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In-vitro Mutagenesis Induction to Improve Abiotic Stress in Tissue Cultured Plantlet of *Picrohiza kurroa* Royle ex. Benth: An Endangered Plant of Western Himalayas, India

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Abstract

The study is focused to establish an improved salt and drought tolerant *Picrohiza kurroa Royle* ex Benth, an endangered medicinal plant of Western Himalayas through mutagenesis system in conjunction with *in- vitro* regeneration technique. Regeneration using leaf as explants from mature plant of characterized germplasm is beneficial because the source plant is not damaged. Efficient callus formation i.e., 85% was achieved in modified MS medium at 2,4-D at 3.5 mg L^{-1}. Ethyl methane sulfonate (EMS) a chemical mutagen was used to induce mutation in the callus biomass under *in-vitro* condition. Relatively decreases in callus biomass were observed as the dose of mutagenic chemical increased from 0.0 to 0.8 mM. Selection of mutants callus lines were also investigated against salt (NaCl) and drought (mannitol) tolerance level by using its various concentrations i.e., 50 to 250 mM L^{-1}. In mutagenized callus (MC) under both stress responses there was increase in callus biomass with the successive increase in concentration of NaCl and Mannitol till 100 mM L^{-1}, after that it started decline. Stress tolerated mutant callus line were also characterized by the accumulation of proline and glycine betaine (GB) content. At 100 mM L^{-1} concentrations of NaCl and mannitol, higher proline and GB content were accumulated in the mutagenized callus i.e., 5.23 and 5.18 µmol g^{-1} FW and 11.23 and 11.98 µg g^{-1} FW respectively which is significantly higher in comparison to non-mutagenized callus (UM). For shoot proliferation in mutant callus line, various concentrations with combination of plant growth regulators (PGR's) were used in treatments (T1, T2 and T3). Invariably, in treatment T3 the concentration of 1.0+0.5 mg L^{-1} resulted in highest shoot regeneration i.e., 85% while minimum 20% was obtained in T1 at concentration 0.5+0.1 mg L^{-1}. NAA fortified MS medium was found superior to IAA and IBA with respect to the induction of roots. The mutant and stress selected grown through tissue engineering were evaluated for their ex situ agronomic performance in saline as well as drought condition for 30 days. Under both stress condition the mutant plant revealed a remarkable increase in all the parameters studied i.e., shoot and root length, fresh and dry root, shoot biomass and number of leaves with respect to normal plant (control).

Keywords: *Picrohiza kurroa*; Mutagenesis; Callus; Growth regulators; Abiotic stress

Introduction

Picrohiza kurroa Royle ex. Benth commonly known as Kutki, is an important temperate medicinal plant species found in western Himalayas. It is a perennial herb and also the principle source of glycoside that is Picroside-I, Picroside-II and Kutkoside which are extracted from dried rhizomes and roots of 3 year old plants [1,2]. It is extensively used in both modern as well as traditional systems of medicine for its invaluable medicinal properties being stomachic, cathartic, cholagogue, blood purifier and useful in treating jaundice, asthma, flatulence, cardiac complaints, antifungal and chronic hepatitis as its active constituents are apocynin, drosin, and nine cucurbitacin glycosides [3-5]. Now a day's researches are more focused on evaluating its hepatoprotective, anticholestic antioxidant and immune modulating activity [6,7]. In the past few decades there has been a resurgence of interest in the study and use of medicinal plants in health care and in recognition of the importance of medicinal plants to the health system [8].

Crop productivity is challenged by abiotic stress factors mainly extreme temperatures (heat, cold, and freezing), drought, high salinity, heavy metals, etc., which limit plant growth and development [9]. Due to the continued reduction of arable land, reduction of water resources and increased global warming trends and climate change [10], It is essential required to develop salt-resistant by conventional, mutational, and biotechnological approaches [11]. Methods of chemical induced *in-vitro* mutagenesis have been successfully used to improve agronomic traits including salinity and drought tolerance in several crop plants [12]. It has been extensively studied that many chemicals such as ethyl methanesulfonate (EMS), diethyl sulfate (DES) and dimethyl sulfate (DMS) act as alkylating agents. In contrast, EMS treatment mostly causes random G/C to A/T transitions and the frequency of truncated mutations is under 5% [13]. Also, Rengaswamy stated that approximately 19.5% of the irrigated soils in the world have elevated concentrations of salts either in the soil or in the irrigation water, damaging both the economy and the environment [9]. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors [14]. Somaclonal variation has contributed to the development of abiotic and biotic stress resistant varieties in major crops [15]. Use of *in-vitro* mutagenesis strategies, especially for vegetatively propagated crops including the major world crops potato and banana, combined with *in-vitro* selection for isogenicity with the parental line have significantly

improved the efficiency of mutation techniques in breeding [16]. Although very little information are available in developing stress tolerant medicinal plant varieties.

