Improvement of safflower (*Carthamus tinctorius* L.) for salinity tolerance under *in vitro* condition

ABSTRACT

Aim- The primary aim of the present study was to screen the salt tolerant calli and optimization of *in-vitro* regeneration protocol from selected screen calli.

Methodology- The cotyledonary leaf explants was sterilized by using 1 % Bavistin, 0.1% Mercuric chloride and 70 % ethanol followed by washing with distilled water. Sterilized explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentration of NaCl (i.e. 0, 50mM, 100mM, 200mM, 300 mM and 400 mM) to check the salinity tolerance ability of PKV pink genotype. In vitro screening of callus was studied by morphological characters like colour and texture of callus, callus growth percentage, relative water contained, cell survivility and proline content from saline stress and unstress calli to confirm the saline tolerance pressure to regenerate the PKV Pink safflower genotype.

Results- MS medium supplemented with 150 mM NaCl showed 50% survival of calli, whereas no growth was obtained in high concentration of NaCl. Moreover, biochemical assay like proline estimation was done for its confirmation from different NaCl stress and unstress calli. The proline accumulation found to be highest callus grown on MS media supplemented with 150 mM NaCl as compared to control. Also studied the morphological observation i.e. colour and texture of calli, callus growth percentage, relative water content and cell viability under different NaCl stress to select the saline tolerance pressure to regenerate the tolerance line in PKV Pink. The saline tolerance shoots failed to produce *in vitro* rooting on the standardized rooting medium. So, the different approach like higher auxin shock and grafting experiment were attempted to overcome the rooting problem. Higher auxin shock experiment failed but grafting approach found satisfactory to overcome the rooting in saline tolerance shoot and for development of saline tolerance line in PKV Pink safflower genotype.

Conclusion- The development of abiotic stress tolerance plants found the better understanding of physiological and biochemical changes in plants under *in vitro* stress conditions.

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Key words: Safflower, Regeneration, Salinity tolerance, Screening

INTRODUCTION

Salinity is one of the major problems to affect plant growth and development in arid and semi arid regions [1]. The reasons of the formation of the salty soil are climatic factors, weathering of parental rocks, oceans and using salty water for irrigation [2]. It is estimated that 20% of the irrigated land in the world is presently affected by salinity. So, tolerance to salinity stress is a key topic to consider for crop improvement. Salinity reduces plant growth and leads to physiological, biochemical and metabolic changes depending on severity and duration of the stress, and ultimately inhibits crop production. Plants develop various physiological and biochemical mechanisms in order to survive in soils with high salt concentration. It has been widely reported that plants subjected to salt stress usually generate different types of reactive oxygen species (ROS) including superoxide, hydrogen peroxide, hydroxyl and singlet oxygen radicals [3, 4, 5]. In addition, accumulation of osmolytes such as proline, glycinebetane, sugars and sugar alcohols also play a substantial role in plant protection against salinity stress [6].

In vitro physiological features provide the basis for effective germplasmic salt testing procedures [7]. This approach is based on the introduction of genetic variation between cells, tissues or regenerated plants [8] Plant culture studies also allow for the differentiation and selection of salt-tolerant and drought-tolerant lines to determine how cell tolerance works at the cellular level. Genetic manipulation and selection of desirable breeds are additional tools in traditional breeding to produce plants that are resistant to stress [9] *in vitro* conditions. *In vitro* selection of salt-tolerant cell cells has been reported in several varieties such as wheat [10], sugarcane [11] and fennel [12]. Safflower (*Carthamus tinctorius* L.) is a well quality oilseed crop [16]. Safflower is a multi-purpose oilseed crop rich in polyunsaturated fatty acids with a moderate salt tolerance and long rooted crop [13]. Although the ancient reproductive capacity has been attributed to high safflower crops, *in vitro* technology may be the catalyst for genetic testing for genetic stress. To date, *in vitro* studies have only been performed to test drought tolerance in safflower [14]. Therefore a test of salt tolerance in safflower is still not reported. In addition, much of the tissue culture acting on safflower is dedicated to increasing direct plant regeneration [15, 16] rather than developing salt-tolerant genotypes.

