 days. There was an initial increase in the per cent dry matter content upto 40 days of culture followed by its gradual decrease till 80 days of callus growth. The optimum callus growth, for all the three parameters studied, was observed between 20 and 40 days of callus cultures.

The variability for in vitro callus proliferation in terms of callus fresh weight, callus growth rate and callus production in lucerne was also found by Brown and Atanassov (1985) in corroboration to the present study. They evaluated numerous lucerne cultivars to determine the role of genetic background in the in vitro cultures of Medicago. A wide range of callus yield was observed among cultivars ranging from 62 mg for cv. Chilan hypocotyl explant to 720 mg for cv. Angus cotyledon. Wide variation for callus growth among genotypes, within some cultivars was also detected. Mroginski and Kartha (1984) also observed that callus initiation and in vitro callus proliferation were dependant upon several factors including genotype, explant tissue, culture medium, the environment under which the explant tissue was grown and other in vitro culture conditions. They observed that genetic variability existed for both callus initiation and in vitro callus proliferation within plants species including M. sativa. In an evaluation of several M. sativa cultivars by Matheson et al. (1990) indicated that all the plants within these populations were not capable of callus formation. The importance of the genetic variance for determining callus production has also been noted in other species such as Zea mays (Beckert and Qing,
5.1.2. Morphogenetic responses of callus differentiation and plant regeneration:

Morphogenetic differentiation towards somatic embryogenesis in *M. sativa* was first reported by Saunders and Bingham (1972) in one plant of cultivar Saranac. Since then, regeneration of somatic embryos in alfalfa has been achieved from cells in suspension cultures (McCoy and Bingham, 1977), long term callus and suspension cultures (Stavarek *et al.*, 1980) and protoplasts (Dos Santos *et al.*, 1980; Kao and Michayluk, 1980; Johnson *et al.*, 1981 and Mezentsev, 1981). In the present study, based on callus induction response, callus quality and callus growth rate, the calli from hypocotyl, epicotyl and cotyledon explants of all the eight genotypes of lucerne under study were cultured on regeneration media for inducing *in vitro* regeneration. The experiments for *in vitro* regeneration were conducted following two pathways, namely, 1) Morphogenetic response of calli directly on the regeneration media and 2) Morphogenetic response of calli on the regeneration media following auxin shock treatment.

5.1.2.1. Morphogenetic response of calli directly on the regeneration media:

MS and SH basal media with different concentrations of auxins and cytokinins were used for studying morphogenetic response of the calli. In total, 48 each of different MS and SH based regeneration media supplemented with kinetin (0.0 mg/l – 8.0 mg/l), BAP (0.0 mg/l – 4.0 mg/l), NAA (0.0 mg/l – 1.0 mg/l) and IAA (0.0 mg/l – 1.0 mg/l), in various combinations were used. The selected calli from various explants of all the genotypes under study were transferred to various regeneration media for inducing morphogenesis towards regeneration.
Different types of morphogenetic responses of callus differentiation and the quality of calli developed on various 96 combinations of MS and SH based regeneration media were observed. Most of the media combinations yielded only different types of callus quality ranging from white, light yellow, yellow, yellowish green, yellowish white and green to little bit brown callus in colour and nodular to friable in texture.

Morphogenetically differentiating types of calli of different colours ranging from white, light yellow, yellowish green, greenish white and green were developed on certain MS and SH based regeneration media combinations containing 0.5 mg/l - 1.0 mg/l kinetin alone or 0.5 mg/l kinetin with 0.5 mg/l BAP together with 0.5 mg/l - 1.0 mg/l NAA or 0.5 mg/l - 1.0 mg/l IAA. MS and SH based regeneration media containing higher concentrations of kinetin ranging from 4.0 mg/l - 8.0 mg/l with or without addition of 1.0 mg/l - 2.0 mg/l BAP together with 0.5 mg/l - 1.0 mg/l NAA or IAA also led to the formation of morphogenetically differentiating types of calli from all the explants in most of the genotypes. However, when MS and SH medium without containing any growth regulators were used as regenerating media, the calli of all the three explants of all the eight genotypes under study turned brown within 20 days of their transfer to these regeneration media without showing any morphogenetic differentiation of any type except in case of the callus of the cotyledon explant of LLC-3 genotype only. This callus from the cotyledon explant of LLC-3 genotype when placed on MS basal regenerating medium, developed somatic embryos within 3-4 weeks after inoculation. These somatic embryos developed through various stages of somatic embryogenesis, however, their subsequent the growth and regeneration into plantlets were arrested.