Artificial propagation techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement. Large-scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemical independent of plant availability and more consistent product quality [17]. *In-vitro* propagation refers to the true-to-type propagation of selected genotypes using *in-vitro* culture techniques. It is an alternative method of propagation [18] and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. *In-vitro* propagation has been achieved in several medicinal plants using tissue culture techniques [19]. Plant regeneration from *kurroa P* has earlier been reported using shoot tips [20]. As this plant possesses inherent proliferation capacity and offset planting capabilities to reproduce it. Including, its propagation through seeds is also poor. Therefore, it is extremely important to explore the different methods of propagation and conservation of *P. kurroa* [21]. The gradual decline in the population of *Picrohiza kurroa* Royle ex. Benth demand launching of conservation effort so as to ensure continuous and ample supply by establishing a balanced cycle of harvest and renewal. Such conservation efforts would ensure continuous and ample supply of this valuable material which is in great demand by the pharmaceutical industry.

In the light of above facts the present investigation was undertook to alleviate the salt stress response in highly endangered and medicinally economically valuable *Picrohiza kurroa* Royle ex. Benth by fusing dual approaches i.e., mutagenesis system and regeneration technique under *in-vitro* condition.

Materials and Methods

Sterilization of explants

Briefly, the *Picrorhiza kurroa* juvenile leaf explants of size (2-5 mm) in length was collected from the Division of Medicinal and Aromatic Plants (MAP), UUHF, Bharsar, Pauri, India at latitude *29°* 45' to 30° 15' and *78°* 24' to 79° 23' E longitude. Initially, the explants were washed under running tap water to remove dust particles for 30 min, and treated with liquid detergent for 10 min, and rinsed three times with sterilized distilled water (SDW). After this the explants was treated with an antifungal agent (Bavistin 0.2%) for 1 hr and the again rinsed three times with SDW. Further, sterilization treatments were conducted under a laminar-flow chamber. The explants were then disinfected with 0.1% (w/v) mercuric chloride for 3 min under aseptic conditions. After this these explants were then thoroughly washed 3-4 times with SDW to remove the traces of mercuric chloride. Damage parts were aseptically trimmed with sterilized surgical blade.

In-vitro callogenesis

Explants were cultured on slight modified MS basal medium consist of macro and micro elements according to Murashige and Skoog with Meso-inosotol (100 mg L^{-1}), Thiamine-HCL (0.5 mg L^{-1}), pyridoxine-HCL (1 mg L^{-1}), Nicotinic acid (0.5 mg L^{-1}), coconut water (10%) and sucrose (30 g L^{-1}), solidified with (0.8%) agar [22]. For efficient callus induction the MS media were amended separately with different plant growth regulators (PGR) i.e., auxins [indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), 2,4 dicholoro phenoxyacetic acid (2,4-D)] and cytokinin [furfurylamino purine (kinetin)] at varying concentration i.e., 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}. The pH of media were adjusted to 5.8 ± 0.1 prior to autoclaving at 121 °C at 15 lbs pressure for 20 min. Culture were incubated under 16 h photoperiod with light intensity of 55 $\mu molm^{-2}s^{-2}$ provided by cool white fluorescent lamps (Phillips, India) at 24˚C ± 2 °C, 60 to 70% relative humidity in culture room. All the cultures were transferred to fresh medium after 2- 3 week duration. The percentage of explants formed was evaluated after 3 weeks of inoculation.

Mutagenic treatment

The chemical mutagen EMS was applied to fresh callus culture at four different concentrations (0.0, 0.2, 0.4 and 0.8 mM) for 6 h treatments [23]. Callus culture were immersed in a 50 ml conical flask containing 20 mL of the MS medium supplemented with appropriate concentrations of EMS [13], after which each flasks were kept in a shaker at 100 rpm. The effectiveness of EMS treatment onto callus was determined by measuring the callus biomass after 6 h. Following EMS treatment, the survived callus i.e., mutagenized callus were rinsed three times in sterilized water and then transferred onto maintenance MS medium for further process.

Development of mutants for abiotic stress tolerance

Mutatagenized callus (MC) and non-mutagenized callus (UM) cultures were subjected to treatments with different concentrations of salt (NaCl) and mannitol stress (0, 50, 100, 150, 200, and 250 mM L^{-1}) to study salt and drought stress effects and identify the optimal concentration of NaCl to be used in the MS medium. After four weeks of treatment, the response was recorded using accumulation of proline and glycine betaine (GB) content.

Determination of free proline concentration

Proline content was estimated using the standard protocol as described by Finnegan and McElroy with minor modifications. MC and UM callus obtained through abiotic stress tolerance were homogenized in 10 mL of 3% sulfosalicylic acid [24]. The homogenate was centrifuged at 15,000 rpm. 2 mL of the supernatant was mixed with 2 mL of 0.2% ninhydrin and 2 mL of glacial acetic acid. Further the mixture was incubated at 100˚C for 1 h and the reaction was terminated on ice. Extraction of Proline was done by 4 mL of toluene. The OD of the supernatant was measured at 520 nm and proline concentration was determined from the standard curve made by using the purified L-proline (Sigma, MO, USA) and calculated on a FW basis ($\mu mol\ g^{-1}$).

