Chapter – 2

In vitro Regeneration

2.1 DIRECT ORGANOGENESIS

2.1.1 INTRODUCTION

Safflower has attracted very little attention as far as tissue culture is concerned. Recent advances made in the field of tissue culture have brought about new emerging technologies for crop improvement. It has the potential to produce numerous genetically identical plants selected for special attributes. The mode of regeneration in general is through direct or indirect organogenesis (Walia *et al.*, 2005; Saikant Gantait, 2010). All these factors drive the tempo of *in vitro* organogenesis and regeneration of complete plants by integrating the individual organs (Gantait and Mandal, 2010). Recently, (Kothari *et al.*, 2010) emphasized on the synergistic obligation of both cytokinins and auxins for initiation of cell division and growth in plant tissue cultures; where an array of experiments have fundamentally recognized the continuation of antagonistic as well as additive interactions involving these two types of PGRs. The tissue culture of safflower has been the subject of several studies focusing on establishment of *in vitro* propagation. It offers a method to increase valuable genotypes rapidly and expedite release of large number of plantlets. Rapid regeneration of plants directly from explants presents an effective strategy to avoid or substantially reduce somaclonal variation as it minimizes culture duration and eliminates or minimizes callus formation in culture (Lakshmanan *et al.*, 2006). Genetic improvement of this crop for agronomical attributes is constrained by the modest levels of variability available in the cultivar germplasm. Plant regeneration from different plant species is influenced by plant growth regulators including the ratios of cytokinin and auxin (Rout *et al.*, 2001; Liao *et al.*, 2004). The suitable ratio of cytokinin and auxin for plant regeneration of safflower depends on the used cultivars. George and Rao (1982) reported the induction of shoots from cotyledons on Murashige and Skoog (1962) salts as the basal medium supplemented with BA (6-Benzyl Adenine), but regeneration frequency was low and complete plants were rarely obtained. Direct shoot regeneration and inflorescence induction has been achieved from cotyledon on MS medium

containing BA or kinetin in combinations with NAA (α napthaleneacetic acid) (Tejovathi and Anwar, 1984; 1987). In addition, primary seedling explants were used on MS medium consisting of BA and NAA for direct as well as indirect organogenesis of safflower cultivars (Ying *et al.*, 1992, Orlikowska and Dyer, 1993, Sujatha and Suganya, 1996, Nikam and Shitole, 1999). Cotyledonary explants cultured on MS medium supplemented with BA and kinetin induced shoot organogenesis (Walia *et al.*, 2005). In earlier reports (Vijaya Kumar and Ranjitha Kumari, 2005) also confirmed that primary seedling explants (cotyledonary and stem node) on MS salts and B5 vitamins medium comprised of BA and kinetin found to be the most effective for shoot organogenesis. In many cases TDZ (Thidiazuron) (1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea), a substituted phenylurea compound was shown to possess potent activity as a cytokinin in the regulation of shoot organogenesis in several plant species (Huetteman and Preece, 1993; Mithila *et al.*, 2001; Murthy *et al.*, 1998). In safflower, shoot regeneration frequencies on medium with TDZ/NAA were high and shoot buds were induced on a wide range of concentrations (Radhika *et al.*, 2006). A major problem associated with *in vitro* culture is the occurrence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues or organs (Larkin and Scowcroft, 1981; Gould, 1986). Recently, the addition of amino acids to the tissue culture medium promoted high frequency of plant regeneration in many plants species including *Artemisia vulgaris* L. (Pradeep kumar and Ranjitha Kumari, 2010). However, there is only one report being described for safflower plant regeneration in amino acids supplemented medium (Walia *et al.*, 2005). The objective of the investigation reported here was to develop a rapid and reproducible *in vitro* regeneration system from different explants of safflower for mass propagation of selected elite clones. All the explants on cytokinins BA, KIN (kinetin) and TDZ alone or in combinations with CPPU N-(2-chloro-4-pyridyl-N'-phenylurea) supplemented medium were tested to improve adventitious and multiple shoot regeneration of safflower cv. NARI-6. This technique provides a rapid reliable system for the production of large number of genetically uniform disease free plantlets. The development of an efficient regeneration protocol is also a prerequisite for tissue culture studies for raising plants of this valuable oilseed crop plant.

2.1.2 MATERIALS AND METHODS

Chemicals and Culture Vessels

Chemicals and growth regulators were used for the preparation of stock solutions of media salts; growth regulators and gelling agent (agar) for tissue culture were obtained from Hi-Media, Mumbai, India. Glassware (Borosil, India) such as culture tubes (25 x 150 mm), conical flasks (250, 500 ml and 1litre), petridishes (20 x 150 mm) were used for *in vitro* regeneration studies. Glasswares were rinsed with distilled water and dried up in hot air oven at 100 °C. Culture tubes and conical flasks were plugged with non-absorbent cotton covered with cotton gauze. The petridishes were covered with parafilm.

Preparation of Media

The demand by plants for nutrients is diverse and different plant species require specific nutrient elements at specific stages. In fact, each of the tissue culture media that have been published in the literature was developed on the basis of a specific genotype of a particular plant species (Lin and Zhang, 2005). MS medium is a universal medium used in plant tissue culture, was prepared separately and stored in brown bottles. Growth regulators were dissolved in respective solvents and final volume was made up with distilled water. All the solutions were stored in refrigerator. Stock solution were pipetted out and final volume was made up with distilled water with or without the addition of growth regulators, amino acids and other additives depending on the experimental set up. The pH of the medium was adjusted to 5.6 - 5.8 using 1 N NaOH or 1N HCl solutions prior to autoclaving at 121°C for 15 min. The medium was gelled with 0.8 % agar (w/v) in case of solid medium.

Plant Materials

Safflower (*C. tinctorius* L.) seeds were procured from the Nimbhkar Agricultural Research Institute (NARI), Maharashtra, India were used for present study. This cultivar was selected based on their area of cultivation, extent of resistance to diseases, agro climatic conditions etc.