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The objectives of the present study were to assess the safflower explants for callus production and shoot induction obtaining the most suitable explant for *in vitro* study. *In vitro* salt tolerance of safflower var PKV Pink. The safflower genotypes that display an increased tolerance to *in vitro* salt-stress could be an important genetic material for salt stress improvement at the whole plant level

MATERIAL AND METHODS

Plant material

In present investigation, experimental material was obtained from Oilseed Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India.

Explants preparation

Mature seeds of safflower of PKV pink washed with tap water for 5 min followed by washed few drops of Tween 20 for 5 min. followed by washed with distilled water for 3-4 times. The seeds surface-sterilized with 70% ethanol for 60 sec. and rinse with steriled distilled water for 3-4 times. Seeds again treated with Bavistin (0.8%) to reduce the fungal contamination. The washed seeds then treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min followed by rinse with steriled distilled water. Further drying of seeds on tissue paper in laminar air flow and used for further work. Surface sterilized seeds were germinated on half strength solid MS medium at 26±2°C with 16 hours light (2000 Lux) and eight hours dark period for 12 days. Cotyledonary leaf was excised from 10 to 12 days old germinating seedling and cut into 0.5-1cm^2 segments and explants were placed on previously standardized callus induction medium [6].

Methodology for *in vitro* regeneration studies

***In vitro* screening of calli on different concentration of NaCl**

Fully grown calli was screened on MS medium containing different concentration of NaCl (0, 100, 200, 300 and 400 mM NaCl) and callus growth was recorded up to 3-4 weeks.

Optimization of selection pressure for salinity tolerance

Morphological characters of calli:

After the placement of calli on different concentration of NaCl (0 to 200mM) for screening of salt tolerance observe the morphological characters of calli i.e. colour and texture.

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Callus growth percentage (CGR)

The percentage of callus growth along with different salinity concentration should be calculated by using following formula, [18]. CGR= [(W2-W1)/W1] ×100, whereas, whereas, W1 = initial weight, W2 = final weight weights, respectively.

RWC (Relative Water Content)

The relative water content (RWC) is a parameter reflecting cellular osmotic stress. Callus samples of known fresh weight were dried in an oven set at 70°C for 48 hrs to calculate callus dry weight. After which they were re-weighed and the difference in the initial and final mass determined. RWC was calculated by formula [19]. RWC= [(FW-DW)/DW] ×100, whereas, FW and DW are the callus fresh and dry weights, respectively.

Proline estimation of different *in vitro* saline stress calli

0.5 g of callus samples was homogenized in 2 ml of 3 % (w/v) sulphosalicylic acid using mortar and pestle. 1.5 ml of extract was taken in test tube and to it 2 ml glacial acetic acid and 1.5 ml of ninhydrin reagent was added. The reaction mixture was boiled in water bath at 100°C for 1 hour. After an hour, the reaction was stopped in ice and finally 4 ml of toluene were added. The two layer forms, the pink colour developed at the top and colourless layer at the bottom. Collect the pink layer and read the OD value in spectrophotometer at 520 nm. The amount of proline in the stress and unstress calli were calculated using a standard cure prepared from L-proline by using the following equation: Proline in μ mol.g $^{-1}$ FW = (Abs $_{extract}$ – Blank)/ slope x Vol $_{extract}$ / Vol$_{aliquots}$ x 1/ FW

Where, Abs $_{extract}$ is the absorbance determined with the extract, Blank (expressed as absorbance) and slope (expressed as absorbance nmol^{-1}) are determined by linear regression, Vol $_{extract}$ is the total volume of the extract, Vol $_{aliquot}$ is the volume in the assay, FW (expressed in mg) is the amount of plant material extracted.

