In vitro selection, regeneration and characterization of NaCl-tolerant plants of Sapindus trifoliatus: an important multipurpose tree

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Abstract

The present study demonstrates the selection of NaCl tolerant callus line and regeneration of NaCl tolerant plants of *Sapindus trifoliatus* L. from selected callus via somatic embryogenesis. Callus induced from leaf disc explants was subjected to the selection of NaCl-tolerant callus line on MS medium containing NaCl. The exposure of embryogenic callus to NaCl (0-250 mM) allowed the selection of callus to tolerate 100 mM NaCl, and it confirms with stability test by the three consecutive transfers of 100 mM NaCl tolerant callus to a medium devoid of NaCl and re-transfer to 100 mM NaCl supplemented medium. NaCl selected and non-selected embryogenic callus lines were characterized by the analysis of callus growth, tolerance index, and accumulation of both inorganic (Na^+ and K^+) and organic (proline, glycine betaine and soluble sugars) content. 100mM NaCl-tolerant callus showed the formation of globular structure, which differentiated different stages of somatic embryos on a maintenance medium containing NaCl (0-200mM). Mature cotyledonary stage somatic embryos developed from 100 mM NaCl tolerant globular structure were germinated on germination medium containing NaCl. About 67 and 38% NaCl tolerant somatic embryos were germinated on a medium without NaCl and containing 100 mM NaCl, respectively. NaCl tolerant plants developed on different germination medium with NaCl survived when transplanted to 0.2 or 0.3% (w/w) NaCl mixed potting mixture. Six-month-old established 100 mM NaCl tolerant plants were characterized by analyzing various physiological and biochemical parameters i.e., chlorophyll, carotenoid, L-proline content and antioxidative (MDA, SOD, CAT and APx) activities. The results suggest that in vitro selection technique offers an efficient way to develop NaCl tolerant plants of *S. trifoliatus*.

Introduction

Salt stress is a key abiotic factor that restricts plant growth, development and productivity. According to a recent estimate, approximately 20% of cultivated lands are adversely affected by salinity, which poses a significant threat to global sustainable food security (Kumar and Sharma 2020). Developing salt-tolerant plants and their establishment in salt-effected areas is an effective and economical way to utilize salinized land and reduce the adverse impact of salinity. The traditional breeding approach is less efficient in generating new salt-tolerant varieties in many crops because the salt-tolerant genetic resources in existing cultivated genotypes are limited (Harfouche et al. 2014; Alzahrani 2021). Alternatively, the use of in vitro culture techniques to select salt-tolerant cell lines from non-tolerant ones has been widely investigated. Many studies reported that biotic and abiotic stress tolerance in plant cell culture could be acquired by exposure to a stress-causing selective agent i.e., NaCl, PEG, mannitol, fungal toxin or culture filtrate etc. for several passages (Rai et al. 2011; Suprasanna et al. 2012). The gradual exposure to salt stress could promote adaptation to salinity (Singh et al. 2003; Zair et al. 2003). The first evidence of plant formation from selected salt-tolerant cell lines is *Nicotiana tabacum* (Nabors et al. 1980). Since then, in vitro selection techniques for the selection of salt tolerance cell lines using NaCl as a selective agent have been reported in several plant species like colt cherry (Ochatt and Power 1989), wheat (Barakat and Abdel-Latif 1996; Zair et al. 2003), potato (Ochatt et al. 1998; Queiros et al. 2007),

tomato (Hassan et al. 2008), rice (Basu et al. 2002; Abdelnour- Esquivel et al. 2020), bamboo (Singh et al. 2003), sunflower (Davenport et al. 2003), sugarcane (Gandonou et al. 2006; Nikam et al. 2014), mungbean (Hassan et al. 2008), *Chrysanthemum morifolium* (Hossain et al. 2006, 2007), Sweetpotato (He et al. 2009), *Zoysia matrella* (Chen et al. 2012), *Medicago truncatula* (Elmaghrabi et al. 2013), *Guizotia abyssinica* (Ghane et al. 2014). Other than salt stress, in vitro selection technique has also been widely used to develop drought, heavy metal tolerance, or disease-resistant plants in many plant species like *Passiflora edulis* (Flores et al. 2012), *Saccharum Officinarum* (Rao and Jabeen 2013), *Citrus sinensis* (Ge et al. 2015), *Daphne jasminea* and *Daphne tangutica* (Wiszniewska et al. 2015), *Cenchrus ciliaris* (Carloni et al. 2017), *Punica granatum* (Kumari et al. 2017), *Solanum tuberosum* (Ashrafzadeh and Leung 2017), *Vanilla planifolia* (Ramirez- Mosqueda et al. 2019), *Eucalyptus tereticornis* (Singh et al. 2020), *Beta vulgaris* (Yerzhebayeva et al. 2019), *Withania somnifera* (Chakraborty et al. 2020), *Zingiber officinale* (Sharma et al. 2020), *Malus* sp. (Verma et al. 2021), *Mangifera indica* (Pradhan et al. 2021). Using this technique, several useful somaclonal variants have been selected in many agricultural crops with abiotic stress tolerance and disease resistance traits, few of them are released for commercial plant production (Rai 2021).

The ability of trees to respond to abiotic stresses is of utmost importance because they have substantial effects on the local climate and provide a variety of products for human requirements, including wood, medicine, biomass, fuel, fodder, and of many industrial and commercial importance (Estravis-Barcala et al. 2020). Trees are more capable of responding to several abiotic stresses through the adoption at a cellular, physiological and molecular level compared to annual agricultural crops, particularly herbs. However, many abiotic stresses, especially salt and drought affect the physiology and metabolism of trees, which can negatively influence their growth and development. In addition, trees exist for years, decades, and sometimes centuries; therefore, they evolved several complex cellular, biological and molecular mechanisms in response to changes in environmental conditions (Harfouche et al. 2014; Zhang et al. 2021). Abiotic stress tolerance is usually achieved by several biochemical modifications of the cellular metabolism, like ion homeostasis, accumulation of osmolytes as an osmoprotectant, and enzymatic and non-enzymatic antioxidative activities for scavenging of reactive oxygen species (ROS) (Munns and Tester 2008; Harfouche et al. 2014; Estravis-Barcala et al. 2020; Zhang et al. 2021; Détain et al. 2022).