In Vitro Seed Germination

Safflower seeds were soaked with 1.0 % Teepol (commercial bleach solution, 0.6 % sodium hypochlorite, Rockitt Benckiser (India) Ltd., Kolkata, India) solution for 30 sec, kept under tap water for 20 min to remove detergent. Further processes were carried out under aseptic conditions by treating with 70 % ethanol (v/v) for 1 min and rinsed twice with sterile distilled water for 2 min followed by 0.1 % $HgCl_2$ (mercuric chloride) for 2 min. The surface sterilized seeds were implanted in Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 3.0 % sucrose (w/v) and 0.8 % agar (w/v) (Hi-media laboratories limited, Mumbai, India). All the cultures were initially incubated in darkness for 24-hr at 23-25 °C and later transferred to 16/8-hr light/dark conditions at a light intensity of 15 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Philips, India) (Fig 2.1). Hypocotyl explants from 7-9 days old *in vitro* seedlings were cut into 0.5-1.5 cm length. Hypocotyl explants were placed vertically in contact with medium region facing up.

Direct Organogenesis

All the hypocotyl explants on culture tubes and bottles each containing 10 ml and 20 ml MS salts, 3.0 % sucrose (w/v) medium supplemented with individual growth regulators (BA and KIN) ranging from 1.0 – 6.5 µM alone or in combinations with various concentrations of CPPU (0.5 - 2.5 µM) is supplemented in medium for adventitious shoot induction. All cultures were incubated under 36 µmol $m^{-2}s^{-1}$ in 16 h photoperiod provided by cool white fluorescent tubes at 25 ± 2 ºC. Each experiment was repeated thrice. Subcultures were done at two weeks interval.

Shoot Elongation, Rooting and Acclimatization

Individual shoots (0.5 – 1.0 cm in length) were carefully excised from the shoot clumps and were transferred to MS basal medium supplemented with GA_3 (Gibberellic acid) (0.5– 2.0 µM) alone or in combinations with 1.5 – 5.5 µM ranges of IBA or CPPU for frequency of shoot elongation. After shoot elongation, the individual green healthy shoots (<1.0 cm long) were excised and transferred to half strength MS basal medium

supplemented with $AgNo_3$ (0.1– 0.5 µM) alone or in combinations with IBA (0.5 – 2.5 µM) for scoring frequency of rooting 5 weeks after culture initiation. The rooted plantlets were removed from the culture tubes and washed in running tap water to remove agar gels from the roots and were successfully transplanted into 6.0 cm diameter plastic pots mixed sterile red soil, garden soil and sand in a ratio of 1:2:1. Each pot was then enclosed in a clean polythene bag, maintained under 16 h photoperiod at a light intensity of 15 µmol m^{-2} s^{-1} provided by cool white fluorescent tube (Philips, India) for first two weeks *in vitro* state and then transferred to field conditions for development into mature plants.

Statistical Analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. All experiments were conducted with Complete Randomized Block Design (CRBD) and factorial with different growth regulators as independent variables. Each experiment was repeated thrice. Regeneration frequency and the number of shoots obtained during initial culture and subsequent transfers were tabulated. The data pertaining to frequencies of shoot induction, root induction and number of shoots/cultures were subjected to analysis of variance test. Mean separation was carried out using Duncan's Multiple Range Test (DMRT).

2.1.3 RESULTS

Shoot Multiplication Response

Hypocotyl Explant

Adventitious shoot buds were initiated from the meristematic region of Hypocotyl explants (Fig 2.2a). Microshoot buds that initiated in the initial culture grew into tiny visible shoots in the medium containing growth regulators. Genotype dependant response was observed in all the experiments. In MS salts with 3.0 % sucrose (w/v), 0.8 % agar medium supplemented with combination of IBA (5.0 µM) and GA_3 (2.0 µM) induced 27.0 number of shoots after 55 days of culture. BA (2.5 µM) and CPPU (1.5 µM) in combination induced 19.8 numbers of shoots. However,

maximum of 34.2 average number of shoots were formed on medium fortified with BA (3.5 μM) and KN (1.5 μM) and also with maximum 97 % of organogenesis were recorded**.** (Table.2.1).

Shoot elongation

Although shoot organogenesis from hypocotyl explants were observed, it is necessary to establish a shoot elongation. In this case, individual microshoots placed on MS salts with 3.0 % sucrose medium supplemented with GA_3 in combinations with BAP were highly suited for the frequency of shoot elongation. BAP (3.5 μM) in combinations with GA_3 (1.5 μM) induced maximum shoot length (3.7 cm in length) with high frequency. Minimum of (0.3 cm) shoot elongation with TDZ (6.0 μM) and GA_3 (2.5 μM) supplemented medium (Table 2.3).

Rooting and Acclimatization Response

Root initiation was observed when the elongated shoots (>0.5) were excised and placed directly on root induction medium for frequency of rooting. In this case, individual excised shoots subcultured on Half strength MS medium supplemented with 3.0 % sucrose and IBA (2.0 μM) in combination with $AgNO_3$ (2.0 μM) induced 57.7 % rooting response with more callus interspersion was observed after 3 - 4 weeks of culture. (Table 2.4; Fig. 2.2 c,d). Rooted plants were transplanted into potting mix (Fig. 2.2 e) and were grown under green house condition after 14 –21 days. The plant survival rate decreased from 97 - 55 % after 2-10 week of acclimatization under green house condition and were substantially grown in the field (Fig. 2.2 e). Regenerated plants did not show any morphological abnormalities compared to donor plant during the maturation period in the field conditions.

2.1.4 DISCUSSION

A successful micropropagation protocol proceeds through a series of stages, each with a specific set of requirements. These are (i) initiation of aseptic cultures, (ii) shoot multiplication, (iii) rooting of microshoots, and (iv) hardening and field transfer of plants raised from tissue culture (Patip *et al.*, 2006). This work placed stress on those micropropagation stages and also paid more attention to the culture factors promoting successful shoot multiplication, such as medium, carbon source and growth regulators. The rate of shoot multiplication differed based on growth regulators and explants type. Both Auxin–Cytokinin Interaction regulates major roles on Meristem Development (Xian-Sheng Zhang *et al.*, 2011). In general, BA and 2-ip is the most commonly used cytokinin for direct regeneration. BA or 2-ip tested medium induced minimum number of shoots and did not support the promotory effect. At higher level of BA more callus formation took place at the cut ends in all the explants and reduction in number of shoots per explants culture was observed (Nikam and Shitole, 1999). However, shoot induction was drastically increased without callus formation in TDZ alone supplemented medium in first set of experiment. In other hand, all the explants cultured on BA, 2-ip and TDZ along with CPPU supplemented medium highly supported to increase the number of adventitious shoots. Maximum number of adventitious shoots was formed in optimum level of TDZ and CPPU along with amino acids such as proline, aspartic acid, phenylalanine and glutamine supplemented medium. However, number of shoot induction was significantly increased in TDZ and CPPU along with Aspartic acid comprised medium. The use of higher level of plant growth regulators along with amino acids resulted in browning of explants. Earlier there are few reports describing shoot organogenesis of safflower from primary seedling explants. George and Rao (1982) reported direct shoot regeneration from cotyledon explants on BA supplemented medium, but regeneration frequency was low and complete plant was rarely obtained. Tejovathi and Anwar (1984; 1987) also reported plant regeneration with *in vitro* capitula induction from cotyledon explants in BA or KN along with NAA supplemented medium. Orlikowska and Dyer (1993) observed multiple shoots regeneration from primary seedling explants and immature embryos of American