Estimation of Glycine betain (GB) concentration

GB concentration was determined by the method of Grieve and Grattan [25]. Similarly, MC and UM callus obtained through abiotic stress tolerance was mechanically shaken with deionized water for 24 h at 25 °C. Samples were filtered and the filtrate was diluted (1:1) with 2 mM L^{-1} H_2SO_4. The extract was cooled in ice and mixed with 200 µL of I2-KI reagent (a mixture of 20% potassium iodide and 15.7% iodine). The tubes were gently mixed and stored at 4 °C for 16 h followed by centrifugation at 10,000 × g for 15 min at 0 °C. Periodide crystals were dissolved in 9.0 mL of 1, 2-dichloroethane, and after 2 h, absorbance was measured at 365 nm. GB concentration was determined from a

standard curve prepared using standard glycine betaine and expressed as µg g^{-1} FW.

Plant regeneration from promising mutant callus culture lines

Only selected MC culture explants having the capability to tolerate the effective salt and mannitol concentration including those found with profuse callusing was cut into small segments and transferred into MS medium (MSB5) also fortified with optimum Nacl as discussed above, B5 vitamins (2 mg L^{-1}) and sucrose (30 mg L^{-1}). In addition for efficient shoot proliferation and multiplication the modified MS medium (MSB5) were also supplemented with different concentrations (0.5, 1.0, 1.5 and 2 mg L^{-1}) of each plant growth regulator were combined together therefore a total of 60 different combinations were examined [26]. The following treatments are as follows.

T1- 6-Benzylaminopurine (BAP)+kinetin (Kn) of concentration in between 0.5 - 2.0 mg L^{-1}

T2- BAP+NAA of concentration in between 0.5 - 2.0 mg L^{-1}.

T3- BAP+IBA of concentration in between 0.5 - 2.0 mg L^{-1}.

T4- devoid of growth regulator.

All the callus cultures were maintained for 3 weeks in growth room at 22 °C, 70% humidity and 16 h light photoperiod. All the treatments were conducted in triplicates..Morphological changes such as shoot initiation, number of shoot per explants and length (cm) were recorded at 15 days intervals. Sub culturing was done after every 1-2 week. Mutant shoots (3-4 cm) with two or three leaflets derived from proliferating cultures were excised with sterilized scalpels and implanted onto half strength MS medium containing 1.5 mg L^{-1} NAA including 30 g sucrose or IAA or IBA separately for rooting. The rooted shoots were transferred to MS medium for further elongation of the roots. For each treatment, three replicates with 3 to 5 explants were carried out and the experiment was repeated twice. Data collected in the experiments were analyzed using SPSS Version 17.0. The means and the differences within the treatments were compared using one-way analysis of variance (ANOVA).

Acclimatization of mutated plant for agronomic performance under stress and drought condition

Rooted plants were taken away from culture media and washed with SDW to remove media from rooting surface then plants were transplanted in plastic pots containing sterilized compost soil (1:1 mixture of peat substrates and potting soil) and were keep moisten regularly by irrigated with Nacl and mannitol containing water at 3 days interval. The plants were acclimatized in room condition at 25 ± 3 °C 16/8 h photoperiod [27,28]. The survival capacity of mutated plants were experimented under salt and drought condition by experimenting following set of treatments (i) Mutatagenized regenerated plants irrigated with water consisting 100 mM L^{-1} NaCl (under salt stress) (ii) Mutatagenized regenerated plants irrigated with water consisting 100 mM L^{-1} mannitol (under drought stress) (iii) Normal parent variety irrigated with water consisting 100 mM L^{-1} NaCl (under salt stress) (iv) Normal parent variety irrigated with water consisting 100 mM L^{-1} mannitol (under drought stress). During cultivation of mutant regenerated plant, the minimal and maximal temperature ranged from 18 to 6 °C respectively and experiment were conducted from January to March, 2014. Effect on various parameters of mutated plant were observed viz. shoot length, root length, fresh shoot and root biomass, dry root and shoot biomass, stem girth and number of leaves were measured after 30 days and data were analyzed statistically by using ANOVA two way variance, to find out the significance levels.

Results

In-vitro callus formation

Callus formation was observed in 15 days after inoculation form the leaf explants on modified MS medium amended with individual different concentration of PGR i.e., 0.5- 4.0 mg L^{-1}. Maximum 85% of callus formation was achieved in 2,4-D at 3.5 mg L^{-1} (Figure 1A) while lowest 5% was observed in NAA at 1.0 mg L^{-1}. However no callus induction %age was recorded in the concentration of IBA up to 2.5 mg L^{-1} after that callusing initiated (Table 1). MS medium devoid of PGR showed no callus formation.