Sapindus trifoliatus L., commonly known as Soapnut tree, is a medicinally and commercially important tree. It has very high economic value in the saponin industry as its fruit contains saponins. Saponins from soapnut trees are used mainly as an environmentally friendly herbal detergent. Traditionally, its fruits are used to treat asthma, diarrhea and cholera (Asthana et al. 2011). Earlier, no information is available on the physiological responses of *S. trifoliatus* to salt stress, neither at the seedling stage nor at the mature phase. Since this tree has great medicinal and commercial value, it is necessary to elucidate the stress tolerance mechanisms and explore in vitro culture techniques for selecting salt-tolerant plants in *S. trifoliatus*. During the past years, few papers have been published on in vitro plant regeneration in *S. trifoliatus* using different explants via organogenesis and somatic embryogenesis (Desai et al. 1986; Unnikrishnan et al. 1991; Asthana et al. 2011, 2017).

The main aim of the present study was the in vitro selection and regeneration of NaCl-tolerant plants of *S. trifoliatus* via somatic embryogenesis from the selected NaCl-tolerant callus. This is the first report on the selection and regeneration of NaCl-tolerant plants of *S. trifoliatus* using in vitro selection technique. The physiological and biochemical characterization of NaCl tolerant and non-tolerant callus and further in vitro selected NaCl tolerant and non-tolerant plants were also analyzed.

Material And Methods

Plant material and embryogenic callus induction

The embryogenic callus was induced from leaf discs obtained from the mature tree, as described by Asthana (2010). In brief, leaf discs were cultured on callus initiation medium (CIM), i.e., 0.8% agar-solidified full-strength MS (Murashige and Skoog 1962) medium + 5.0 mg l^{-1} 2, 4- D + 0.01 mg l^{-1} Kin + 3% sucrose, for the callus induction. After three weeks of inoculation, explants were transferred to growth regulators (PGRs) MS basal medium and subcultured two to three times again on the same medium to raise the appropriate amount of actively growing embryogenic calli for conducting experiments on the selection of NaCl tolerant callus.

Selection of NaCl tolerant callus and stability test

The callus was subjected to screening for selection of NaCl tolerant callus line on MS basal medium containing different concentrations (25, 50, 100, 150, 200 or 250 mM) of NaCl. The medium without NaCl served as a control. The procedure for selection of NaCl tolerant callus line is depicted through a flow chart (Supl. Figure 1). The criteria for selection of NaCl tolerant callus was based on callus growth without necrosis. On each concentration of NaCl, the callus was subcultured at regular intervals (four weeks) till the callus grew without necrosis. During subcultures, the necrotic part of callus was removed, and only healthy growing callus was transferred to a fresh medium with the same concentration of NaCl. The maximum level of NaCl at which callus growth became normal after a few subcultures, was identified as a tolerant level of NaCl. The normal growing callus on such medium was subcultured once or twice and thereafter subjected to stability test. Since 100 mM NaCl was the maximum level of NaCl at which necrosis was not observed after a few subcultures, hence, the callus growing on 100 mM NaCl was referred to as the stable and tolerant line for further experimentation.

Characterization of NaCl tolerant callus line

For characterization, both the control calli (developed and growing on medium without NaCl) referred to as Callus I and NaCl tolerant embryogenic calli (developed and growing on 100 mM NaCl containing medium) referred to as Callus II were transferred separately on MS basal medium supplemented with NaCl as well as without NaCl in order to study various physiological and biochemical variations existing amongst them.

Fresh weight and dry weight measurement

The callus growth rate was examined in terms of fresh weight (FW) and dry weight (DW) of the control callus in the medium without NaCl and tolerant callus in the medium with 100 mM NaCl. To measure the fresh weight, the callus was taken out from the culture tube and blotted to remove the water droplets sticking to the external surface of the callus. For dry weight measurement, callus of NaCl tolerant and non-tolerant was dried in a hot air oven at 80^0C for 24h and the dry weight was subsequently measured. The FW and DW were measured at 1, 7-, 14-, 21- and 28-day intervals after the subculture of callus on fresh medium.

Tolerance index (TI)

The tolerance index of control and 100 mM NaCl tolerant callus lines was compared by measuring FW of embryogenic calli after 30 days of subculture on MS basal medium having various concentrations (25, 50, 100, 150 and 200 mM) of NaCl and without NaCl using the following formula (Dutta Gupta et. al. 1995).

$$TI = \frac{\text{FW on medium having NaCl}}{\text{FW on NaCl free medium}} \times 100$$

Analyses of Na^+ and K^+ content

Approximately 1.0 g callus was taken in a 100 ml conical flask containing 20 ml of acid mixture (concentrated nitric acid and hydrogen peroxide in the ratio of 9:1), heated softly on a hot plate until the solution became clear and colorless and left for cooling. The Na^+ and K^+ contents were estimated by reading the absorbance at 589 and 766.5 nm using Atomic Absorption Spectrophotometer (AAS) (Perkin Elmer AAS, model-2380). The Na^+ and K^+ contents were calculated using the following formula:

$$\underset{(\mu g\ g^{-1}\ FW\ of\ plant\ material)}{Na^+\ or\ K^+} = \frac{ppm\ (AAS) \times V}{W}$$

Where V = volume of extract, W = weight of plant material

After the computation of Na^+ and K^+ contents, a ratio of K^+/ Na^+ was determined.

Estimation of organic contents

Total free proline content in NaCl tolerant and non-tolerant callus was assessed by the standard procedure of Bates et al. (1973) using the approx. one-gram fresh weight of embryogenic callus. The glycine betaine content in NaCl tolerant and non-tolerant callus was measured by the procedure of Grieve and Grattan (1983) using approx. 500 mg fresh weight of callus. Total soluble sugar content was determined according to the method of McCready et al. (1950) using glucose as standard.

Regeneration of NaCl tolerant plants from NaCl tolerant calli

Callus proliferation and development of globular structure (GS) from 100 mM NaCl tolerant callus

For callus proliferation and the formation of globular embryogenic structures, the NaCl tolerant (100mM) embryogenic calli were subcultured regularly at an interval of four weeks on maintenance medium, i.e., MS basal medium containing 3% (w/v) sucrose containing different concentrations of NaCl (25, 50,100, 150 or 200 mM). Embryogenic calli induced on a medium devoid of NaCl were subcultured on a maintenance medium without NaCl. The frequency of embryogenesis (FE) was calculated as percent cultures showing the formation of globular embryogenic structures from NaCl tolerant embryogenic callus on maintenance medium containing different concentrations of NaCl (25, 50, 100, 150 or 200 mM).