safflower (*Carthamus tinctorius* L.) cv. Centennial on BA or TDZ and NAA fortified medium. Moreover, (Radhika *et al.*, 2006) reported that TDZ and NAA combinations facilitated plant regeneration from hypocotyls, cotyledon, primary leaf and root explants of safflower genotypes (cv. Manjira, A-1, HUS-305). In earlier study, shoot node and cotyledonary node explants cultured on BA and KN supplemented medium showed shoot organogenesis of safflower cv. NARI-6 (Vijaya Kumar and Ranjitha Kumari, 2005). In many cases, the hormone concentrations cannot be the sole mechanism controlling *in vitro* developmental processes. It may be related to differences in tissue and cell differentiation and organization in all the explant culture. NARI-6 cultivar showed more response favorably with highest regeneration frequency of 97 % in hypocotyls explants, 92 % in leaf. Similarly, the addition of amino acids into the medium was found to be more beneficial for maximum shoot bud induction in other plant species (Milazzo *et al.***,** 1998; Vila *et al.*, 2003; Selvaraj *et al.*, 2002; Baskaran and Jayabalan, 2005; Vasudevan *et al.*, 2004; Mao *et al.*, 2006). Adventitious shoot buds induced from explants remained stunted and failed to elongate rapidly. Isolated microshoots from shoot clumps were transferred to shoot elongation medium where high frequency of elongation and inflorescence head induction was observed. GA_3 plays a vital role in shoot elongation and has a stimulatory effect on the growth and internodal elongation many plant species. Shoot elongation was decreased slightly with increasing cytokinin concentration, has been observed in other plant species (Tang and Guo, 2001; Nirmal Babu *et al.*, 2003; Xinping and Deyue, 2006).

In present study, high frequency of shoot elongation was observed in MS medium supplemented with BA and GA_3, but further increase in plant growth regulators concentration promoted shoot induction. Rooting of *in vitro* plants is genotypically dependent. However, in the present observation, shoots cultured on IBA and $AgNO_3$ supplemented medium induced efficient rooting. The reduced concentration of salts, sucrose could be supportive to promote rooting in NARI-6. Differences in genotype for *in vitro* response have been previously reported in many cultivars (George and Rao, 1982; Orlikowska and dyer, 1993; Rani *et al.*, 1996; Nikam

and Shitole, 1999; Walia *et al.*, 2005; Radhika *et al.*, 2006). The regeneration ability was dependent on the nature of genotype of the cultivars within the species.

2.1.5 SUMMARY

- Hypocotyl explants cultured on BA+KN treated medium was optimized for more number of adventitious shoot regeneration in NARI-6
- Other growth regulators BA+ 2, 4-D showed slow response for adventitious shoot regeneration in hypocotyl explant culture.
- Maximum shoot induction and proliferation was observed in hypocotyls explants cultured on optimum level of BA (3.5 μM) and KN (1.5 μM) with mean no. of shoots (34.2).
- Regenerated plants placed on shoot elongation medium containing BAP (3.5 μM) and GA_3 (1.5 μM) found better shoot elongation with the maximum (3.7 cm).
- Elongated shoots cultured on root induction medium containing IBA (2.0 μM) and $AgNO_3$ (2.0 μM) showed better response of frequency (57.7 %).
- The green healthy rooted plantlets were maintained in the green house condition and were grown to mature plants in the field.

2.2. INDIRECT ORGANOGENESIS

2.2.1 INTRODUCTION

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissue (Thorpe, 1998; Victor *et al.*, 1999). An efficient plant regeneration system is a pre-requisite for development of disease free plants through tissue culture technology with or without using the techniques of genetic engineering (McNicol and Graham, 1990). In order to utilize the advantages of *in vitro* propagation, true-to-type reproduction is highly desirable. In many cases, cultures of isolated plant cells and tissues in artificial nutrient media have become enormously important for fundamental and practical applications, particularly in the clonal propagation industry. Cell division in *in vitro* culture depends on a large number of factors. These factors include inorganic ions, amino acids, nucleic acid base adenine, plant growth regulators like cytokinins, auxins and gibberellic acid as well as well cultural conditions such as light and temperature, physiological state and age of explant and genotypes.

Auxins and cytokinins are the main plant growth regulators concerning cell division and differentiation related to induction and development of somatic embryogenesis and to induce adventitious shoot organogenesis (Feher *et al.*, 2003, Ramage and Williams, 2004).The balance between auxin and cytokinin plays an important role in determining the morphological development of an explant in culture (Ammit Rao, 1983; Gaspar *et al.*, 1996). A high cytokinin to auxin ratio promotes shoot formation, a low cytokinin to auxin ratio results in root formation and a balance between the two factors promotes callus formation (Ammit Rao, 1983; Gasper *et al.*, 1996; Ramage and Williams, 2004). Manipulation of the composition and the ratio of these plant growth regulators is often the primary empirical approach to optimize *in vitro* development (Ochatt *et al.*, 2000; Pan *et al.*, 2003). Any type of living vegetative cell, a group of cells, a portion of organ in the culture medium, produce the undifferentiated mass of parenchyma cells known as callus. The response of explants to initiate callus vary with the type and the concentrations of plant growth regulators used. Plant regenerated from organ cultures, calli, protoplasts and via somatic embryogenesis