Growth regulator	Concentration (mg.L^{-1})	No. of explants inoculated	No. of explants showed callus	% of explants with callus induction
IBA	0.5	20	-	-
	1	20	-	-
	1.5	20	-	-
	2	20	-	-
	2.5	20	-	-
	3	20	1	20
	3.5	20	2	10
	4	20	2	10
NAA	0.5	20	-	-
	1	20	1	5
	1.5	20	2	10

	2	20	2	10
	2.5	20	2	10
	3	20	1	15
	3.5	20	2	10
	4	20	2	10
2,4-D	0.5	20	-	-
	1	20	-	-
	1.5	20	-	-
	2	20	3	15
	2.5	20	9	45
	3	20	15	75
	3.5	20	17	85
	4	20	8	40

Table 1: Callus induction from leaf explants at different concentration of growth regulators in *Picrohiza kurroa*. " -"= No callusing; "Poor callusing" = 20-50%; "Considerable callusing"=51-85%; "Intensive callusing"= 86^{-1}00%.

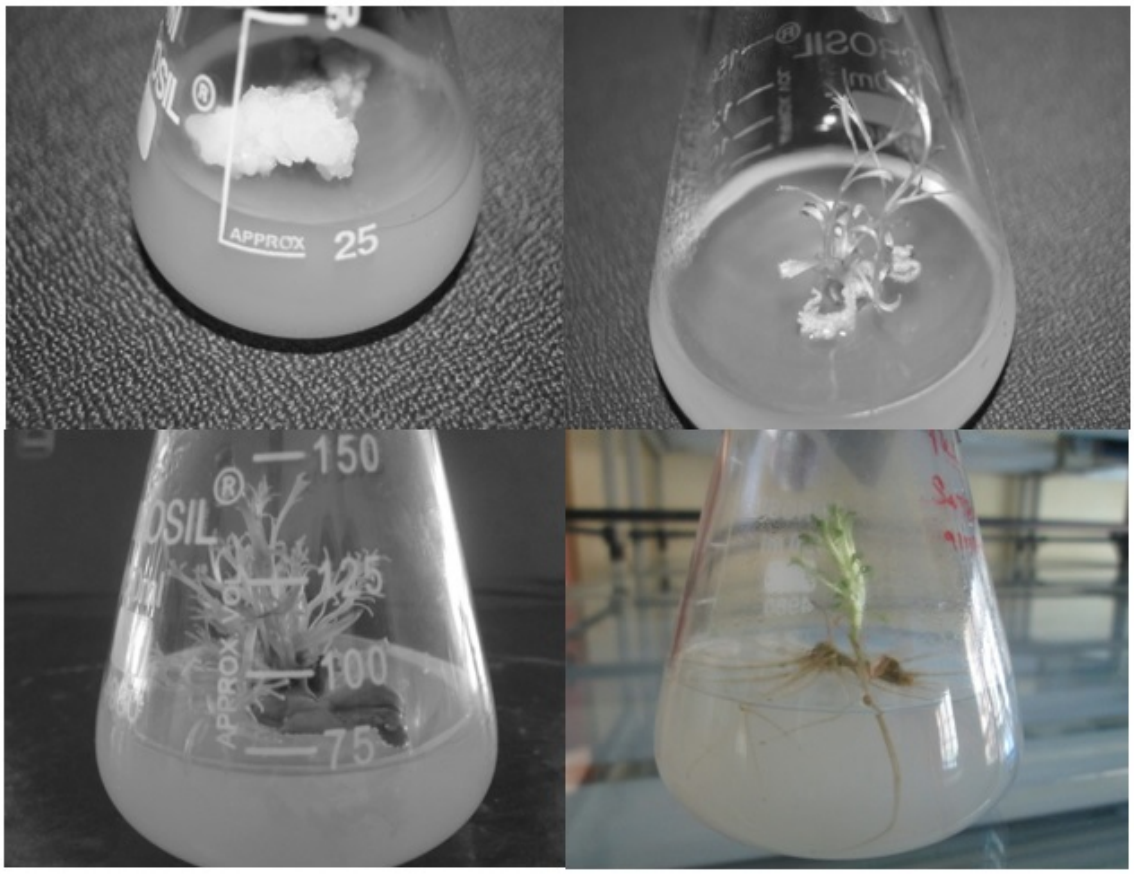

Figure 1: Organogenesis through tissue culture technique (A) Callus induction (B) Shoot proliferation from mutant callus line. (C) Multiple shoots elongation from mutant line at optimum phytohomone concentration with combination. (D) Rooting formation.

Mutagenized callus culture for osmotic stress response

Fresh, actively proliferating callus showed more sensitivity to EMS treatment than the control. Decreases in callus biomass were observed as the dose of mutagenic chemical increased (0.0, 0.2, 0.4 and 0.8 mM) (Figure 2). 50- 83% reduction in biomass were recorded at low concentration of EMS i.e., 0.2 - 0.4 mM with respect to control while >90% reduction was observed at high concentration. The color of callus was also affected at high concentration of EMS as it gets brown. The abiotic stress i.e., salt and drought were also experimented in MC and UM callus at varying concentration of NaCl and Mannitol (0 - 250 mM L^{-1}). In MC condition for both the stress responses there was increase in callus biomass with the successive increase in concentration of NaCl and Mannitol till 100 mM L^{-1}, after that it started decline (Figure 3). Significant enhancements of 20 - 35% were observed in mutagenized callus over to unmutagenized (control). This test also identify that 100 mM L^{-1} is the optimum salt concentration which can be used for further proliferation of shoot in respective MS medium. The salt and mannitol pooled data also suggest that above this optimum concentration the callus growth was inhibited.