Cell viability Test

Tetrazolium test was used to check the cell viability. The calli of different stress medium is used to check the viable or live cells. Tetrazolium choride is a dye primarly used to check the seed viability by staining the live cells in tissue giving red colour.5-7 cutted pieces of calli of about 0.2 cm^2 were soaked in 0.1 % tetrazolium chloride for 24 hrs and appearances of red colour were recorded for each different saline stress calli.

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Methodology for regeneration from selected salt tolerant screened calli

After the optimization of *in vitro* salinity tolerance pressure, the screened calli from 45 day old were further transferred on previously optimized medium used for regeneration i.e. formation of shoot induction, multiple shoots formation and shoot elongation. The observation was recorded at 15 to 20 days of interval.

Grafting approach (*in vivo and in vitro)* to develop saline tolerant safflower plant

Grafting practices were attempted to overcome the rooting problem and development of saline tolerance line in PKV Pink.

***In vitro/ in vivo* grafting**

Seed germination was carried out in small plastic bags filled with soil and for *in vitro* grafting, seed germination done on half strength of solid MS medium grown in beaker. Just prior to grafting, decapitate the seedling grown in soil and half solid MS medium respectively. Give V shape cut to decapitate seedling i.e. root stock of age 15 and 30 days. Placed the matching scion (1-2cm) and parafilm was wrapped around the graft point. Grafted seedlings were covered with polythene bags/ plastic cups with holes to maintain the humidity up to 70-80% and grown at 26±2ºC with 2000 lux at 16hr (day)/8hr (night) photoperiod for 1 month and regular observation were recorded.

RESULTS AND DISCUSSION

***In vitro* selection of calli for salt tolerance pressure:**

After 3-4 weeks of inoculation, full callus growth was observed on MS with 2, 4-D and KIN (1mg/L) each. The uniform size of callus excised and placed on callusing medium containing different concentrations of NaCl (0, 100, 200, 300 and 400 mM NaCl) screening for salinity as shown in Plate 1.

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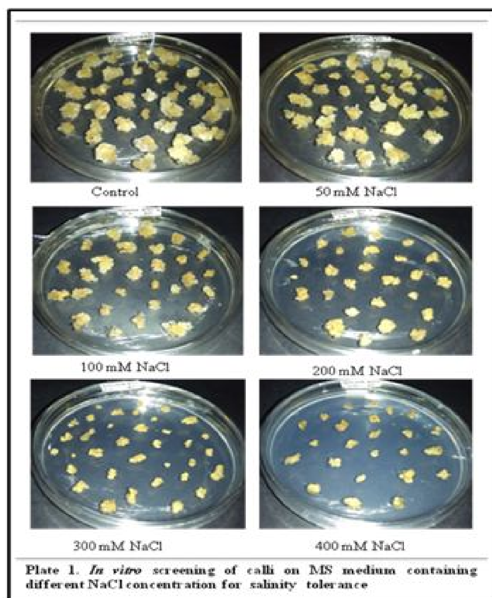

Plate 1. *In vitro* screening of calli on MS medium containing different NaCl concentration for salinity tolerance

Morphological observations for different salinity stress:

After 3-4 weeks, the morphological characters i.e. callus colour and texture, visible callus growth, callus growth percentage, Relative water content of callus (RWC), cell viability with tetrazolium chloride (TTC test) and proline were estimated (Table 1). The two different colors and texture were observed in different saline stress callus growth. Yellowish and whitish color of callus was observed in control and stress upto 100mM NaCl and the nature found was fragile (quietly easy to cut). While hard texture and pale yellow colour of calli from 200mM to 400mM NaCl medium. The hard texture of callus might be due to increase in the NaCl in the medium. Woodward [20] stated that callus production was so variable that it was not possible to produce callus of consistent texture, colour and growth under salinity condition.