often show phenotypic and DNA variation, a phenomenon which has been termed somaclonal variation (Larkin and Scowcroft, 1981). Assessment of genetic stability of a genotype throughout a given tissue culture would be a tremendous advantage for *in vitro* regeneration and genetic engineering. In earlier studies of safflower, attempts have been made for capitulum induction (Baker and Dyer, 1996; Ying *et al.*, 1992, Orlikowska and Dyer, 1993, Mandal *et al.*, 1995, Sujatha and Suganya, 1996, Tejovathi and Anwar, 1993; Nikam and Shitole, 1999, Mandal and Gupta, 2003; Vijaya Kumar and Ranjitha Kumari, 2005; Radhika *et al.*, 2006) was reported by many authors. However, in plants sugars are not only the source of a carbon skeleton and energy, but are also regulators in many aspects of life's activities. These include metabolism, assimilating partitioning and transporting, growth and development, stress responses and others. Sugar regulates these activities by enhancing or repressing expression of relevant genes (Koch, 1996; Smeekens, 2000; Rolland *et al.*, 2002). Sucrose has been the most tested carbon source for induction of somatic embryogenesis. It has been used mostly at 3 % of the culture medium in a wide range of plants including peanut (Little *et al.*, 2000), *Helianthus maximiliani* (Vasic *et al.*, 2001) *Eucalyptus globulus* (Pinto *et al.*, 2002) and African violet (Mithila *et al.*, 2003).

In the present study an efficient protocol for plant regeneration through intermediate callus culture of safflower was standardized. The influence of various auxins (IAA, NAA, IBA and 2, 4-D (2, 4-dichlorophenoxyacetic acid) and cytokinin (TDZ) and the culture conditions are required for inducing callus and shoot organogenesis.

2.2.2 MATERIALS AND METHODS

Plant Materials

Safflower (*C. tinctorius* L.) seeds of NARI-6 cultivar were procured from Nimbhkar Agricultural Research Institute (NARI), Maharashtra, India were used for the present study. Seeds were soaked with 4-5 drops of 1.0 % Teepol (commercial bleach solution, 0.6 % sodium hypochlorite, Rockitt Benckiser (India) Ltd., Kolkata, India) solution for 30 seconds and kept under tap water for 20 min to remove detergent. Further processes were carried out under aseptic conditions by treating with 70 %

ethanol (v/v) for 1 min and rinsed twice with sterile distilled water for 2 min. followed by 0.1 % $HgCl_2$ (mercuric chloride) for 2 min. The surface sterilized explants were implanted in Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 3.0 % sucrose (w/v) and 0.8 % agar (w/v). All the cultures were initially incubated in darkness for 24-hr at 23 - 25 °C and later transferred to 16/8-hr light/dark conditions at a light intensity of 15 μmol m^{-2} s^{-1} provided by cool white fluorescent tube (Philips, India).

Leaf explant Preparation and Culture Condition

The regeneration experiments were carried out using (leaf explants) which were excised from 6-9 days old *in vitro* seedlings. All explants were dissected into 0.5-1.0 cm length by using a surgical blade. Leaf lobe explants were placed with the right side up (abaxial surface into the medium) or upside down (adaxial surface into the medium), and leaf explants were placed horizontally into culture tubes (25×150 mm) each containing 15 ml MS basal salts, 3.0 % (w/v) sucrose medium supplemented with different types of plant growth regulators and were solidified with 0.8 % (w/v) agar (Hi-media laboratories limited, Mumbai, India). The medium was autoclaved at 121 ºC at 15 lbs pressure after adjusting the pH to 5.7 with 0.1 N NaOH. All the cultures were incubated under 15 μmol m^{-2} s^{-1} in 16 h photoperiod provided by cool white fluorescent tubes (Philips, India) at 25 ± 2 ºC. The culture conditions remained the same for all the experiments unless otherwise specified.

Callus Induction

The calli were initiated from both adaxial, abaxial surface and cut end of explants within 2-3 weeks of culture on MS salts with 3.0 % (w/v) sucrose medium supplemented with different ranges (0.5 – 2.5 μM) of individual auxins IAA, IBA, NAA and 2,4-D. Calli were subcultured at 2 week intervals. There were 50 explants/treatment and the experiments were carried out three times. Callusing efficiency was defined as the percentage of explants that produced callus per culture tube.

Adventitious Shoot Induction

After calli initiation, small piece of calli were isolated and subcultured on MS salts with 3.0 % (w/v) sucrose medium supplemented with different ranges (1.5 – 7.0 µM) of TDZ in combinations with individual concentrations of auxins IAA, IBA, NAA and 2,4-D ranges from 0.5-2.5 µM for calli proliferation and adventitious shoot regeneration. Individual microshoots were isolated from the callus clumps after initial 4 weeks of culture and subcultured on medium supplemented with optimum levels of TDZ (4.5 µM) and IBA (1.5 µM) in combinations for microshoot proliferation. There were 60 explants/treatment and the experiments were carried out three times. Data for shoot induction frequency (percentage of explants producing shoot buds) were recorded every week end after culture initiation.

Rooting and Hardening of Plants

Individual plantlets were trimmed to above 1.0 - 2.0 cm length and placed on half strength MS salts with 3.0 % sucrose medium supplemented with IBA (0.5 – 2.5 µM) and NAA (0.5 – 2.5 µM)in combination with KN or $AgNO_3$ for the frequency of root induction. There were 60 plantlets/treatment and the experiments were carried out 3 times. Data were recorded on per cent of root induction after 5 weeks of culture initiation. For first stage of hardening, plantlets with well developed roots were carefully removed from the culture tubes and washed under running tap water to remove agar gels from the roots and were transplanted onto 6.0 cm diameter plastic cups containing the mixture of sterile red soil, garden soil and sand in the ratio of 1:2:1. To maintain high humidity the plastic cups were covered with polythene bags. Plantlets were irrigated with 5 ml of half-strength MS inorganic salts and subsequently with distilled water during the first two weeks. The polythene bags were then removed and plants were acclimatized in earthen pots containing soil mixture in the second stage of hardening. All acclimatized plants were maintained under 15 µmol $m^{-2}s^{-1}$ in 16-hr photoperiod provided by cool white fluorescent tubes at 25 ± 2 ºC for another 2 weeks before the plants were transferred to the greenhouse. Data for frequency of survival rate of potted plants were recorded after 3 - 5 weeks.

Statistical Analysis

All experiments were conducted with Complete Randomized Block Design (CRBD) and factorial with different growth regulators as independent variables. Each experiment was repeated three times. Callus growth and the number of shoots obtained during initial culture and subsequent transfers were tabulated. Mean separation was carried out using Duncan's Multiple Range Test (DMRT).