5.1.2.2. Morphogentic reonse of calli on regeneration media following auxin shock treatment:

The selected calli from different explants of various genotype under study were transferred on auxin shock treatment media for four days following the method of Romagnoli et al. (1996) and then transferred and grown subsequently on four
different regeneration media sequences, namely, SHKI, MSKI, SHPKI and MSPKI, separately for regeneration. The calli grown on SHKI and MSKI regeneration media sequences, developed into various types of quality of calli ranging from yellowish white, yellowish green, light green to yellowish brown and did not show any kind of regeneration response. The effect of basal media was evident on these calli. Greening of calli was observed in MSKI whereas the calli turned yellowish white and subsequently became brown and non regenerative on SHKI media sequence. However, after auxin shock treatment when calli were transferred to proline supplemented MS or SH media (MSPKI, SHPKI), globular shaped somatic embryos were differentiated within 6-7 days of culture and the cultures were full of these globular structures within 18-20 days. These globular shaped somatic embryos also remained arrested at the stage only but when these calli were transferred to kinetin and IAA sequence of MSPKI and SHPKI media separately, the greening of these callus was observed on MS based medium while the callus remained yellowish and became brown on SH based regeneration medium. The green calli of all the genotypes subsequently developed shoot primordia on 4.0 mg/l kinetin and 1.0 mg/l IAA step of MSPKI regeneration sequence. On further increasing the kinetin to 8.0 mg/l in the next step of MSPKI regeneration sequence, shoot bud organogenesis was observed in the calli of hypocotyl explant of the genotypes IL-75, C-10, IG-1212 and AL-95-12, cotyledon explant of A-3 and LLC-3 and epicotyl explant of IL-75 only. At this higher level of kinetin in MSPKI regeneration sequence, the shoots started turning yellowish brown instead of further development of shoots. But, when these developing shoots were transferred to reduced kinetin concentration (2.0 mg/l), healthy green shoot development and shoot bud multiplication was observed. The shoot bud development was improved on their transfer to MS basal medium of MSPKI media sequence. The proline supplementation and the effect of basal medium were found critical as regeneration occurred on proline supplemented MSPKI regeneration sequence only and no regeneration was observed on MSKI or SHKI sequences which were devoid to proline. or even on proline supplemented but SH based SHPKI regeneration media sequence. The regeneration on MSPKI media sequence occurred mostly through shoot bud organogenesis and varied among different explants and genotypes. The regeneration of shoots per calli was maximum
form the calli of cotyledon explant of LLC-3 genotype followed by hypocotyl explant of IL-75. The minimum efficiency of regeneration was observed from the calli of epicotyl and cotyledon explants of IL-75 and A-3 genotypes respectively. The calli from hypocotyl explant showed maximum response for regeneration followed by that of cotyledon and least response for regeneration was exhibited from the calli of epicotyl explant.

Several authors (Meijer and Brown, 1985 and Chen and Marowitch, 1987) have also reported that only a few genotypes in certain cultivars have been found to possess the regeneration capacity. The plant regeneration studies from in vitro cultures have shown that not all lucerne germplasm were able to produce somatic embryos and plantlets, the frequency of induction of somatic embryogenesis also varied among various cultivars (Bingham et al., 1975 and Brown and Atanassov, 1985). This variation was observed even among the genotypes of a single cultivar (Kao and Michayluk, 1981; Phillips, 1983 and Mitten et al., 1984). Even though the frequency of regenerating genotypes within a cultivar was high, much variation existed in the efficiency of regeneration among them (Mitten et al., 1984). This was attributed to the intervarietal and intravarietal heterogeneity in alfalfa. Fuentes et al. (1993) evaluated in vitro embryogenic response in 9 lucerne varieties and found that all the varieties, except San Joaquin II, gave a positive response in one or more of the protocols tested. Arcioni, et al. (1989) suggested that it was necessary to identify and isolate regenerating genotypes before using M. sativa in plant genetic manipulation studies owing to the higher degree of genotypic variation for regeneration. The range of germplasm from which plant regeneration has been reported is, however, vary narrow. The numbers of regenerative genotypes from which plant populations have been established and evaluated are even more limited. The clones from cultivar Regen S developed by Bingham et al. (1975) have been used almost exclusively for regeneration studies (Walker et al., 1978, 1979; Hartman et al., 1984 and Stuart and Strickland, 1984). The regeneration frequency was affected by the interactions, medium x genotype (Brown and Atanassov, 1985) and explant x genotype (Novak and Koncena, 1982). Brown et al. (1984) reported wide variation among cultivars in the ability to form somatic embryos. Somatic
embryo formation occurred only in 34 per cent of cultivars, when 5 medium protocols using 4 cultivars were compared by them. However, Chen et al. (1987) observed that some genotypes produced somatic embryos regardless of medium protocol or explants source. Suginobu et al. (1991) concluded that genotype background was a more critical factor for somatic embryogenesis than either the medium or the nature of the explant. Varga and Badea (1992) suggested that the application of tissue culture techniques for alfalfa improvements required screening within specific cultivars in order to identify genotypes capable of regenerating entire plants.