Table 1. Morphology of calli on different saline tolerance stress

Sr. no.	Treatments	Observation recorded after one month	
		Colour	Texture
1.	Control (CIM+ 0 NaCl)	Yellowish and whitish	Fragile
2.	CIM+ 50mM NaCl	Yellowish and whitish	Fragile
3.	CIM+ 100mM NaCl	Yellowish and whitish	Fragile
4.	CIM+ 200mM NaCl	Pale yellow	Hard
5.	CIM+ 300mM NaCl	Pale yellow	Hard
6.	CIM+ 400mM NaCl	Pale yellow	Hard

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(Where, CIM is callus induction medium containing MS with 2, 4-D and KIN (1mg/L) each.)

Relative water content (RWC) of different *in vitro* stress calli:

The percentage of relative water content in callus showed directly proportional relationship with *in vitro* saline stress up to certain limit i.e. 200 mM, Above 200mM saline stresses the relative water content remains constant. Relative water content is directly related to the growth of callus. As the saline stress increases the growth of callus decrease. The saline stress reduces the availability of nutrient in the medium and finally decrease in the RWC of different stress callus as shown in the Fig 1A. The similar findings were observed by Kakaei et al. [18] they found the highest and lowest RWC was recorded for cultivar in culture medium containing 20 mg-1 and 160 mg-1 NaCl, respectively, working on NaCl and PEG induce osmotic stress on callus growth parameters in two safflower cultivars. Soheilikhah et al. [21] observed the decrease of callus water content was under both NaCl- and mannitol-induced stress.

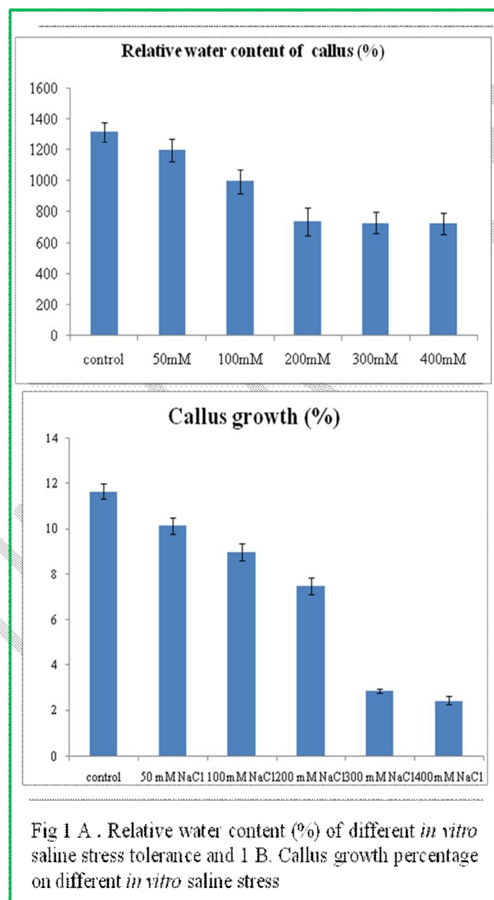

Fig 1 A . Relative water content (%) of different *in vitro* saline stress tolerance and 1 B. Callus growth percentage on different *in vitro* saline stress

Visible callus growth and callus growth percentage:

The calli (3-4 weeks) when inoculated on different saline stress medium was weight and again weight after 4 weeks and calculated as per the formula**.** The percentage of callus growth on different saline stress shows as the salinity stress increases the relative callus growth percentage is decreasing, up to certain level, i.e. 200mM (Fig 1B). The similar findings were observed by Kakaei et al. [18] they found the highest RGR and CGR were recorded in calluses grown on MS medium without NaCl and 20 mg^{-1} NaCl, while the lowest RGR and CGR were observed in media containing 160 mg^{-1} NaCl, working on NaCl and PEG induce osmotic stress on callus growth parameters in two safflower cultivars.