Differentiation of NaCl tolerant somatic embryos from 100 mM NaCl tolerant callus

For differentiation of somatic embryos, the globular structures produced from 100 mM NaCl tolerant embryogenic calli were transferred to differentiation medium i.e., MS basal medium containing NaCl (25, 50, 100, 150 or 200 mM). The number of somatic embryos of each stage produced per globular structure was recorded after eight weeks of transfer.

Germination of NaCl tolerant somatic embryos

The mature cotyledonary staged somatic embryos tolerant to 100 mM NaCl were harvested and transferred to germination medium i.e., full-strength MS medium and 2% (w/v) sucrose containing 25, 50 or 100 mM NaCl or without NaCl. The somatic embryos differentiated and developed on a medium without NaCl, i.e., produced from control calli were also harvested and transferred to a germination medium without NaCl. The data for the percent germination of somatic embryos was recorded after four weeks of transfer of somatic embryos to the germination medium.

Acclimatization of NaCl tolerant plants

The NaCl tolerant (100 mM NaCl) plants (developed from *in vitro* selection technique) and non-tolerant plants (developed from somatic embryogenesis through leaf callus on the media devoid of NaCl) having well-developed roots and shoots were selected for acclimatization and hardening. For transplantation of NaCl tolerant plants, the tolerant plants obtained from 25, 50 and 100 mM NaCl containing germination medium were transferred to potting mixture containing 0.2, 0.3 or 0.5% (w/w) NaCl. Non-tolerant plants were transferred to potting mixture devoid of NaCl. The experiments of acclimatization of tissue culture raised plants of *S. trifoliatus* was performed as described by Asthana et al. (2011, 2017). Once plants were acclimatized in laboratory conditions, the plants were established initially in earthen pots containing garden soil and the same concentration of NaCl and later in field conditions. Data for the percent of established plants were recorded after four weeks of transfer to earthen pots.

Physiological and biochemical analysis of NaCl tolerant and non-tolerant plants

About six-month-old hardened NaCl tolerant (100 mM NaCl) plants were compared with the non-tolerant plants and analyzed for various physiological as well as biochemical parameters. The well hardened potted plants of non-tolerant and salt tolerant lines were irrigated once every two-day interval with 50 ml of 50, 100, 150 or 200 mM NaCl solution prepared in double distilled water for one month. Non-tolerant and tolerant plants rinsed with double distilled water without NaCl served as control. After one month, the leaves of plants were harvested to determine different biochemical parameters.

The leaf tissue of the NaCl tolerant as well as non-tolerant plants was harvested and used for the determination of various biochemical parameters such as chlorophyll (*a* and *b*) content, carotenoid content, proline content, and parameters of oxidative stress i.e., malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APx). Proline content was analyzed according to the procedure described above. The chlorophyll and carotenoid contents were calculated using Spectronic-20 colorimeter (Bausch & Lomb) according to the standard procedure used by Witham et al. (1971). Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid (TBA) method (Heath and Packer 1968). Total Superoxide dismutase (SOD) activity was analyzed according to the method described by Giannopolitis and Ries (1977). Catalase (CAT) activity was determined by following the consumption of H_2O_2 at 240 nm according to Aebi (1984). Ascorbate peroxidase (APx) activity was measured according to the method given by Nakano and Asada (1981).

Experimental design and data analysis

For callus induction, selection and plant regeneration experiments, 24 replicates were used while the experiments on the characterization of salt tolerant callus lines and plantlets were performed with six replicates. All the experiments were conducted thrice. Data were subjected to analysis of variance (ANOVA), and comparison among means of specific treatment pairs was made by the least significant difference (LSD) test. The significant difference among means was analyzed using Duncan's multiple range tests (DMRT) at $P < 0.05$.

Results And Discussion

Callus induction and in vitro selection of NaCl tolerant callus lines

Leaf discs obtained from mature trees cultured on callus initiation medium (CIM) showed the induction of friable and cream-colored callus within two to three weeks of culture. After three-four weeks of induction, callus was transferred to PGRs free medium i.e., callus proliferation medium (CPM), for their growth and proliferation (Fig. 1A). A similar trend of induction of callus and further somatic embryogenesis was also observed in *S. trifoliatus* when sepal used as an explant (Asthana et al. 2017). Earlier, the potential of leaf explants for somatic embryogenesis has also been reported in *Sapindus* sp. (Desai et al. 1986; Unnikrishnan et al. 1991; Asthana 2010; Kim et al. 2012; Singh et al. 2015).

Actively growing and proliferated calli on CPM transferred to medium containing different concentrations of NaCl for the selection of salt-tolerant callus line showed the growth of callus except on medium supplemented with 250 mM NaCl. Except for control and medium containing 25 mM NaCl, browning (necrosis) on the growing callus was observed on medium supplemented with 50–250 mM NaCl (Fig. 1A-F). Calli transferred to medium supplemented with 50 mM NaCl showed only a few necrotic spots (Fig. 1B). However, at higher concentrations of NaCl (100, 150, 200 mM), a large portion of the callus showed necrosis (Fig. 1C-E). On 250 mM NaCl-supplemented medium, the necrotic callus became dark brown and subsequently died after four weeks of transfer. During the in vitro selection process and after two to three subcultures, calli of 150 and 200 mM NaCl were completely necrotic and could not survive. 100 mM NaCl was identified as a tolerant level of NaCl because it was the highest concentration of NaCl at which necrosis was not observed after the third and subsequent subcultures. Hence, the callus growing on 100 mM NaCl-containing medium was subjected to stability test.

The results presented in this study reveal that the embryogenic callus on NaCl medium allowed its growth but with necrosis of different degrees depending on NaCl level in the medium. The callus necrosis could be due to their inability to accumulate osmotic solutes, thereby restricting uptake of water molecules from the medium. The exposure of embryogenic calli of *S. trifoliatus* to a lethal concentration of NaCl allowed the selection of callus having the ability to tolerate 100 mM NaCl. This may have become possible due to the spontaneous adaptation of callus cells to the NaCl environment and the adaptability of callus cells to the readjustment of metabolic processes and compensation for osmotic and ionic changes (Singh et al. 2003; Queiros et al. 2007).