In lucerne, various explants for callus induction and regeneration from different genotypes have been tried by different workers. Many of them have reported the superiority of petiole and hypocotyl explants with respect to regenerability (Piccioni et al., 1996). Lupotto (1983) induced cyclic production of embryoids through secondary embryogenesis from cells derived from the hypocotyl callus. The high probability of predicting of embryogenic genotypes based upon cotyledon callus suggested that cotyledons were a good explant source to screen for embryogenic genotypes (Chen et al., 1987). Okumura et al. (1993) reported that shoot tip explant had better ability to undergo somatic embryogenesis. However, the competence of the different tissues in the regenerative pathway has not been completely understood (Finstad et al., 1993). Scarpa et al. (1993) found that hypocotyl derived callus were the best regenerating tissue in *Medicago polymorpha*. The concentration and combination of growth regulators have been known to govern plant regeneration. For most the legumes, the early stages of somatic embryogenesis have been induced by exposure of tissues to 2,4-D with or without association of other growth regulators (Ammirato, 1983), which was in corroboration with present study. In many legumes, as was observed in the present study also, the removal of 2,4-D and exposure to media lacking hormones or with various combinations of cytokinin and other auxins has led to maturation of embryoids and shoot formation. The inductive role of 2,4-D in regeneration of organogenic shoot buds and somatic embryo formation has been demonstrated by several authors (Saunders and Bingham, 1975; Walker, et al., 1979 and Walker and Sato, 1981).
The induction periods varied with the concentration of 2,4-D and plant species which ranged from 6 months Mitchell cultivar of soybean (Christianson et al., 1983) to as little as 3 to 4 days for alfalfa (Walker and Sato, 1981 and Brown and Atanassov, 1985). Finstad, et al. (1993) also demonstrated the requirement for the acquisition of competence prior to the induction of embryogenic pathway. In a study by Shetty and McKersie (1993), somatic embryogenesis was induced in petiole derived culture in the presence of 1mg 2,4-D and 0.2 mg/l kinetin and embryo elongation occurred when embryogenic calli were transferred to hormone free medium. Lupotto (1983) showed that although the initiation of somatic embryogenesis started on simple hormone-free medium, a more complex medium with yeast-extract was needed to propagate embryogenic cultures. However, the induction phenomenon was not found specifically to 2,4-D only (Kao and Michayluk, 1981) as NAA has also been observed replacing 2,4-D in several cases. Won et al. (1999) found that somatic embryos were formed from callus obtained from hypocotyl explants of M. sativa cv. Vernal on MS medium containing 4 mg/l 2,4-D and 0.1or 0.5 mg/l kinetin. Nolan et al. (1989) reported that induction of embryo formation occurred on a medium containing 10µM NAA and 10µM BAP and embryo maturation was promoted after transfer to a medium containing 1µM NAA and 10 µM BAP and shoot development or occasional plantlet development occurred on a subsequent transfer to 0.1 µM NAA and 1µ M BAP. Zhang et al. (1995) cultured callus of M.sativa cv. Jining in liquid MS medium containing 4 mg 2,4-D and 1 mg BA/litre. Leaves and buds were induced after subculturing. Takamizo et al. (1991) concluded that the UM medium containing 4mg/l 2,4-D supplemented with BAP rather than kinetin was more effective for somatic embryogenesis in hypocotyl derived calli of M.sativa cv. Tachiwakaba. Parrott and Bailey (1993) produced somatic embryos in five genotypes out of the callus cultures of 300 genotypes of M.sativa on Blaydes medium containing 10.74 µM NAA, 11.42 µM IAA and 9.29 µM kinetin from leaf, petiole and internodes explants. Shao et al. (2000) developed two protocols for the regeneration of tetraploid lines of alfalfa. In the first regeneration system, leaf explants were
incubated on MS medium supplemented with 2,4-D and kinetin for callus formation and subcultured onto growth regulator-free MS medium to induce direct somatic embryogenesis. In the second regeneration system, the inoculated explants were incubated on B5 medium with hormones to produce somatic embryos via embryogenic callus. Saunders and Bingham (1972) regenerated plants in alfalfa from callus tissue initiating from hypocotyl, internode and immature ovary through both organogenesis and embryogenesis following a two step method. The calli were developed on B5 basal medium in lucerne, morphogenesis was induced on the same medium containing benzyl adenine phosphate with subsequent placement on a hydrate with growth regulators which ultimately led to formation of shoots (Kraic et al. 1994). Saunders and Bingham (1972) observed that the callus on its transfer to a medium containing inositol and yeast extract differentiated into shoot buds profusely and most of this grew into plants. According to Romagnoli et al. (1996) observed that the best media for somatic embryo production was MS medium + 10 μM 2,4-D + 4.6 μM kinetin. After the auxin shock, when the calli were transferred to MS medium containing 10-20 mM NH₄⁺ and 30 mM proline, a large number of somatic embryos were produced which were grown to plants on MS or half strength of MS medium. Addition of 10-25 mM proline and 0.1-0.5 mM thioproline in the medium stimulated 2,4-D induced somatic embryogenesis. Stuart and Strickland (1984 a, b) also found that the addition of L-proline to SH medium increased embryoid formation. Further development of embryoids occurred in media supplemented with either yeast extract or in MS based media containing high levels of nitrogen. Present study has been in confirmation on with that of Stuart and Strickland (1984) who also observed that the proline-enhanced regeneration of somatic embryos. The Optimum regeneration occurred when 10mM proline and 25mM ammonium was added to the regeneration medium. Walker and Sato (1981) suggested that exogenously supplied ammonium ions were critical to in vitro morphogenesis. Shetty and McKersie (1993) suggested that addition of proline, thioproline and potassium enhanced the size of embryogenic callus significantly subsequent embryo formation along with higher number of cotyledonary embryos.
In corroboration to the present investigation, Walker et al. (1978) also obtained regeneration through shoot bud organogenesis in *M. sativa* L. cv. ‘Regen’ by transferring callus from induction medium containing growth regulators to a regeneration medium lacking of growth regulators. They suggested that the determination of organ type occurred principally on regeneration medium. During organogenesis in alfalfa, the process of organ induction might initiate on induction medium and the determination of organ type had taken place on regeneration medium. Churova (1981) observed that growth centers were induced and stalk meristems differentiated and developed into rootless plants (shoot stocks) in MS medium as in case of present study also. However he also successfully attempted to induce shoot bud organogenesis in callus cultures of *M. sativa* on Blaydes medium. Musiyaka et al. (1998) cultured callus of *M. sativa* on B5 medium with 0.5mg/l 2,4-D and 50mg NAA/l and restored morphogenetic potential. Buds and roots formed after 30 days of culture on a medium inducing morphogenesis. Nikolic et al. (1985) observed that callus induction, bud formation, shoot elongation and rooting in lucerne were obtained on sequence of media with different hormone additions.