2.2.3 RESULTS

Callus Induction

For callus initiation, the immature leaf segments were placed in contact with the solidified MS salts with 3.0 % sucrose medium containing different auxins (Fig. 2.3a). The resulting callus was allowed to grow up to 45 days. In our study, individual auxins NAA, IAA and IBA induced light green nodular calli in immature leaf. However, maximum callus with 92 % of callusing response from leaf explants of NARI-6 cultivar in combination of (4.5 μM TDZ + 1.5 μM IAA) noticed after 45 days of culture (Table 2.2).

Elongation of Shoots

Although shoot organogenesis from leaf derived calli was observed, it is necessary to establish a shoot elongation. In this case, individual microshoots placed on MS salts with 3.0 % sucrose medium supplemented with TDZ and GA_3 combination were highly suited for the frequency of shoot elongation. Although the presence of BAP (3.5 μM) in combinations with GA_3 (1.5 μM) induced minimum shoot length (0.2 cm in length). Whereas maximum of (2.8 cm) shoot elongation with TDZ (3.5 μM) and GA_3 (1.5 μM) supplemented medium (Table 2.3).

Root Induction and Acclimatization

Shoots produced from callus were placed on rooting medium supplemented with IBA and NAA. However, the use of half strength MS salts with 3.0 % sucrose medium supplemented with NAA and KIN (kinetin) or $AgNO_3$ produced more extensive root formation and induction of adventitious roots. However, maximum of 53.8 % rooting was achieved on half strength MS salts with 3.0 % sucrose medium

supplemented with combination of IBA (2.0 μM) and NAA (0.5 μM) (Table 2.4; Fig.2.3 d).The fully regenerated plants were acclimatized without growth chamber facility and successfully transferred to soil. After 4-10 week of acclimatization under greenhouse conditions. (Fig.2.3 e).

2.2.4 DISCUSSION

The present study focus on callus production and shoot organogenesis from leaf explants are the best known explants for *in vitro* culture. Growth regulators in the medium result in variable callus induction in different explants. In previous studies on the effect of cytokinins and auxins on initiation of aseptic cultures from rose nodal stem segments (Davies, 1980; Vijaya *et al.*, 1991; Carelli and Echeverrigaray, 2002), BA (4.4–13.2 μM/l) as a major PGR or in combination with low NAA concentration (0.02–0.54 μM/l)) was found suitable for shoot initiation. Other researchers have reported a high percentage of bud break on hormone-free medium within 10–12 days, but the growth rate was very low in roses (Rout *et al.*, 2004). In the present study different auxins tested primarily for frequency of calli induction of safflower (2.5 μM) NAA showed an increase frequency for callus initiation and induction as compared to other auxins (IAA, IBA and 2,4-D). Individual auxins induced calli subcultured on MS basal with 3.0 % sucrose medium supplemented with TDZ in combinations with different auxins influenced the conversion and proliferation of organogenic calli from which adventitious shoot regeneration was observed. In earlier studies for shoot multiplication, sucrose (3 %) has commonly been used as carbon source. Murashige and Skoog (1962) stated that 3 % sucrose is generally better than 2% or 4 % for tissue culture. On medium supplemented with 3 % sucrose almost all the new leaves turned brown, which inhibited shoot growth. *In vitro* shoot multiplication relies largely on medium formulations containing BA as the major PGR in combination with a low concentration of NAA (Syamal and Singh, 1996; Carelli and Echeverrigaray, 2002). The explants grew well at different PGR concentrations without visible morphological abnormalities but also without increased proliferation, but on MS medium without growth regulator the growth rate was lower and the induced shoots were seriously vitrified. When the culture medium contains NAA at higher concentration the bud can

form more callus from the base section, which will greatly affect the young seedling's absorption of water and nutrition, and thus inhibit its growth.

In the present study the adventitious shoots at the basal edge of the explants sources that occurred through an indirect pathway after callus formation. This adventitious shoots appeared on the surface of the calli, and later originated from internally formed meristematic centers**.** It is well known that the types of explant, the physiological state of the donor plant, mineral uptake, and distribution patterns (Ramage and Williams, 2004). Observations on the number of explants responding to callus induction and shoot regeneration were made at weekly intervals and tabulated four weeks after culture initiation. Data on the number of shoots per regenerating explant was not recorded as the shoots were innumerable on medium supplemented with TDZ + NAA and the propensity to regenerate continued even after transfer of the material to subculture medium. Rooting of *in vitro* regenerated shoots of safflower has been consistently problematic, thus reducing the overall efficiency of whole plant regeneration (George and Rao 1982, Nikam and Shitole 1999). Less than 10 % of shoots regenerated from cotyledons rooted on hormone-free MS medium with 6 to 8 % sucrose (George and Rao 1982) or 9 % sucrose (Tejovathi and Anwar 1987). Most of the other reports define the use of growth regulator free MS medium or medium supplemented with NAA for rhizogenesis but the frequency of rooting was not up to the desired extent (Sujatha and Suganya 1996). In the present investigation, a high frequency shoot elongation was achieved in the presence of GA_3 and BAP treated on MS medium containing 3.0 % sucrose. Rooting of *in vitro* regenerated shoots is a major problem in safflower. However, a higher frequency rooting was observed in 'NARI-6' on NAA and $AgNO_3$ combinations in the tested medium. In the earlier studies of safflower, shoots regenerated from explants and callus, produced roots at 21.7 and 17.3 % in low level of NAA or IBA or IAA treated medium (Nikam and Shitole, 1999). Moreover, Sujatha and Suganya (1996) reported that elongated shoots subcultured on half-strength MS medium with 3.0 % sucrose and low level of NAA induced high frequency of rooting. In the present study, a controlled callus interspersion around the shoots with high frequency rooting was observed on a half strength MS medium supplemented with NAA and IBA in NARI-6. Rooted plants were transferred to the

potting mix and were grown under green house condition. Micropropagation of safflower provides an opportunity to harvest virus-free material and conserve important germplasm resources *in vitro.* It furnishes material for genetic transformation and for employing molecular techniques in breeding.