Stability test for NaCl tolerance and characterization of NaCl tolerant callus lines

The three successive transfers (each at four weeks interval) of 100 mM NaCl tolerant callus on medium without NaCl and re-transfer to 100 mM NaCl supplemented medium showed growth without necrosis. This observation suggests that the selected NaCl tolerant callus at 100 mM NaCl is stable due to probably some permanent genetic changes.

Different physiological and biochemical parameters were analyzed to characterize non-tolerant callus that developed on medium without NaCl (Callus I) and NaCl tolerant calli, which developed on 100 mM NaCl containing medium (Callus II).

Fresh weight and dry weight measurement

ANOVA of data showed a significant increase in growth rate (in terms of FW, DW and FW/DW ratio) in both Callus I and Callus II (Table 1). The callus I grown on medium without NaCl exhibited linear increase in FW with increasing culture period but the FW of the tolerant callus induced on medium containing 100 mM NaCl was slow during the initial seven days of subculture and the tolerant calli resumed their normal growth after 14 days of culture. Such a growth pattern was also seen in *Dendrocalmus strictus* (Singh et al. 2003). Except time interval of seven days, DW of Callus II measured after different time intervals was

higher than that of Callus I (Table 1). In comparison to non-tolerant callus (Callus I), low FW/DW ratio was observed in tolerant callus (Callus II) during their growth on medium with 100 mM NaCl (Table 1). The slow growth rate of callus in salt stress conditions is a common adaptive phenomenon and is associated with an osmotic adjustment by accumulating various solutes, which are essential for balancing the water potential inside the cells compared to the surrounding media (Singh et al. 2003; Queiros et al. 2007).

Table 1
FW, DW and FW/DW ratio of the two callus lines, Callus I and Callus II after different time interval

Duration	Callus I			Callus II		
(days)	FW (mg)	DW (mg)	FW/DW	FW (mg)	DW (mg)	FW/DW
1	200.0 ± 4.16e	17.73 ± 1.58e	11.32 ± 1.36b	200.0 ± 4.16e	19.27 ± 1.12e	10.45 ± 0.67b
7	497.66 ± 40.52d	44.13 ± 3.50d	11.41 ± 1.25b	371.60 ± 5.24d	35.53 ± 0.76d	10.47 ± 0.38b
14	995.53 ± 87.29c	65.20 ± 6.92c	15.37 ± 0.71a	953.67 ± 65.91c	84.32 ± 8.69c	11.51 ± 1.33b
21	1980.93 ± 57.88b	126.67 ± 11.20b	15.80 ± 0.94a	1972.30 ± 37.54b	129.83 ± 3.53b	15.20 ± 0.33a
28	2362.60 ± 68.72a	135.60 ± 1.37a	17.44 ± 0.69a	2305.60 ± 50.83a	147.43 ± 2.81a	15.65 ± 0.53a
LSD ($P \leq 0.05$)	92.819613	19.42276	3.238563	129.02591	13.93188	2.345556
ANOVA	$F_{4, 10}$ = 251.840	$F_{4, 10}$ = 69.940	$F_{4, 10}$ = 6.941	$F_{4, 10}$ = 530.522	$F_{4, 10}$ = 161.926	$F_{4, 10}$ = 11.911
Callus I = Control callus developed and maintained on 0.8% agar solidified full-strength MS medium + 3% sucrose						
Callus II = 100 mM NaCl Tolerant callus developed and maintained on 0.8% agar solidified full-strength MS medium + 3% sucrose + 100 mM NaCl						
Values represent the Mean ± SE of three repeated experiments, each with twenty-four replicates.						
Mean values followed by the different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.						

Tolerance Index (TI)

The TI of both the callus lines slightly increased at 25 mM NaCl supplemented medium and decreased thereafter with an increase in NaCl concentrations in the medium (Fig. 2A). The higher TI of NaCl tolerant callus than the control callus observed in the present study demonstrates former to be more resistant to NaCl than the latter. A similar observation was also reported in *Dactylis glomerata* (Dutta Gupta et al. 1995) and *Dendrocalamus strictus* (Singh et al. 2003).

Na^+ and K^+ content

The level of Na^+ increased significantly ($P \leq 0.05$) with increasing concentrations of NaCl in both the callus lines i.e., Callus I and Callus II. However, Na^+ content was significantly higher in Callus II as compared to Callus I at all the concentrations of NaCl tested as well as on medium without NaCl (Table 2). Similarly, K^+ content and K^+/Na^+ ratio was significantly lower in Callus II compared to Callus I at all concentrations of NaCl (Table 2). Accumulating inorganic ions in response to salt stress is a common osmotic adjustment process. In the present investigation, the application of NaCl significantly increased intracellular Na^+ content in both control and tolerant callus. Our result is in accordance with the observation of Singh et al. (2003) in another woody species *D. strictus* who also found high Na^+ accumulation and low K^+ content in NaCl tolerant and the control calli. It is well known that Na^+ and K^+ homeostasis is essential for cellular functions because both compete for the active site of enzymes. A balanced cytosolic K^+/Na^+ ratio is a significant factor for salt tolerance in plants, and an imbalance in K^+/Na^+ ratio in a cell can disrupt various enzymatic processes (Munns and Tester 2008; Zhang et al. 2021).

Table 2

Effect of different concentrations of NaCl on ionic content (Na^+ and K^+ ion) and of K^+/Na^+ ratio in Callus I and Callus II