Bingham et al. (1975) observed that embryogenic capacity was controlled by two genetic loci and it was possible to incorporate it into cultivars by means of recurrent selection. Increase regeneration was found in the Regen S cultivar of alfalfa, developed by two cycles of recurrent selection for regeneration capacity. Reisch and Bingham (1980) found that in diploid alfalfa, bud differentiation from callus by controlled by two dominant gene and both were required to present in order to obtained more than 75 % regeneration. Wan et al. (1988) studied 7 tetraploid lucerne cultivars and they also suggested that regenerability in this system was controlled by 2 complementary dominant genes, both were necessary for regeneration. Gene dosage influenced regeneration efficiency and cytoplasmic effects influenced the interaction between callus induction medium and regenerability. Walton and Brown (1988) found evidence of cytoplasmic inheritance for extent of embryogenesis in 2 reciprocal crosses of *M. sativa*.
5.1.2.3. Histology of morphogenetic responses

In the present study, morphogenetic potential towards regenerability of different types of calli was observed and it was found that only whitish or greenish white and granular or nodular types of calli were capable of regeneration. The cell masses among the calli potentially capable of morphogenetic differentiation were composed of compact and round shaped cells of comparatively small and uniform size with densely staining cytoplasm. These regenerating cells were without intracellular spaces and exhibited meristematic activity. These types of cells were also noticed in regenerating calli by Kohlenbsch (1978) and Vasil and Vasil (1982). Vasil and Vasil (1981), observed that embryogenic calli were characteristically compact, much organized and white to pale yellow in colour. These regenerating calli when induced for morphogenetic differentiation on various regeneration media exhibited both pathways of regeneration, that is, via somatic embryogenesis and shoot bud organogenesis. The most frequently noted morphogenetic response in the present study was the organogenic induction of shoot buds while somatic embryogenesis was quite ephemeral. The histological examination for occurrence of somatic embryogenesis and shoot bud organogenesis in the present study revealed that the developing somatic embryos exhibited bipolar meristematic structures with a plumule region at the distal end and radical region at the proximal end of these structures. Such somatic embryos were largely globular, heart shaped or torpedo shaped, having cotyledonary protuberances. The most commonly occurring and stable morphogenetic response towards the regeneration from the regenerating calli was through somatic bud organogenesis. The histological by such unipolar meristematic outgrowths appeared on the surface of these calli during organogenesis which soon assumed the structure of shoot buds. The regenerating shoot buds were typically by similar to the vegetative shoot apex in their morphology and histology examination having clearly discernible tunica-corpus organization and bearing laterally subtending leaf primordia. Such shoot buds developed in to vegetative shoots bearing leaves laterally and were like shoot stumps, which had vary little or no shoot primordia. Similarly, morphogenetic structures developing in the regenerating calli were also observed by Bond and
Webb (1989) in their study with *Trifolium repense*, Ammirato (1983), also found the formation of bipolar structures, resembling zygotic embryos, with both a shoot and root meristem during their study of somatic embryogenesis in lucerne as opposed to organogenesis in which only shoot and root formation was induced. Initially, somatic embryos were thought to arise exclusively from single cells (Haccius, 1978). Histological examination of embryogenic tissues showed that the embryos arose from single cells of the parental explant (dos Santos et al., 1983). Dos Santos et al. (1983) also suggested that embryoids were organized from groups of apparently homogenous meristematic cells on organised callus, white or friable callus and were originated from single embryogenic cells distinguishable from the nonembryogenic cells by staining reactions. They provided evidence for the origin of embryoids from single cells in the epidermis of cotyledons, hypocotyls and roots of induced plantlets as well as in friable callus of *M. sativa*. Lantcheva et al. (1999) developed somatic embryos from *M. truncatula, M. littoralis, M. murex* and *M. polymorpha* and confirmed the nature of the directly formed somatic embryos by histological analysis.