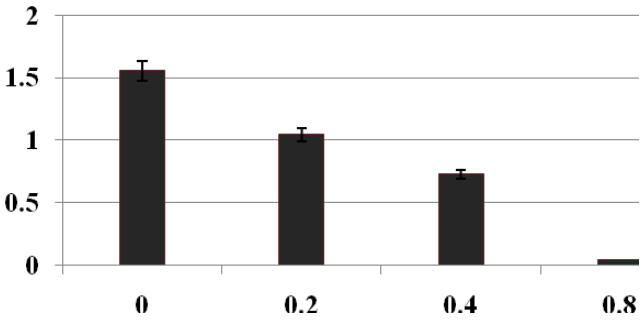

Figure 2: Effect on callus biomass of genotype *Picrohiza kurroa* after treatment with various concentration of EMS mutagen. (Concentration of EMS (%) on X-axis)

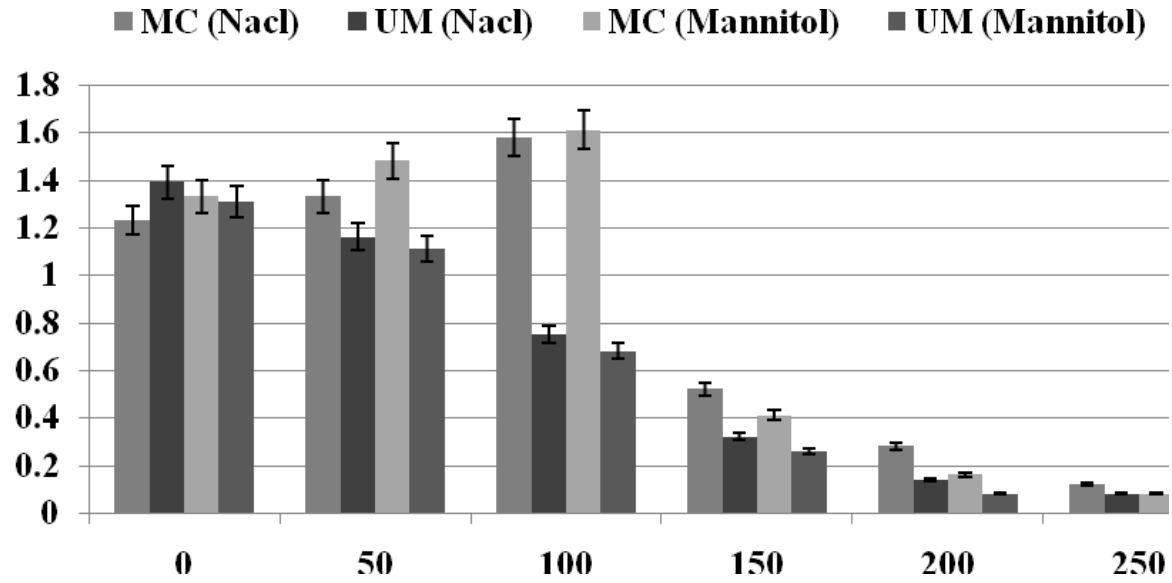

Figure 3: Salinity and drought tolerance pattern of mutagenized callus (MC) and unmutagenized callus (UM) lines at varying concentration. (Concentration of stress chemical used ($mM.L^{-1}$) on X-axis)

Quantitative determination of proline and GB content

Quantitatively the proline and glycine betaine content in both MC and UM calluses were also estimated under different concentration of salt and drought stress chemical (0 - 250 mM L^{-1}). The MC callus in both responses showed maximum amount of proline accumulation (5.23 and 5.18 µmol g^{-1} FW) which is significantly higher in comparison to UM calluses (3.23 and 3.18 µmol g^{-1} FW) respectively. In both the cases at 100 mM L^{-1} resulted in higher amount of proline content (Figure 4). Similarly the level of GB content also found in higher concentration (11.23 and 11.98 µg g^{-1} FW) at 100 mM L^{-1} under salt and drought stress (Figure 5). As in case of proline accumulation the GB content also showed a decreasing trend in its quantity.