Callus cell viability under stress and unstress condition:

After calculating the percentage of relative water content of different saline stress, the calli were used to check the cell viability. 5-7 cut pieces of calli of about 0.2 cm^2 were soaked in 0.1 % tetrazolium chloride for 24 hours and appearances of red color were recorded for each different saline stress calli. Tetrazolium chloride is a dye primarily used to check the seed viability by staining the live cells in tissue giving red color. We use this dye to check the callus cell viability in different saline stress medium. The stained cell with tetrazolium (0.1%) with red color was considered as viable. While unstain with no red colour was consider not viable cell under different stress. In unstressed medium, high no. of callus cell stain with Tetrazolium chloride giving red color. As the *in vitro* NaCl stress increases the number of callus stain decreases upto 200mM NaCl. Above 200mM NaCl stess i.e 300 mM and 400mM NaCl stress, no callus cell stain to red colour indicated that in high saline stress above 200mM the cell are not viable as shown in Plate 2. The similar findings were observed by Kakaei et al. [18] they found callus cell viable upto 160 mg/L *in vitro* NaCl stress, working on NaCl and PEG induce osmotic stress on callus gowth parameters in two safflower cultivars.

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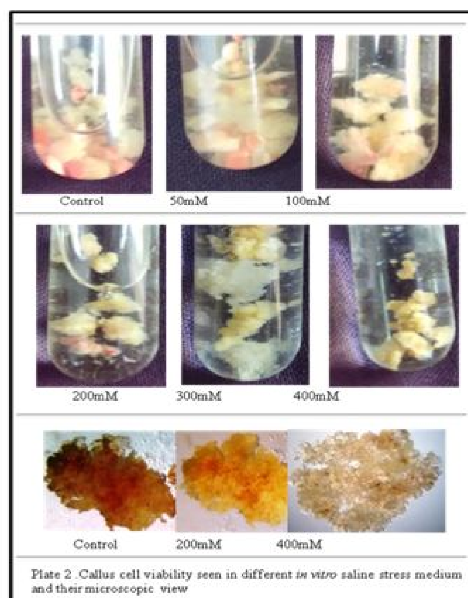

Plate 2 .Callus cell viability seen in different *in vitro* saline stress medium and their microscopic view

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Proline Content

Preparation of Standard curve:

To determine the amount of proline in µmol. g^{-1}. The standard proline curve (20µM to 100µM) was estimated to determine the amount of proline in different stress calli. The average absorbs with different known concentration.

Estimation of proline of different saline stress calli:

From the above studied visible parameters, i.e. callus growth, relative water content and cell viability of different *in vitro* stress calli showed moderate growth in between 100mM to 200mM. The proline content was estimated from different salt stressed tolerant calli (28 days old) grown up to 3-4 weeks on CIM with different NaCl (100mM, 125mM, 150mM, 175mM and 200mM) stress medium as shown in Plate 3. Under control conditions the proline accumulations found less (0.245 µmol. g-1 FW) and increases upto 150 mM NaCl i.e.1.669 µmol. g^{-1} FW and further the accumulation starts declined with increase in NaCl concentrations. The directly proportional relatioship up to 150 mM was seen in the proline content of stress tolerance calli. Thus, the results clearly indicate that salt stress has a significant effect on proline accumulation. The accumulation was higher up to 150 mM NaCl stress that might be due to stress tolerance further decreased due to break of defense mechanism. So, we select 150mM NaCl to grow the safflower tolerance line shown in Fig 2 B. The similar finding of proline was observed by Rahman et al. [22] while working on NaCl stress

callus culture of *Datura metel* and *D. Stramoniu* and Elyasi et al. [23] working on drought stress of immature embryo and callus culture of wheat. Other than callus culture proline estimation Pagariya et al. [24] also found increases proline, malondialdehyde and enzyme activity assays like catalase, peroxidise,superoxide dismutase content in salt stress working on sugarcane plants for differential expressed candidate genes.