5.1.2.4. Rhizogenesis:

The shoots regenerated on MSPKI regeneration medium sequence from the calli of various explants in six genotypes, LLC-3, IL-75, A-3, C-10, IG-1212 and AL-95-12 lacked roots, possessing only shoot stumps at their basal ends which were surrounded by the callus from which they arose. These shoot stumps along with a little bit a surrounding callus on their transfer to the same hormone free MS medium showed very poor development of roots which was limited to LLC-3 and IL-75 genotypes only. Hence, they were transferred on eight different rooting media, 4 each based on MS and SH basal salts supplemented with 0.5 mg/l kinetin, 2.0 mg/l NAA and with or without 2.0 mg/l IAA and with or without 3 g/l activated charcoal. The best response for root induction and development was observed in the shoots of LLC-3, IL-75, IG-1212 and C-10 genotypes on the SH based root induction medium containing 0.5 mg/l kinetin, 2.0 mg/l NAA, 5.0 mg/l IAA and 3g/l activated charcoal. The developments of roots were comparatively vigorous in LLC-3 and IL-75 than IG-1212 and C-10 genotypes and no rooting response was
observed in the genotype A-9 and AL-95-12 in any of the eight rooting media. Kraic et al. (1994) could induce the roots on the in vitro developed shoots on rooting medium in lucerne. Liu et al. (1993) could produce adventitious roots along the buds on the differentiation media from the calli derived from hypocotyl and cotyledon of M. sativa. Scarpa et al. (1993) observed rooting and plantlet development on a medium containing 2-ip and IAA during regeneration of plants from hypocotyl derived calus in M. polymorpha. However, Iantcheva et al. (1999) observed in M. truncatula, M. littoralis, M. murex and M. polymorpha, that the shoots regenerated in vitro easily develop root system on a medium with reduced level of macro elements and sucrose.

5.1.2.5 Survival of plantlets:
Habituation of the in vitro grown plantlets to the external harsh conditions as been observed as a gradual process. In the present study, the regenerated plantlets with well developed shoots and roots were taken out of the culture flasks and after washing their roots thoroughly with sterile water they were transferred in small plastic pots filled with autoclaved soilrite and well soaked with ¼ strength MS basal salt solution containing no growth regulators and sucrose. Sufficient moisture regime and controlled temperature and photoperiodic conditions were maintained around the pots for few days. The surviving plants were then transferred to larger pots containing soil, sand and FYM (1:1:1 v/v) and kept in diffused light at room temperature for 8-10 day before their transfer to field. The plants with well developed roots and shoot were obtained in LLC-3, IL-75, C-10 and IG-1212 genotypes only. In genotypes C-10 and IG-1212, none of the in vitro regenerated plants could with stand even the first step of hardening. However, in case IL-75, out of 30 regenerated plantlets, only 12 could survive in the first step of hardening and none of could survive in the subsequent stage of hardening and acclimatization. Whereas, Out of 60 regenerated plants of LLC-3, 52 survived in the first step of hardening and only 42 plants could develop to maturity in the field condition. All these 42 in vitro regenerated plants belonged to LLC-3 genotype regenerated from cotyledon explant. Nagarajan et al. (1986) also established regenerated lucerne plants in soil. Rooted plantlets were also transferred in to soil and adapted to field
condition gradually by Kraic and Hzik (1994). However, regenerated plantlets of lucerne were transplanted into soil acclimatized in a grown chamber for two weeks until the plants reached 20 cm and then moved to the green house (Moursy et al., 1999), whereas embryo were planted directly into soil plotting mix under non sterile condition without exogenous nutrients (Fujii et al., 1999).