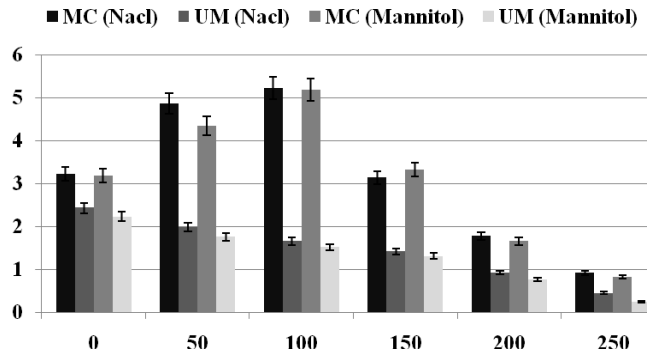

Figure 4: Quantitative estimation of proline content in mutagenized callus (MC) and unmutagenized callus (UM) lines under varying concentration of abiotic stress regime. (Concentration of stress chemical used ($mM.L^{-1}$).

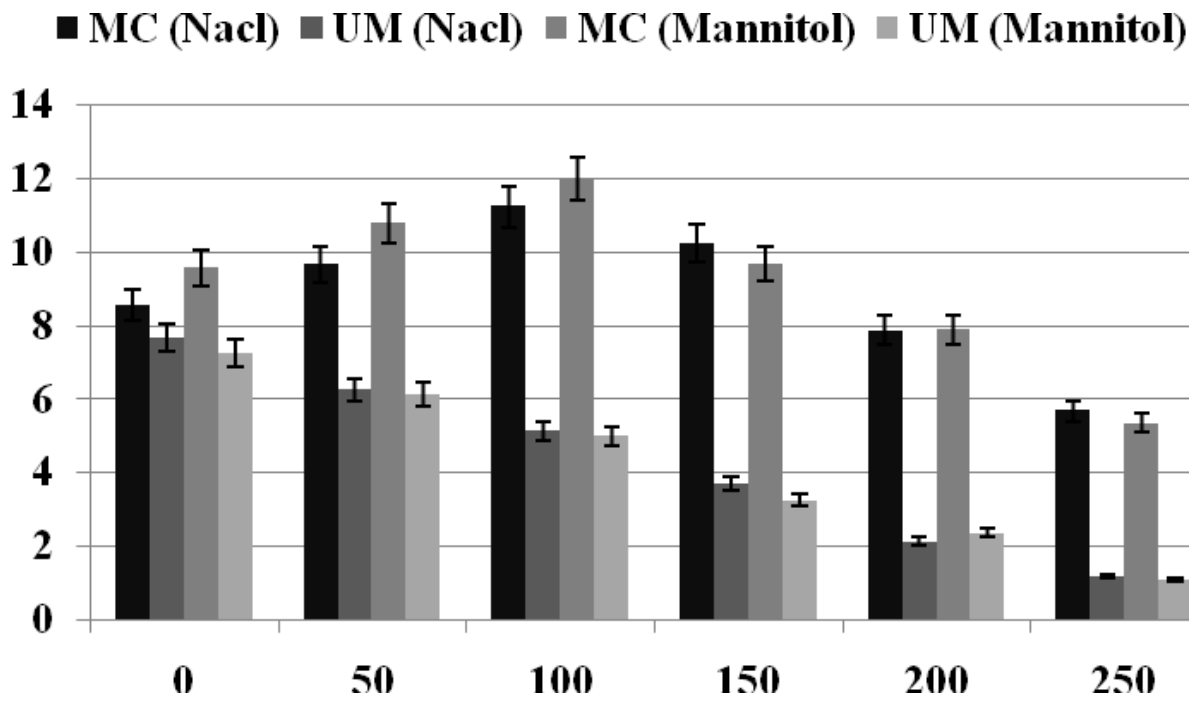

Figure 5: Determination of glycine betaine (GB) level mutagenized callus (MC) and unmutagenized callus (UM) lines under varying concentration of abiotic stress regime. (Concentration of stress chemical used (mM.L^{-1}) on X-axis)

In-vitro shoot and root proliferation from callus

Based on the salt and drought tolerance level, proline and GB content the MC culture explants were selected for further shoot proliferation studies. After 2-3 weeks from culture initiation, shoots appeared and increased on subsequent sub-culturing (Figure 1B). MSB5 medium devoid of growth regulator did not initiate shoot differentiation. Numbers of shoots per explants among different concentrations in combinations of growth regulators were significantly facilitated the shoot differentiation (Figure 1C). Among the various concentration with combination of phytohormones treatments (T1, T2 and T3). In treatment T3 the concentration of 1.0+0.5 mg L^{-1} resulted in highest shoot regeneration i.e., 85% whereas minimum 20% was obtained in T1 at concentration 0.5+0.1 mg L^{-1}. Additionally, the maximum the no. of shoot/explants and shoot length were recorded in T2 and T3 treatment of values 12 and 5.7 cm respectively at concentration 1.0+0.5 and 1.0+0.1 mg L^{-1} (Table 2). The regenerated shoots were excised and transferred to MS basal medium supplemented with auxins viz. IAA, IBA or NAA, for root formation. NAA fortified MS medium was found superior to IAA and IBA with respect to the induction of roots. Maximum of 95% shoots were rooted with an average of 15.1 roots per shoot and average length of 4.9 cm on modified MS medium supplemented with 1.5 mg.L^{-1} NAA (Figure 1D).