2.2.5. SUMMARY

- Leaf explants showed higher frequency of calli production in different auxins (TDZ, IBA, IAA, NAA and 2,4-D) tested medium.
- Although conspicuous callus mass was obtained from immature leaf explants cultured on TDZ + IAA (4.5 µM -1.5 µM) supplemented medium showed 92 % of organogenesis.
- All the explants derived calli were isolated and subcultured on plant regeneration medium containing TDZ along with low level of different auxins (IAA, IBA, NAA and 2,4-D) induced adventitious shoot regeneration.
- Although, maximum number of shoots were formed from leaf explants cultured on TDZ (4.0 µM) and IBA (1.5 µM) fortified medium showed (28.5) mean no. of shoots with 78 % of organogenesis.
- Regenerated plants placed on shoot elongation medium containing TDZ (3.5 µM) and GA_3 (1.5 µM) found better shoot elongation with (2.8 cm).
- Elongated shoots placed vertically on suitable rooting medium containing half strength MS salts, 3.0 % sucrose, IBA (2.0 µM) and NAA(0.5 µM) from which 53.8 % frequency of rooting was noticed.
- Rooted plants with subsequent branching was observed and transferred into plastic pots were survived under green house condition.

Table 2.1 Effect of growth regulators on shoot regeneration from Hypocotyl explants of safflower (*C. tinctorius* L.) cv. NARI-6.

MS medium composition (µM)		Hypocotyl (Direct)	
		Organogenesis (%)	Mean No. of shoots
BA+CPPU			
1.0	0.5	20±0.7 de	6.7±1.4 cd
2.0	1.0	48±0.2 cd	14.2±1.0 c
2.5	1.5	62±1.0 bc	19.8±2.6 b
3.0	2.0	59±1.8 c	19 ±3.2 b
4.0	2.5	50±1.0 c	17.3±0.5 bc
BA+KN			
1.5	0.5	32±1.4 d	8.2±0.8 cd
2.0	1.0	65±2.0 bc	17.5±1.3 bc
3.5	**1.5**	**97±1.3 b**	**34.2±1.0 ab**
5.0	2.0	66±0.5 bc	22±1.0 ab
6.5	2.5	62±0.9 bc	20±1.5 b
IBA+GA_3			
1.5	0.5	51±1.9 c	9.7±1.0cd
3.0	1.0	80±2.4 ab	19±2.1 b
4.5	1.5	87±1.2 a	21.5±0.7a
5.0	2.0	90±1.8 a	27±0.3 ab
BA+2,4-D			
1.5	0.5	5.0±0.1 f	3.0±0.5 f
3.0	1.0	10±0.5 e	0.5±0.3 e
4.5	1.5	22±1.7 de	1.8±0.4 de
5.0	2.0	31±2.1 d	3.6±0.1 d

Means with common letters within a column are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test (DMRT).

Table 2.2 Effect of growth regulators on callus and shoot regeneration from leaf explants of safflower (*C. tinctorius* L.).

MS medium composition (µM)	Leaf Explants (In direct)		
	Organogenesis (%)	**Mean No. of callus**	**Mean No. of shoots**
TDZ+IAA			
1.5 0.5	18.50±1.7 e	4.1±1.7 e	3.1±1.2 e
3.0 1.0	40.0±1.8 cd	10.5±1.9 d	9.5±1.9 d
4.5 1.5	**92.0±1.5 a**	**45±0.7 a**	27.0±0.7 a
6.0 2.0	62.0±0.3 ab	14.6±2.2 b	11.6±2.2 b
7.0 2.5	55.0±2.3 c	13±2.7 bc	10.2±2.7 bc
TDZ+NAA			
1.5 0.5	42±2.2 e	2.0±0.3 e	2.5±0.3 e
3.0 1.0	57±1.0 d	11.1±0.4 cd	10.3±0.4 cd
4.0 1.5	62.4±0.7 bc	22.5±2.5 a	21.7±2.5 a
5.5 2.0	66.8±1.0 ab	12.2±3.5 b	11.8±3.5 b
7.0 2.5	70±0.4 a	10.4±0.8 bc	9.4±0.8 bc
TDZ+IBA			
1.5 0.5	24.6±1.0 e	8.0±1.5 e	5.0±0.5 e
3.0 1.0	63±1.0 b	15.0±0.8 d	14.1±0.7 cd
4.5 1.5	**78±1.4 a**	18.0±0.2 ab	**28.5±2.5 a**
5.0 2.0	60±0.8 cd	21.5±1.1 a	17.2±3.5 b
7.0 2.5	51±0.4 d	16.4±3.9 bc	14.4±0.8 bc
TDZ+2,4-D			
1.5 0.5	0.8±0.0 e	0.6±0.0 e	0.5±0.1 e
3.0 1.0	8.8±3.0 d	0.7±0.5 d	0.8±0.2 d
4.5 1.5	22±0.5 bc	0.9±1.5 bc	1.3±1.3 bc
6.0 2.0	17±0.3 a	1.9±0.4 a	1.5±0.6 a
7.0 2.5	15.0±1.1b	1.4±2.2 b	1.2±2.4 b

Means with common letters within a column are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test (DMRT).

Table 2.3 Effect of growth regulators on shoot elongation in Hypocotyls and leaf explants of safflower (*Carthamus tinctorius* L).

MS medium composition (µM)		Shoot length (cm)		
		Mean No. of responsive plantlets	Hypocotyl explants	Leaf explants
BAP + GA_3				
1.0	0.5	16.2±1.0c	1.8±1.2 d	0.6±1.0 ea
3.0	1.0	19.0±1.3bc	2.6 ±0.5 bc	1.0±1.0 eb
3.5	**1.5**	**26.0±1.7 a**	**3.7±1.4 a**	0.9±1.0 ed
5.0	2.0	21.0 ±1.2b	2.5±1.0 bc	1.1±1.0 ec
6.0	2.5	18.3±1.1bc	2.4±1.3 bc	0.2±1.0 ed
TDZ + GA_3				
1.0	0.5	14.6±0.6 e	0.5±1.0 ea	1.2±1.0 d
3.0	1.0	11±1.0 b	0.9±1.0 eb	2.1 ±0.3 bc
3.5	**1.5**	**18±1.4 a**	0.8±1.0 ed	**2.8±1.4 a**
5.0	2.0	10±0.8 cd	1.1±1.0 ec	2.1±1.0 bc
6.0	2.5	08±0.4 d	0.3±1.0 ed	1.7±1.3 bc