Plantlets were transferred to soil and maintained for 3 weeks in a grown cabinet (216μ mol m⁻² s⁻¹, 23 ± 1°C, 12-h photoperiod, 80 % R.H) by Scarpa et al. (1993). However, Nolan et al. (1989) transferred the plantlets to sterile sand supplemented Hoaglands nutrients solution for further growth, where as according to Atanassov and Brown (1984), mist chamber was necessary for plants and after 7-12 days in the mist chamber plants could successfully be potted and moved to the green house. According to Saunders and Bingham (1972), plantlets were potted in sterilized soil inoculated with rhizobium and grown briefly in the green house before transfer to the field.

5.2. Somaclonal variation:

Somaclonal variation commonly appear in in vitro plants regenerated via callus phase. One of the applications of tissue culture is the exploitation of genetic changes occurring in plants regenerated from callus and protoplasts for producing agronomically useful variants (Larkin and Scowcroft, 1981 and Evans and Sharp, 1983). Plants regenerated from tissue culture might quite often vary from the tissue donor plant in one or more characteristics. This variation was termed as “somaclonal variation” by Larkin and Scowcroft (1981). There has an immense utility of somaclonal variation for inducing genetic variability in lucerne since a large amount of natural variability is known to be present in alfalfa, but it has been very difficult to improve forage yield potential in comparison to cereals and food crops (Bingham, 1981). Because of these problems, the genetic improvement of this crop adopting conventional breeding methods has been quite difficult and the breeding activities have not resulted in any significant improvement in its yield potential in the last century (Rosellini and Veronesi, 2002). Induction and the possible utilization of
somaclonal variation in the genetic improvement of alfalfa could be derived from comparison of ranges of variations observed in the somaclones and the original populations.

5.2.1. Morphological variations in somaclones:

Among all the somaclones, the plants developed from the callus of cotyledon explant of the genotype LLC-3 only could survive in the field and develop up to maturity. The performance of these forty-two cotyledon derived somaclones of LLC-3 was studied for various morphological traits of agronomic importance as compared to the mean values for the respective trait of the parent and number of superior and inferior somaclones for the respective trait, frequency of somaclonal variation and the frequency distribution of the somaclones for various traits were determined. The regenerated plants, the somaclones, exhibited bi-directional variation for most of the morphological traits. The mean performance of all somaclones deviated considerably for most of the traits as compared to the parent. Overall performance of the somaclones was found better over to the parent with respect to the number of nodes and internodes, stem girth and flower size. The maximum number of superior somaclones was recorded for stem girth followed by for number of nodes and length of internodes. The minimum number of superior somaclones over to parent was observed for per cent dry matter content followed by fresh leaf weight and flower length. The range of bi-directional variation was substantial in case of plant height, branches per plant, fresh and dry weights of leaf and stem, total green weight, total dry weight, dry matter content and flower size. Frequency of variation in the somaclones varied considerably for various morphological characters while for stem girth and dry weight of stem most of the somaclones deviated significantly from the parent and the minimum variation was noticed with regard to per cent dry matter content. The frequency distribution of somaclones for various morphological traits as per the variation occurred in both the directions in terms of the mean values of the parent and the similarity was exhibited in the somaclones with respect to parent for most of the characters under study.
Somaclonal variants for qualitative genetic alternations (Bingham and McCoy, 1986), disease resistance (Latunde-Dada and Lucas, 1983 and Johnson et al., 1984) and quantitative traits such as herbage yield (Johnson et al., 1984 and Pfeiffer and Bingham, 1984) have already been reported in alfalfa. Nagrajan and Walton (1989) found that plant height, stem girth, number of nodes, per stem and internodes length were lower in one somaclone than the original populations. Somaclonal variation is a common phenomenon and an array of variation involving flower colour, leaf morphology, branching patterns, fertility, regeneration ability, vigor and ploidy level has been observed among regenerated plants (Reisch and Bingham, 1981; Bingham and McCoy, 1986 and Groose and Bingham, 1986). As in case of present study variation in morphological and biochemical traits were also studied in regenerated lucerne plants (Deineko et al., 19997). Arcioni et al. (1988) found that somaclonal variation was genotype specific.