Treatments	Concentration (mg.L^{-1})	Shoot regeneration%	Avg. no. of shoot	Shoot length (cm)
T1	0.5+0.1	48	3.5 ± 1	3.1
	0.5+0.2	62	6.4 ± 3	3.3
	0.5+0.5	35	7.2 ± 2	2
	0.5+0.1	20	8.5 ± 2	2.4
	1.0+0.1	60	9.3 ± 3	2.7
T2	1.0+0.2	70	10.6 ± 2	4.6
	1.0+0.5	85	12.3 ± 1	5
	1.0+1.0	32	6.3 ± 2	2.5
	2.0+0.1	45	4.2 ± 1	3

	2.0+0.2	60	3.7 ± 1	4.3
	2.0+0.5	50	2.3 ± 1	3
T3	2.0+0.1	40	2.7 ± 1	2
	0.5+0.1	52	2.2 ± 2	4
	0.5+0.2	55	4.5 ± 1	3.2
	0.5+0.5	62	5.5 ± 2	4.1
	0.5+1.0	70	6.8 ± 3	4.5
	1.0+0.1	72	6.4 ± 2	5.7
	1.0+0.2	80	5.2 ± 1	3.3
	1.0+0.5	85	6.4 ± 2	3.5
	1.0+1.0	45	3.3 ± 1	2.2
	2.0+0.1	40	3.2 ± 1	2
	2.0+0.2	33	2.2 ± 1	2.1
	2.0+0.5	25	2.1 ± 2	2.3
	2.0+1.0	35	1.7 ± 1	2
T4	-	-	-	-

Table 2: Synergism of cytokinin:auxin at the varying concentrations with combination in MSB5 medium on shoot regeneration form the mutagenized callus (MC) tissue of *Picrohiza kurroa*. " -"= no differentiation in shoot. "(-"= no differentiation in shoot).

Field evaluation

Selected salt and drought tolerable mutants callus lines which are regenerated through *in-vitro* proliferation technique were assessed in pots for their agronomic performance under NaCl and mannitol stress condition. In both stress condition the mutant plant revealed a remarkable increase in all the parameters studied i.e., shoot and root length, fresh and dry root, shoot biomass and number of leaves with respect to control. In respective treatments increment varied in the range of 33 to 40%, 11.7 to 20.3% and 19 to 82% were recorded in root, shoot length and fresh and dry root, shoot biomass respectively (Table 3). Moreover the numbers of leaves were also more over to control.

Treatment	Length (cm)		Shoot biomass (g)		Root biomass (g)		No. of leaves
	Shoot	Root	Fresh	Dry	Fresh	Dry	
Mutatagenized regenerated plants irrigated with water consisting 100 mM L^{-1} Nacl.	5.23	3.45	1.42	1.11	0.98	0.81	4
Mutatagenized regenerated plants irrigated with water consisting 100 mM L^{-1} mannitol.	5.25	3.34	1.32	1.03	0.92	0.78	5
Normal parent variety irrigated with water consisting 100 mM L^{-1} Nacl.	3.46	2.12	1.15	0.98	0.54	0.21	2
Normal parent variety irrigated with water consisting 100 mM L^{-1} mannitol.	3.12	1.98	1.08	0.82	0.41	0.14	2

Table 3: In plant performance of mutated plants under salt and drought stress condition after 30 days of plantation.

Discussion

The application of induced mutagenesis with *in-vitro* culture has proved effective in the induction of genetic variation, selection, and multiplication of mutant clones [29]. The combination of chemical mutagenesis of explants and somatic embryogenesis represents an attractive *in-vitro* technique for mutagenesis [30]. Current investigation also exploits the bioengineering approaches i.e., by

amalgamating the vitro mutagenesis and tissue regeneration technique to alleviate the salt stress response in highly endangered and medicinally economically valuable *Picrohiza kurroa* Royle ex. Benth. Effect of varying concentration of PGR's on the induction of callus from leaf explants are presented in Table 1. 2, 4-D promoted efficient callus induction in comparison to other growth regulators (Figure 1A). Similarly Ting stated that inclusion of auxin and cytokinin leads to the development of mass of undifferentiated cell i.e., callus [31]. Earlier also Bisht et al. developed the callus biomass by using different explants i.e., leaf, stem and rhizome of *Podophyllum hexandrum* Royle through micropropagation technology [26].

In this study, different concentrations of EMS mutagen were tested with *Picrohiza kurroa* callus cultures to generate mutant populations (Figure 2). Decreases in callus biomass were observed as the dose of mutagenic chemical increased (0.0 to 0.8 mM). Earlier also, chemical mutagens such as EMS or N-nitroso, N-methyl urea (NMU) have been used to induce mutagenesis to broaden the genetic base of genotypes [13,30], although the precise nature of mutation induction has not been clarified. EMS is one of many stress elements, and its simulative effect on plant regeneration in the culture of somatic tissue has been reported [32]. Several plant genotypes have frequently been used for efficient induction of somatic embryogenesis, organogenesis, and transformation in previous studies [33,34]. Although it is the first attempt to induce chemical mutagenesis system in callus of economically valuable and endangered medicinally *Picrohiza kurroa* Royle plant.