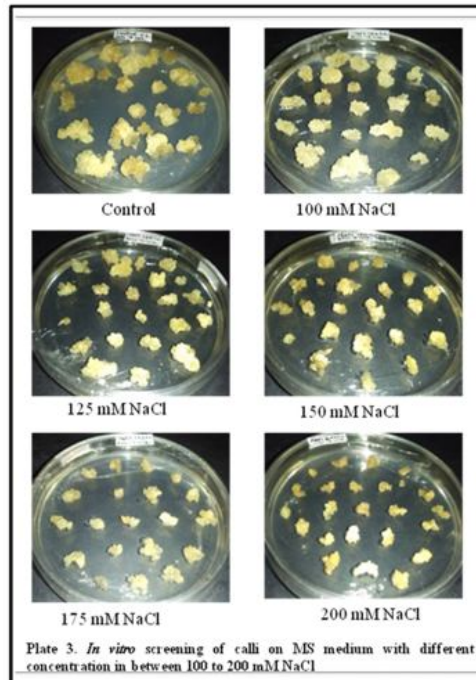

Plate 3. *In vitro* screening of calli on MS medium with different concentration in between 100 to 200 mM NaCl

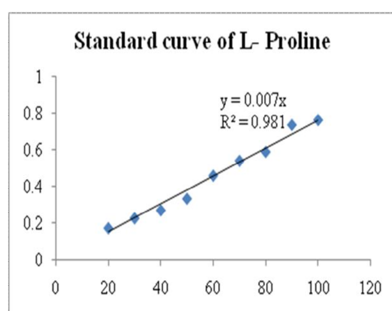

Fig 2 A. Standard curve of L-proline

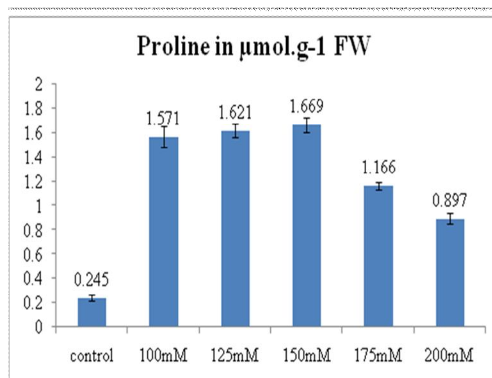

Fig 2 B. Proline content of different stress calli with standard error for each treatment

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Regeneration of salt tolerant screened calli:

After the optimization of *in vitro* saline tolerance pressure ie. 150 mM NaCl. The calli is further transferred to previously optimized medium for regeneration, i.e. formation of shoot induction, multiple shoots formation on 4 mg/L BAP and shoot elongation on the same composition containing 1mg/L GA3 along with 150 mM NaCl in each regeneration steps shown in Plate 4. The similar result was observed by Alagarasan et al. [25] *in vitro* screening of callus under different concentration of NaCl and identification of salt tolerant callus and regeneration of the callus under different concentration of NaCl and development of somaclonal variants from the salt tolerant calli. Saline tolerances shoots were transferred to standardized concentration of rooting medium containing MS medium with NAA (2mg/L). But no rooting was seen to tolerance shoots. The successful rooting in other oilseed and medicinal crop are carried out on plain MS medium given by Sen janyanti et al. [26]. So the attempts for rooting to saline tolerances shoots were done on plain MS medium which failed to root. The

different attempts like higher auxin shock and grafting attempts were carried out. Higher auxin shock i.e. 10 mg/ml auxin (IBA) shock upto 7 days and transferred to further rooting medium and use of activated charcoal found effective to induce rooting in safflower. So, different auxins like IAA, IBA and NAA i.e 10 mg/L stock were used to induce roots in safflower. The 10 mg/L different auxin stock sterilized with syringe filter and placed in a test tube in which autoclaved cotton dipped and saline tolerance shoots was placed for 2-8 days to induce rooting. The saline shoots from higher IAA , IBA and NAA were placed on the optimized rooting medium, i.e. NAA(2mg/L) medium containing additional two content i.e., activated charcoal (1g/L) and phyta gel (2.8%) for 3-4 weeks. No root induction was observed in any combination. Only callus formation in a less and more amount at the base of the shoot as the increasing different auxin shock concentration was observed. Rooting in other oilseed and medicinal plants shown by Senjanyanti et al. [26] on plain MS medium. So the MS medium was also attempted to induce tolerance roots. Callus formed at the bases of shoots grown on NAA (2mg/L) after higher different auxin shock were trimmed out and placed on the Plain MS medium containing the same concentration of Activated charcoal and phyta gel for 3-4 weeks. Again no rooting was seen in any combination.