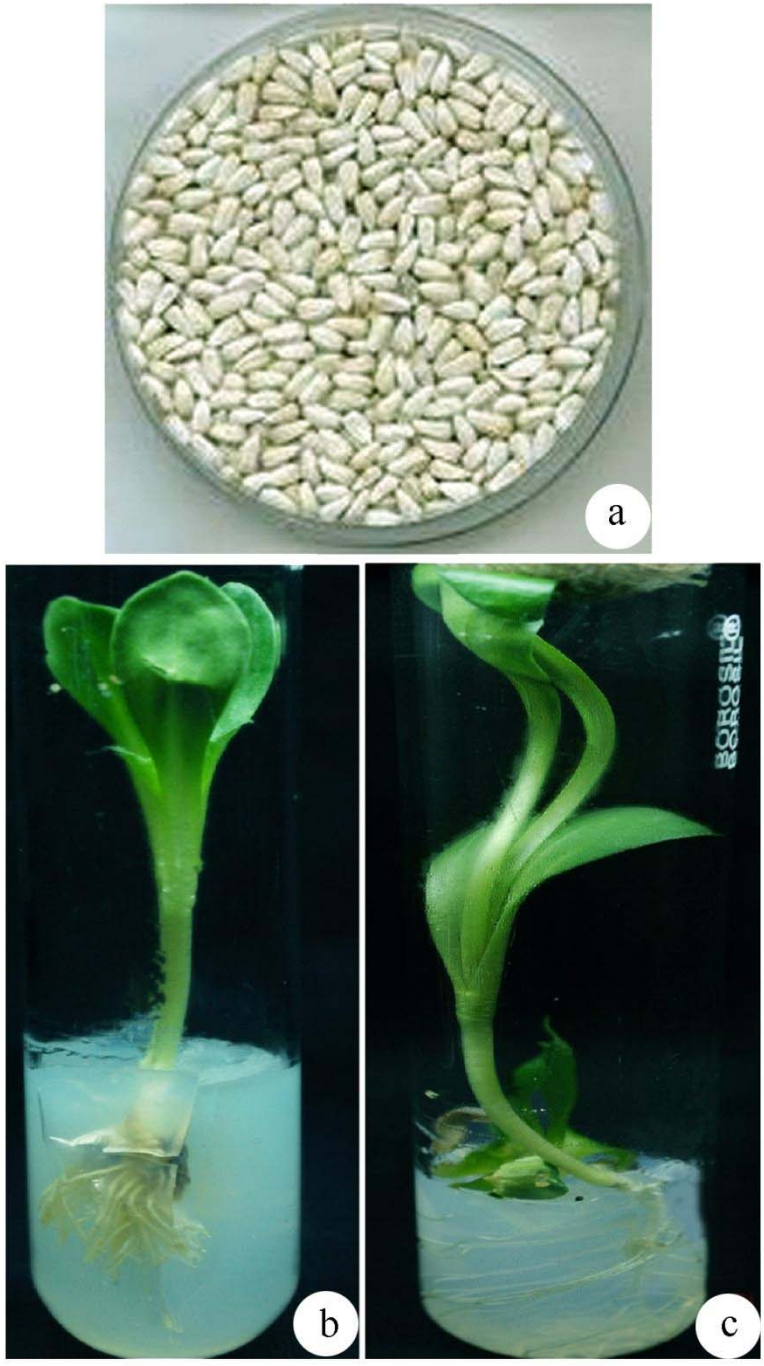
Means with common letters within a column are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test (DMRT).

Table 2.4 Effect of growth regulators on root induction from Hypocotyl and leaf derived plants of safflower (*Carthamus tinctorius* L.) cv. NARI-6

½ MS medium composition (µM)		Rooting frequency (%)	
		Hypocotyl	**leaf**
IBA+$AgNO_3$			
0.5		13.5±1.7d	12±0.5 e
1.0		24.0±1.0 c	15.4±1.6 d
1.5		30.0±0.5b	23.5±2.0 ab
2.0		**57.7±1.6a**	24.7 ±0.7a
2.5		25.2±0.3bc	21±2.1 cb
NAA+ KIN			
0.5	0.3	26±0.2e	16±2.1d
1.0	0.3	40.3±0.9cd	31.7±1.7c
1.5	0.3	42.1±1.8ab	43.5±2.0 ab
2.0	0.3	47.5±1.5 a	46±0.5 a
2.5	0.3	40.8±1.7 c	34±1.2 b
IBA+NAA			
0.5	0.5	33±2.0 cd	25±1.9 c
1.0	0.5	36.2±2.7c	34±1.7 b
1.5	0.5	49.8±0.5 a	47.2±0.1ab
2.0	**0.5**	45.1±0.2 ab	**53.8±0.4 a**
2.5	0.5	43±1.7 bc	33.4±3.1 b

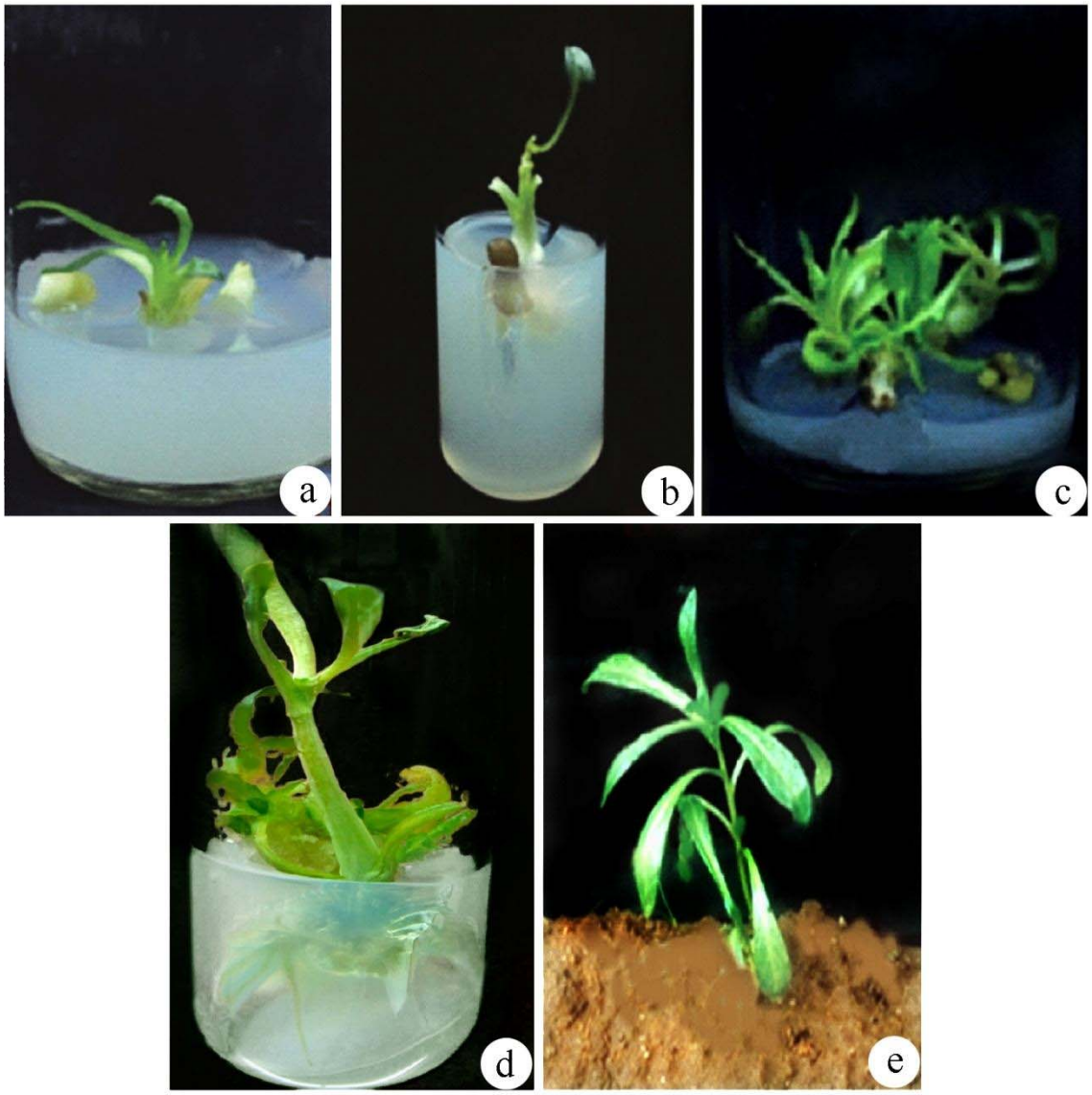
Means with common letters within a column are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test (DMRT).