Present study also studied the *in-vitro* selection of callus culture by inclusion of different concentration of NaCl and mannitol in the selection medium to develop the survival ability against salt and drought stress. Mutagenized callus (MC) revealed that there was increase in callus biomass with the successive increase in concentration of Nacl and Mannitol till 100 mML^{-1}, after that it started decline (Figure 3). This reduction effect presumably arises from dehydration of cells through low water potential or nutritional imbalance because of interference of salt and mannitol ions with essential nutrients [35]. Salt and drought stress also results in oxidative damage to membranes and peroxidation of membrane lipids [36]. The degradation of membranes due to lipid peroxidation also leads to leaching of cellular electrolytes, a response used as an indicator of disturbance of membrane integrity. To avoid such stress effect plant have evolved several defensive mechanisms such as modulated expression in the metabolism and synthesis of osmolytes to counteract stress responses [37]. Screening of mutagenized cultures during dedifferentiation and differentiation stages could be very useful for selection of salt tolerance, as described earlier [10].

Our result clearly indicted the mutagenized callus (MC) accumulated higher level of proline (Figure 4) and GB content (Figure 5) than unmutagenized callus (UM) at 100 mM L^{-1} of Nacl and mannitol. It is well documented that there is a correlation between change in proline concentration with its capacity to tolerate and/or adapt to salinity and drought stress conditions [23]. Gandonou et al. reported that proline accumulation increases in salt-tolerant callus under salinity [38]. The accumulation of proline and GB content are widely used as a selection criterion for salinity and drought tolerance [39]. Higher accumulation has been reported in salt tolerant species, whereas moderately tolerant species accumulate intermediate levels and sensitive species accumulate low or no levels [40].

Jain derived improved plant variety by inducing mutagenesis in conjunction with tissue engineering [41]. The effects of various PGR's on shoot induction, multiplication and rooting, on morphogenesis of callus growth were also assayed. It showed that in different concentrations of PGR's in combinations significantly facilitated the shoot differentiation (Table 2). The synergistic effect of auxin and cytokinin has been demonstrated in several medicinal plants, viz. *Santolina canescens* [42], *Rauvolfia tetraphylla* [43], *Bupleurum fruticosum* [44] and *Rotula aquatic* [45]. In accordance with these reports, the present investigation also exemplifies the positive modification of shoot induction efficiency by an auxin in combination with cytokinin. Root formation was also induced in *in-vitro* regenerated shoots by culturing them on half strength of MS containing IBA, NAA, and IAA separately. Among the three types of auxin, NAA was found to be most effective for root formation (Figure 1D).

In-vitro mutagenesis of cultured explants, cells, and tissue cultures represents a feasible method for induction of genetic variation, which can be subjected at the cellular level to selection for desirable traits [46,47]. However, success of *in-vitro* mutagenesis programs will depend on evaluation of mutant clones under field conditions to confirm their performance for the selected trait of interest. Therefore in this study the mutant and stress tolerated plants derived through tissue culture technique were also evaluated in pots for their agronomic performance based on different phenotypic characters under stress condition. Initially, mutant plants were acclimatized to room temperature i.e., at 25 ± 3°C because direct plantation at high temperature in field could be unfavorable for the establishment moreover against both salinity as well as drought condition the mutant plant indicated a wide range of variations in all the parameters tested i.e., shoot and root length, fresh and dry root, shoot biomass and number of leaves with respect to control (Table 3). Houshmand et al. conducted the field trial on wheat to check its performance under saline and non-saline field conditions [48]. The results suggest that *in-vitro*-induced mutagenesis followed by *in-vitro* selection can be applied to induce genetic variation for salt tolerance, besides improving agronomic characteristics in *Picrohiza kurroa* Royle ex. Benth.

Conclusion

Present investigation has successfully demonstrated that *in-vitro* EMS mutagenesis in establishing the salinity and drought stress tolerance level in callus biomass can be very useful and cost effective protocol to generate mutants lines in an economically valuable endangered medicinal plant i.e., *Picrohiza kurroa* Royle ex. Benth. Our results also suggested that the accumulation proline and GB content plays an important role in osmotic adjustment in plant cells under these stresses. However, concentrations of PGR's were also optimized for efficient shoot and root proliferation in these mutagenized callus and plantlets derived from these callus were acclimatized and evaluated for their agronomic performance under abiotic stress condition. So, the conjunction of these two techniques i.e., mutagenesis and tissue culture can be beneficial in crop improvement programs. This is highly advantageous for the production of salt and drought tolerant genotype of *Picrohiza kurroa* Royle ex. Benth plants for a range of further biotechnological applications.

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