Plate 4. Regeneration of screen calli on optimized medium containing 150 mM NaCl

Grafting

After failing in all the possible methods for rooting of stress tolerances shoot. In order to overcome the rooting problem on stress tolerances shoots, we tried for *in vitro* and *in vivo* grafting method to produce the salinity tolerances line in safflower. Belide et al. [27] firstly developed *in vivo* grafting method to overcome poor *in vitro* root formation in transgenic safflower development.

***In vivo/in vitro* grafting**

The sterilized seed were inoculated on ½ strength of MS media and seedling were used for grafting while, for *in vivo* grafting root stock was grown in soil. Observation was recorded at regular interval after grafting as shown in Table 2. 10% and 12.5% survival frequency of grafted saline tolerant shoots was recorded after one week as shown in Plate 5.

Table 2. *In vitro/ in vivo* grafting of saline tolerant shoots

Sr. No.	Age of root stock (days)	Survival frequency of grafted saline tolerant shoots (scion)	
		In vitro grafting (%)	*In vivo* grafting (%)
1	15	10	12.5
2	30	8	12.5

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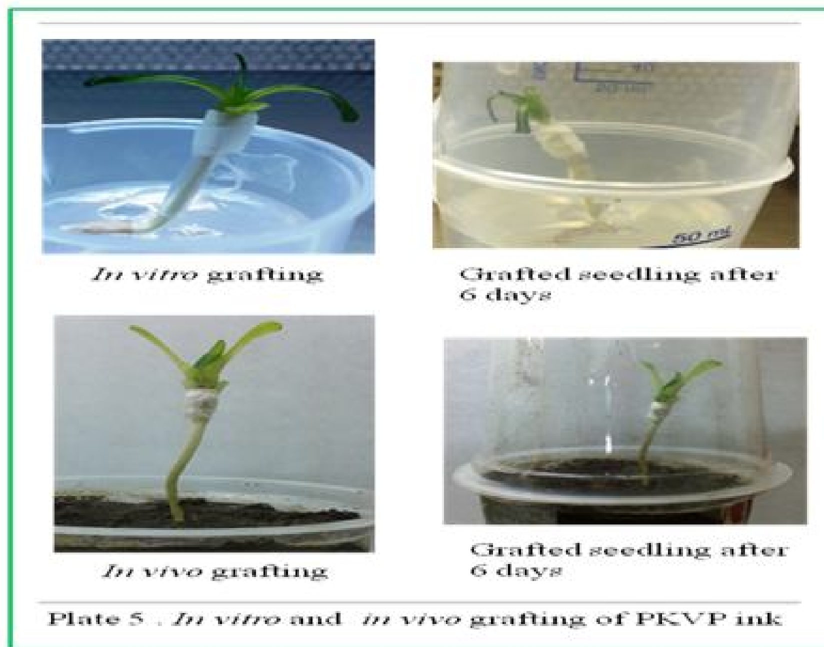

Plate 5 . *In vitro* and *in vivo* grafting of PKVP ink

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CONCLUSION

The optimized protocol for safflower *in vitro* regeneration is effective in developing salinity tolerance safflower line. The aim of this study was to select the salinity tolerant line in safflower. This study will help us to understand the mechanism of cell tolerance to high salinity tolerance pressures and also studies on better understanding of physiological and biochemical changes in plants under *in vitro* stress conditions. *In vitro* selection makes possible to save the time required for developed of abiotic stress tolerant lines in safflower crops and other commercial crops.

Conference disclaimer:

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