In a field study of herbage yield of 32 diploid and 16 tetraploid regenerates of HG2 alfalfa revealed variants for herbage yield (Reisch and Bingham, 1981). These results were in corroboration to the present study. Herbage yield as considered as quantitative genetic trait as controlled by many genes. Somaclonal variants of alfalfa were also noted among the alfalfa plants regenerated from the callus (Saunders and Bingham, 1972). Johnson, et al. (1980) reported that thirty-two regenerated plantlets were phenotypically different from the plant of the lucerne variety Regen S1 from which they were regenerated. Groose and Bingham (1984) obtained plants from hypocotyl derived callus of two tetraploid alfalfa genotypes bred to be heterozygous for four heritable traits and 21 per cent of the regenerants were variants for one or more characters. Hartman, et al. (1984) found that somaclonal variation for disease resistance in alfalfa was genetically transmissible and appeared to be due to dominant mutation. Ptackova et al. (1988) observed significant differences in water holding capacity in 40 somaclones from a single *M. sativa* plant. Some of them combined drought resistance with high chlorophyll content. Safarnejad et al. (1996) observed that one somaclone when compared with the parent line of lucerne showed increased salt tolerance, greater accumulation of proline and a greater increase of antioxidant enzyme, glutathione reductase.
However, Nikolic et al. (1986) observed that the regenerated plants did not differ from normal plants of the variety in their morphological traits and yield.

Mechanisms of somaclonal variation identified in alfalfa include changes in chromosome number and structure, dominant and recessive mutations, transposable elements and changes in chloroplasts and mitochondria. A variant may carry more than one nuclear or cytoplasmic mutation.

5.2.2. Biochemical characterization of somaclones:
Isozymes of PGI, PGM, G6PDH, AAT, SOD and esterase were analysed for 6 somaclones with their parent using PAGE technique. Somaclones differed among themselves with their parent for isozymes of PGI, PGM, G6PDH, AAT SOD and esterase. High level of genetic variability within and between populations of *M. intertexta* than *M. ciliaris* was reported by Cherifi, 1996. In another study on protoplast fusion, the hybrid nature of the selected calli was confirmed by isozyme analysis (Pupilli et al., 1991). Similarly, Pupilli et al. (1992) confirmed hybrid nature of regenerants by isozyme analysis.

This is the first report on somaclonal variants substantiated by isozyme polymorphism in lucerne. Where as several reports have established somaclonal variation on isozyme variation (Mangolin et al., 1997; Binsfold et al., 1996 and Pramanik et al., 1996). However, Richard et al. (1995) reported that results from isozyme system could not differentiate significantly the hybrid larch plants regenerated from tissue culture from their stocklings and the ortet. Pramanik et al. (1996) found a new band of superoxide dismutase in multiplying shootlets which was, according to them, a *denovo* variation or due to post transcriptional modification of the gene. Similarly Mangolin et al. (1994) found that IDH, ACP, PER and EST enzyme systems were good markers for investigating possible genetic variations. However, the present study indicated PGI, PGM, G6PDH, AAT, SOD and EST isozymes as good enzyme systems for detecting somaclonal variation.
Many studies have reported isozymic differences between somaclones developed using various explants. Khavkin (1991) have found that some loci more susceptible to somaclonal variation than others. His data indicated a specific control system whereby ordering tissue and organ specific isozyme spectra might be affected by somaclonal variation at the early stages of a regulatory cascade. Martelli et al. (1993) could distinguish rootstocks and regenerants in apple based on isozyme banding patterns.

**Dendrogram analysis:**

Dendrogram analysis from the data analysed from six isozymes indicated 3 groups of six somaclones and parent based on similarity index. Our study indicated S1 and S2 as closer to parent than others and S5 was the most distant somaclone. This analysis further substantiated somaclonal variation as somaclones clustered into 3 groups. Cherifi (1996) also reported 2 taxa based on cluster analysis between populations of *M. intertexta* and *M. ciliaris*. Whereas, Zhang and Hao (1993) grouped lucerne genotypes grown under different environmental conditions into 3 groups based on banding patterns of peroxidase and esterase isozymes. However, the present study is the first report of such an analysis among somaclones of *M. sativa*. Utility of results of such dendrogram analysis could be to use distant somaclones as targets for cross hybridization.

**5.2.3. RAPD analysis of somaclones:**

To further substantiate the results obtained from isozyme analysis, DNA markers such as random amplified polymorphic DNA were analysed among somaclones of lucerne. Results indicated polymorphism for all the primers used further confirming somaclonal variation. RAPD markers are more efficient and reliable to characterize variation as they indicate genomic differences without getting affected by the environment and post transcriptional modifications. Similar opinion was reported by Echt et al. (1992) who found RAPD markers useful for the rapid development of genetic information in species like lucerne where little information currently exists or it is difficult to obtain. Similarly, Phan et al., 1996 also found extensive DNA
material used to produce protoplasts for the gene transfer.

In the present study, the maximum polymorphism was recorded from OPE-01 primer with respect to number of alleles showed by parent with the somaclones. Yu and Pauls (1993) also identified RAPD markers linked to gene controlling somatic embryogenesis in *M. sativa*. The segregation data indicated that the marker was linked to A locus. However, in this study, segregation analysis of somaclones using RAPD in still to be studied. Another study by Piccioni *et al*. (1997) estimated somaclonal variation in axillary branching propagation and indirect SE by RAPD fingerprinting. The plantlets derived from axillary branching propagation exhibited no variation for any of the 75 RAPD markers obtained from eight different decamers whereas RAPD fingerprints of 9 of 39 plantlets regenerated by indirect embryogenesis differed from that of the donor for at least one primer and one polymorphic amplification product.