Fig. 2.1. *In vitro* Seed Germination



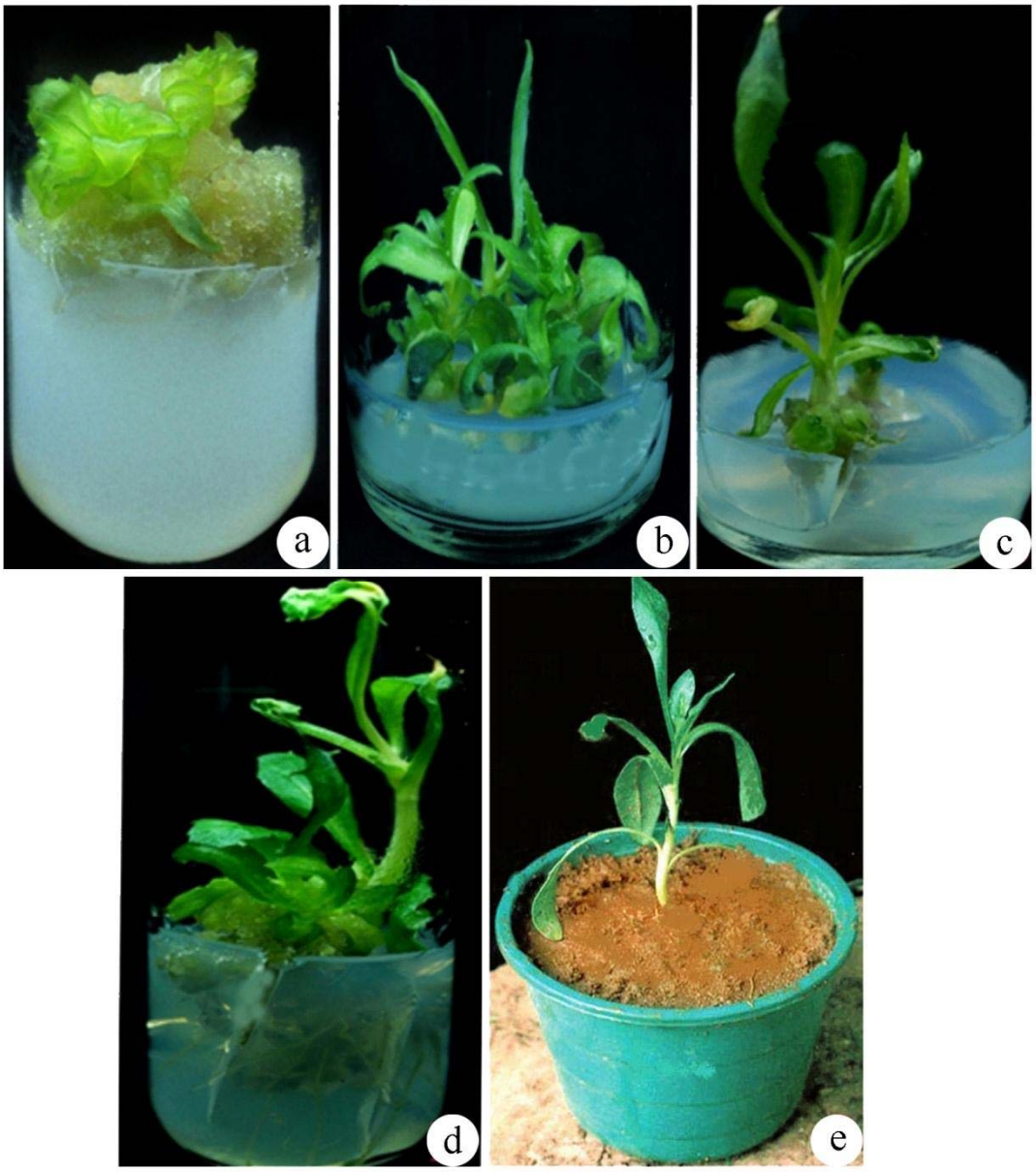
a – Safflower Seeds
b – *In vitro* seed germination
c – Fully germinated Plantlets

Fig. 2.2. Direct Organogensis from Hypocotyl explant



a – Hypocotyl explants initiation
b – Shoot initiation
c – Multiple shoot proliferation
d – Rooting
e – Grown in the field

Fig. 2.3. Indirect Organogensis from Leaf explants



a – Callus initiation from leaf explant
b – Microshoot initiation
c – Shoot proliferation
d – Rooting
e – Hardened in plastic pot