Conc. of NaCl in medium (mM)	Na^+ (mg g^{-1} FW of Callus)		K^+ (mg g^{-1} FW of Callus)		K^+/Na^+ ratio	
	Callus I	Callus II	Callus I	Callus II	Callus I	Callus II
0.0	1.36 ± 0.04d	5.10 ± 0.06f	2.04 ± 0.11d	1.75 ± 0.11c	1.49 ± 0.03ab	0.34 ± 0.02b
25.0	1.98 ± 0.05c	5.73 ± 0.02e	3.26 ± 0.15a	2.70 ± 0.03a	1.65 ± 0.11a	0.47 ± 0.01a
50.0	2.04 ± 0.16c	6.82 ± 0.34d	3.11 ± 0.09ab	2.46 ± 0.10a	1.36 ± 0.07b	0.36 ± 0.03b
100.0	4.24 ± 0.23b	7.82 ± 0.10c	3.04 ± 0.05ab	2.12 ± 0.11b	0.72 ± 0.05c	0.27 ± 0.01c
150.0	5.10 ± 0.04a	8.51 ± 0.11b	2.84 ± 0.09bc	1.98 ± 0.03bc	0.56 ± 0.02cd	0.23 ± 0.01cd
200.0	5.36 ± 0.06a	9.35 ± 0.06a	2.61 ± 0.11c	1.96 ± 0.09bc	0.49 ± 0.02d	0.21 ± 0.01d
LSD ($P \leq 0.05$)	0.378124	0.483344	0.321759	0.260763	0.190740	0.046997
ANOVA	$F_{5, 12}$ = 196.8	$F_{5, 12}$ = 108.9	$F_{5, 12}$ = 18.18	$F_{5, 12}$ = 17.27	$F_{5, 12}$ = 69.23	$F_{5, 12}$ = 40.49
Callus I = callus developed and maintained on medium without NaCl						
Callus II = 100 mM NaCl Tolerant callus developed and maintained on medium containing 100 mM NaCl						
Medium: 0.8% agar solidified full-strength MS medium + 3% sucrose + NaCl.						
Data was recorded after 4 weeks of transfer of calli on medium containing different concentrations of NaCl.						
Values represent the Mean ± SE of three repeated experiments, each with twenty-four replicates.						
Mean values followed by the different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.						

Organic contents

The organic contents i.e., free proline, glycine betaine and total soluble sugar content of both tolerant and non-tolerant callus increased with increasing concentrations of NaCl in the medium (Fig. 2B, C & D). However, all three organic contents were significantly high in Callus II as compared to Callus I at all the concentrations of NaCl (25, 50, 100, 150 and 200 mM) and medium without NaCl. The accumulation of high levels of organic contents like free proline, glycine betaine, and total soluble sugar content in

response to abiotic stresses is well documented in many plant species. The increase in intracellular concentration of free organic solutes in NaCl tolerant calli might indicate an increase in the solutes and osmotic potential. Under abiotic stresses including salt stress, the accumulation of proline and glycine betaines in tissues aids several functions to ameliorate the salt stress i.e., osmotic adjustment, detoxification, stabilization of membranes, protection of chloroplastic and mitochondrial functions, and scavenging of free radicals (Munns and Tester 2008; Rai et al. 2011; Zhang et al. 2021). In agreement with the observations of many other plant species, calli of *S. trifoliatus* subjected to salt stress showed a positive correlation between the accumulation of organic solutes and salt tolerance (Gandonou et al. 2006; Hassan et al. 2008; He et al. 2009; Rai et al. 2010; Chen et al. 2012; Nikam et al. 2014).

Regeneration of NaCl tolerant plants

Formation of globular structure (GS) and somatic embryo production from 100 mM NaCl tolerant callus

Non-tolerant and 100 mM NaCl tolerant both calli gave rise to small, shiny, green-colored globular structures (GS) from which somatic embryos were differentiated in a similar manner as observed by Asthana et al. (2017) from sepal explants (Fig. 3A & B). However, the frequency of embryogenesis (FE) and the average number of globular structures produced per culture were differed in both tolerant and non-tolerant callus culture. Both FE as well as the average number of globular structures produced per culture were maximum in 25 mM NaCl supplemented maintenance medium (FE = 94.19 ± 1.64%; the number of GS = 30.67 ± 1.23). Above 25 mM NaCl, both FE and the average number of globular structures decreased significantly (Table 3). After subsequent subcultures, globular structure formation was observed only in 25, 50 and 100 mM NaCl-containing medium. The potential for the formation of globular structures was lost in the callus cultures of 150 and 200 mM NaCl-containing medium.

Table 3
Effect of NaCl concentration on frequency of somatic embryogenesis (FE) and production of globular structures (GS) from NaCl (100 mM) tolerant callus

Concentration of NaCl (mM)	Percent frequency of embryogenesis (FE) (%)	Average number of globular structures (GS) per culture
0.0	91.55 ± 0.12a	22.75 ± 2.50a
25.0	94.19 ± 1.64ab	30.67 ± 1.23b
50.0	83.33 ± 2.41b	16.33 ± 2.76c
100.0	69.44 ± 3.67c	13.67 ± 1.21cd
150.0	31.94 ± 3.67d	8.28 ± 1.21de
200.0	18.06 ± 2.78e	3.83 ± 0.59e
LSD ($P \leq 0.05$)	8.2697	5.4344
ANOVA	$F_{5, 12} = 144.628$	$F_{5, 12} = 30.468$
Medium: Callus maintenance medium (0.8% agar solidified full-strength MS medium + 3% sucrose) containing different concentrations of NaCl		
Values represent the Mean ± SE of three repeated experiments, each with twenty-four replicates.		
Mean values followed by the different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.		
Inoculum: 200 ± 4.16 mg FW of tolerant callus.		

Globular structure developed from non-tolerant and 100 mM NaCl tolerant calli could differentiate different stages of somatic embryos on MS basal medium with or without NaCl (Fig. 3C-E). Somatic embryos produced on medium with or without NaCl were green or sometimes whitish or yellowish. Somatic embryos produced on 25 mM NaCl-containing medium had remarkably large cotyledons. The number of different stages i.e., globular, heart and cotyledonary of somatic embryos differentiated per globular structure, were maximum on 25 mM NaCl containing medium (Table 4). The number of somatic embryos per globular structure decreased on medium containing 50, 100, 150 or 200 mM NaCl. The somatic embryos induced on 150 or 200 mM NaCl containing medium developed up to the cotyledonary stage and later necrosed and unable to further development and germination. Compared to a medium containing 25 mM or devoid of NaCl, the decline in somatic embryo formation from globular structure at higher concentrations of NaCl may be owing to the non-embryogenic adaptation of callus cells at higher concentrations of NaCl. Similar to our observation, less competence of callus/embryogenic cells to develop somatic embryos on a medium containing higher concentrations of NaCl has also been reported in many species (Patnaik and Debata, 1997; Singh et al. 2003; Rai et al. 2010).