In the study, we scored a maximum of 11 alleles for OPE-01 primer implying maximum polymorphism. Piccioni *et al*. (1997) generated 19 new RAPD markers from 8 primers that were not found in the donor plant fingerprints. Most of the somaclonal variation displayed one to five polymorphic bands. While this study indicated all somaclones to be polymorphic. Piccioni *et al*. (1997) observed only six of the nine somaclones as polymorphic with two or more primers. They also concluded that axillary branching propagation was a safer cloning technique while determining RAPD markers as efficient tools for the early detection of somaclonal variants in tissue culture of alfalfa.

Most of the studies on markers reported so far have included natural propagations (Brummer *et al*., 1995; Barcaccia *et al*., 1994; Kiss *et al*., 1997; Gherardi *et al*., 1998). In most of the cases, high level of genetic variation was found within and between populations while most of the loci were highly polymorphic where as in very few population, specific polymorphisms were identified. Similar results were obtained in our study on somaclones also. Linkage maps have been constructed
Dendrogram analysis:

Phylogenetic relationship with genetic relatedness was studied by dendrogram analysis of polymorphic RAPD marker, among 5 somaclones and their parent. In contrast to dendrogram analysis using isozyme loci, dendrogram from RAPD analysis has revealed a single group implying closeness of somaclones to their parent. This has indicated the superiority of isozyme loci in distinguishing somaclones over RAPD markers. Tavoletti et al. (1998) also used cluster analysis and principal coordinate analysis to characterize genetic diversity in 3 diploid populations of *M. sativa* complex. They also concluded that the high level of genetic diversity could be efficiently applied in genetic mapping of five mutants.

Comparative analysis of two dendrogram:

While two dendrogram generated separately from isozyme and RAPD have similarly implying a trend in genetic relatedness, isozyme seemed to be distinguishing somaclones more authentically than RAPD markers which were randomly selected. Both the dendrograms indicated S5 somaclone most distant from parent while S1 and S3 were the most closest to parent according to isozyme and RAPD analysis, respectively. Major difference in the results appeared to be in the number of clusters that were three and one for isozymes and RAPD analysis respectively. Order of and genetic relatedness of somaclones appeared to be similar between two dendrograms. The minor difference among dendrograms could be ascribed to the area of genome sampled through RAPD and isozymes. Nevertheless, dendrogram generated from isozyme could be considered for breeding purposes in view of large number of loci studied.
5.2.4. Chemical analysis
Nutritional composition of germplasm in any species is very useful to have the first hand knowledge of genetic variability for subsequent development of nutritionally superior/ better cultivars of that particular crop/species. Nitrogen content together with the cell wall contents are the most important factor to the voluntary intake (Van Soest, 1994).

Crude protein content in the germplasm of lucerne under study varied between 24.0-34.6 %. Concentration of cell wall fractions (NDF, ADF. Cellulose and lignin) ranged from 20.4-33.7, 14.2-23.0, 10.8-18.6 and 3.7-5.2 % amongst the evaluated somaclones of lucerne germplasm. Panwar et al. (1992) reported lower CP and higher fiber contents in the two tested varieties of lucerne (T-9 and LH-84). CP contents was 15.66-16.17 %, while NDF and ADF contents were 67.65-70.05 % and 46.47-53.47 %, respectively in these varieties. On the other hand mean contents of different lucerne cultivars for CP, NDF, ADF, cellulose, hemicellulose and lignin (20.30, 43.92, 34.90, 24.41, 9.02 and 9.37 %) reported by Tewatia et al. (1998) are differed than the present findings. Higher CP contents and lower fiber fractions in the somaclones of present study may be attributed either to the differences in the stage of crop harvested for evaluation or due to occurrence of somaclonal variation. Age of crop, environment, genetic variability and nutrition (fertilizer) are some of the factors affecting CP and cell wall contents of a crop. Tewatia et al. (1998) observed lower CP (17.10 %) and higher crude fiber (26.30%) in the hay of lucerne harvested at the mature stage of growth. As crop matured there was a decrease in cell contents (CP, chlorophyll, sugar etc.) and increase in fiber (NDF, ADF, cellulose and lignin) accumulation. Mahanta et al. (1999) evaluated diploid and tetraploid lines of berseem and reported that wide variability existed in different lines in respect of quality traits (CP, NDF, ADF, cellulose and lignin) which confirmed that genetic differences occurs in germplasm of different forage species.