Table 4
Effect of NaCl concentration on number of different stages of tolerant somatic embryos differentiated per globular structure (GS)

Concentration of NaCl (mM)	Average number of different stages of somatic embryos per globular structure		
	Globular embryo	Heart stage embryo	Cotyledonary stage embryo
0.0	8.89 ± 0.24ab	6.69 ± 0.86a	10.17 ± 1.31a
25.0	9.76 ± 0.33a	6.94 ± 0.74a	11.08 ± 1.34a
50.0	7.58 ± 0.46bc	6.15 ± 0.58ab	9.42 ± 0.30a
100.0	7.16 ± 0.55c	5.35 ± 0.81ab	8.89 ± 3.09a
150.0	4.78 ± 0.15d	4.09 ± 0.29bc	2.33 ± 0.67b
200.0	2.58 ± 0.61e	2.24 ± 0.42c	2.28 ± 0.98b
LSD ($P \leq 0.05$)	1.31314	2.00383	4.80370
ANOVA	$F_{5, 12} = 39.39$	$F_{5, 12} = 7.645$	$F_{5, 12} = 6.534$
Medium: Callus maintenance medium (0.8% agar solidified full-strength MS medium + 3% sucrose) containing different concentrations of NaCl			
Values represent the Mean ± SE of three repeated experiments, each with twenty-four replicates.			
Mean values followed by the different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.			

Germination of NaCl tolerant somatic embryos and conversion to plants

The cotyledonary stage somatic embryo (Fig. 4A) tolerant to 100 mM NaCl was isolated individually and transferred to germination medium supplemented with 25, 50 or 100 mM NaCl and without NaCl for germination and plantlet conversion. The tolerant somatic embryos inoculated on a germination medium without NaCl or with 25 mM NaCl showed initiation of germination (Fig. 4B) within a week of inoculation. While on 50 or 100 mM NaCl supplemented medium, germination of somatic embryos was delayed, and it was observed after about two weeks of inoculation. The percentage of germinated tolerant somatic embryos decreased on NaCl-supplemented germination medium as compared to medium without NaCl (Table 5). About 67.57 and 38.89% NaCl tolerant somatic embryos were germinated on medium without NaCl and containing 100 mM NaCl, respectively. The plants produced from NaCl tolerant somatic embryos were healthy with well-developed roots and shoots irrespective of the concentration of NaCl in the germination medium (Fig. 4C). Result of this study clearly indicates that the plants of *S. trifoliatus* retain salt tolerance as mature cotyledonary stage somatic embryos tolerant to 100 mM NaCl able to germinate on medium containing NaCl and further convert into complete plantlets.

Table 5
Germination and plant recovery from NaCl (100 mM) tolerant somatic embryos

Concentration of NaCl (mM)	Percent germination of somatic embryos (%)
0.0	67.57 ± 1.68a
25.0	58.33 ± 2.41ab
50.0	51.39 ± 3.67b
100.0	38.89 ± 3.77c
LSD ($P \leq 0.05$)	9.733
ANOVA	$F_{3, 8}$ = 16.398
Medium: Germination medium (0.8% agar solidified full-strength MS medium + 2% sucrose) containing different concentrations of NaCl	
Values represent the Mean ± SE of three repeated experiments, each with twenty-four replicates.	
Mean values followed by the different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.	

Acclimatization of NaCl tolerant plants

NaCl tolerant plants developed on different germination medium with NaCl survived when transplanted to 0.2 or 0.3% (w/w) NaCl mixed potting mixture (Supp. Table 1). In 0.2% (w/w) NaCl containing potting mixture, 58.40 ± 2.10%, 51.79 ± 5.74% and 43.33 ± 3.33% survival of plants was recorded, when the plants were taken from 25, 50 and 100 mM NaCl supplemented germination medium respectively. In 0.3% (w/w) NaCl containing potting mixture percent survival of plants was decreased when compared to 0.2% (w/w) NaCl containing potting mixture, while None of the plants transferred to 0.5% (w/w) NaCl containing potting mixture survived (Supp. Table 1). The survival rate of the control (non-tolerant) plants was 90.33 ± 3.33%, and they grew luxuriantly as compared to NaCl tolerant plants. After one month of transplantation of NaCl tolerant plant, initiation of new leaves and shoot elongation was observed; however, chlorosis of leaves was also observed. About eight-month-old salt tolerant plants established in earthen pots were successfully transferred to the field (Fig. 4D).

Physiological and biochemical analysis of NaCl tolerant and non-tolerant plants

The six-month-old hardened salt tolerant (100 mM NaCl) plants were compared with the non-tolerant plants by analyzing various physiological and biochemical parameters. NaCl treatment led to gradual reduction in the chlorophyll a and chlorophyll b content in leaves of both NaCl tolerant and non-tolerant plants with increasing concentration of NaCl in irrigation water (Supp. Figure 2A & B). In contrast, carotenoid content was increased with increasing concentrations of NaCl. However, at all NaCl

treatments, the carotenoid content level was higher in salt-tolerant plants than in non-tolerant plants (Supp. Figure 2C). The decrease in chlorophyll content in response to salt stress is associated with oxidative stress, inhibiting chlorophyll synthesis by activating the chlorophyll-degrading enzyme chlorophyllase (Santos 2004). The increase in carotenoids under salt stress was attributed to its role in the non-enzymatic photoprotection of chloroplast against salt stress (Hossain et al. 2006).

In the present investigation, proline was accumulated in both the tolerant as well as non-tolerant plants in response to salinity. However, salt-tolerant plants have a higher level of proline than non-tolerant plants (Supp. Figure 3A). In addition, accumulation of MDA was higher in non-tolerant plants as compared to salt-tolerant plants (Supp. Figure 3B). Salt stress leads to the degradation of cell membranes by peroxidation of membrane lipids. The MDA content is an important index to reflect lipid peroxidation and disturbance to the membrane integrity (Munns and Tester 2008; Rai et al. 2011; Estravis-Barcala et al. 2020; Zhang et al. 2021). Our results are in agreement with Queiros et al. (2007) and Nikam et al. (2014), who also observed an increase in MDA content with the increase in salt stress in potato and sugarcane, respectively.

The present study showed that exogenous treatment of NaCl significantly affected the antioxidative enzyme activity in tolerant and non-tolerant plants. Although, with increasing concentrations of NaCl, all three antioxidative enzyme activity (SOD, CAT and APx) increased in both tolerant as well as non-tolerant plants. However, the activity of SOD, CAT and APx was significantly higher in salt-tolerant plants than in non-tolerant plants (Supp. Table 2).

Under almost all abiotic stresses, plants produce ROS, resulting in oxidative damage and eventually leading to cell damage. In several abiotic stress conditions, many plant species triggers many antioxidative enzyme activities in cell to scavenge and detoxify the reactive oxygen species and protect themselves from salt stress (Munns and Tester 2008; Rai et al. 2011; Harfouche et al. 2014; Estravis-Barcala et al. 2020; Zhang et al. 2021). The present study shows a significant increase in all three antioxidative enzyme activity in both tolerant and non-tolerant plants under NaCl stress. Still, the increase was more significant in the tolerant line than the non-tolerant line, protecting the tolerant plants from damage.

Conclusion

In conclusion, we have successfully developed salt-tolerant plants of *S. trifoliatus* using in vitro selection technique. Based on different physiological and biochemical analyses of tolerant callus and regenerated plantlets, we can conclude that the NaCl-tolerant plants showed all adaptive features towards salt stress. Plants regenerated from selected cell lines through somatic embryogenesis survived in the potting mixture containing 2 and 3% (w/w) NaCl suggesting that salt-tolerant plants developed in this study can survive in salt-rich soil. The selected plants seemed to overcome the effects of NaCl in field conditions. However, the agronomic performance of these plants, particularly saponin contents under salt-stress conditions, should be tested.

Declarations

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Authors Contribution Statement

PA designed and conducted all the experiments and wrote the first draft of the manuscript. MKR helped in data analysis and edited the manuscript. UJ guided the research and edited the final manuscript. All the authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

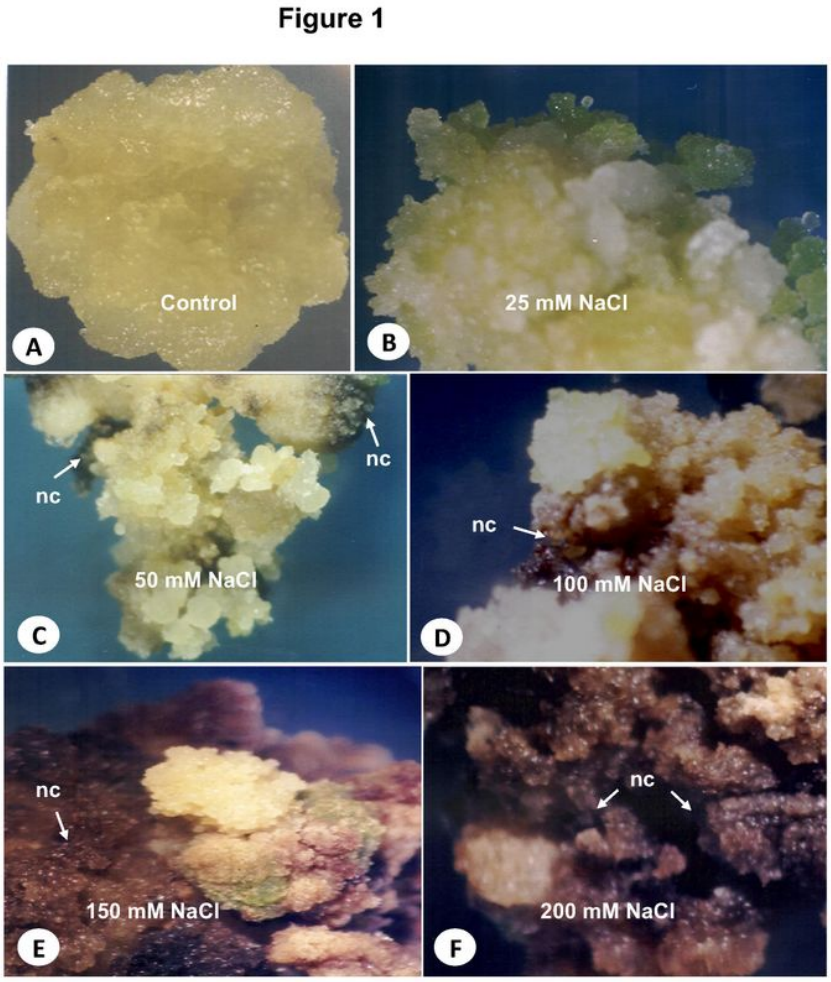

Figure 1

Induction and proliferation of callus in *S. trifoliatus* from leaf disc on

(A) control (without NaCl)

(B) 25 mM NaCl

(C) 50 mM NaCl

(D) 100 mM NaCl

(E) 150 mM NaCl

(F) 200 mM NaCl

Figure 2

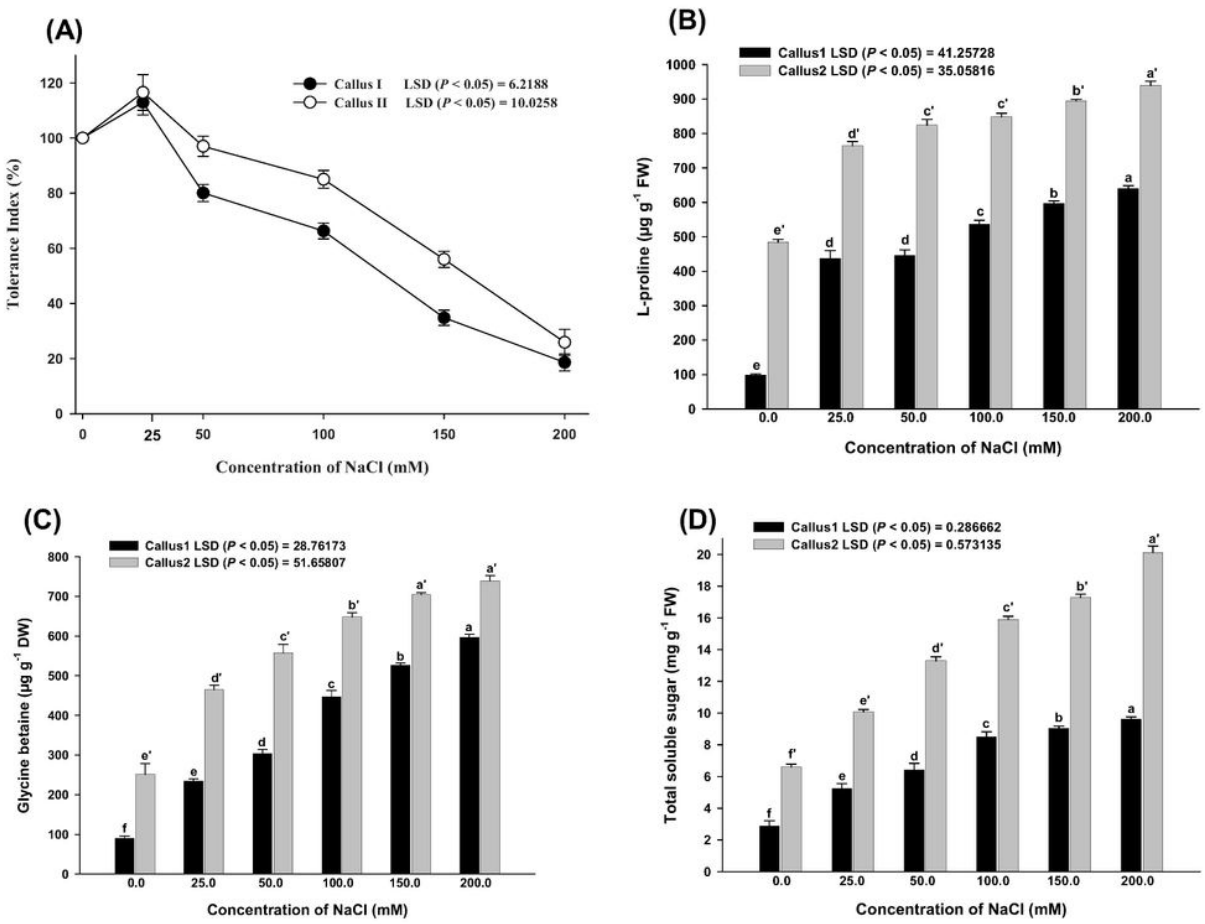

Figure 2

Effect of NaCl on (A) Tolerance Index (%) (B) L-proline (C) Glycine betaine and (D) Total soluble sugar content of callus I and callus II

Callus I = Control callus developed and maintained on medium without NaCl

Callus II = 100 mM NaCl tolerant callus

According to Duncan's multiple range test, mean values with the different alphabets are significantly different from each other at $P \leq 0.05$.

Figure 3

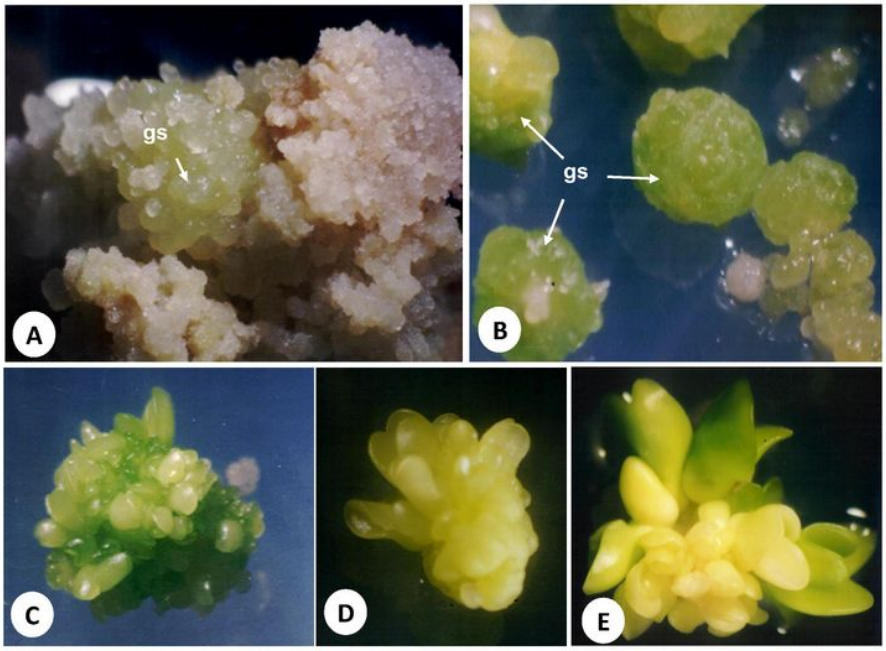

Figure 3

Formation of globular structure (GS) and somatic embryo production from 100 mM NaCl tolerant callus

(A) 100 mM NaCl tolerant callus (B) production of globular structure (GS) from 100 mM NaCl tolerant callus (C-E) Formation of globular, heart and cotyledonary stages of somatic embryos form globular structure

Figure 4

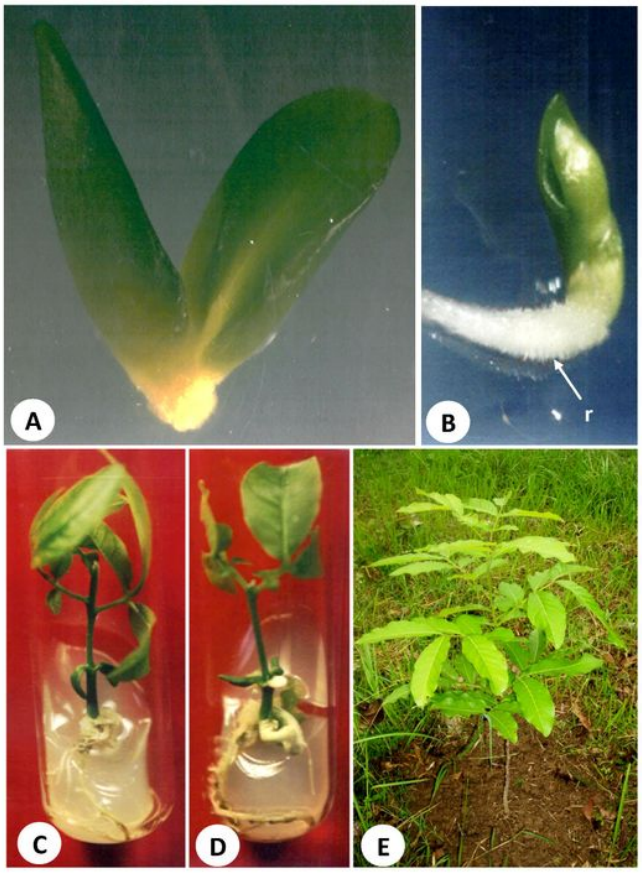

Figure 4

Germination of NaCl tolerant somatic embryos and conversion to plants

(A) A mature NaCl tolerant cotyledonary stage somatic embryo (B) germination of somatic embryo on NaCl containing medium (C) well-developed plants produced from NaCl tolerant somatic embryos with healthy with roots and shoots (D) A well hardened NaCl tolerant plant established in field

Supplementary Files

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