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Radiation-induced in vitro mutagenesis system for salt tolerance and other agronomic characters in sugarcane (*Saccharum officinarum* L.)

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ARTICLE INFO

Article history:
Received 18 June 2014
Received in revised form 11 September 2014
Accepted 28 September 2014
Available online 13 October 2014

Keywords:
Gamma-rays
Mutagenesis
Embryogenic callus
Saccharum officinarum L
Salt tolerance

ABSTRACT

Gamma ray-induced in vitro mutagenesis and selection for salt (NaCl) tolerance were investigated in sugarcane (*Saccharum officinarum* L.). Embryogenic callus cultures were irradiated (10 to 80 Gy) and subjected to in vitro selection by exposure of irradiated callus to NaCl (0, 50, 100, 150, 200, and 250 mmol L^{-1}). Increasing NaCl concentrations resulted in growth reduction and increased membrane damage. Salt-selected callus lines were characterized by the accumulation of proline, glycine betaine, and Na^+ and K^+ concentration. Higher accumulation of proline and glycine betaine was observed in NaCl stressed callus irradiated at 20 Gy. Na^+ concentration increased and K^+ concentration decreased with increasing salt level. Irradiated callus showed 50–60% regeneration under NaCl stress, and in vitro-regenerated plants were acclimatized in the greenhouse, with 80–85% survival. A total of 138 irradiated and salt-selected selections were grown to maturity and their agronomic performance was evaluated under normal and saline conditions. Of these, 18 mutant clones were characterized for different agro-morphological characters and some of the mutant clones exhibited improved sugar yield with increased Brix%, number of millable canes, and yield. The result suggest that radiation-induced mutagenesis offers an effective way to enhance genetic variation in sugarcane.

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1. Introduction

Sugarcane (*Saccharum* spp.) is an important industrial crop, ranking among the ten most planted crops in the world. Besides being the major sugar contributor, accounting for more than 70% of the world's sugar, sugarcane is important as the raw material for sugar-producing and allied industries [1]. Conventional breeding has contributed greatly to the development of agronomically improved varieties; but limitations such as a narrow gene pool, a complex genome, poor fertility, and a long

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Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.

http://dx.doi.org/10.1016/j.cj.2014.09.002
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breeding/selection cycle make it difficult to undertake further improvement. Agronomically improved sugarcane varieties endowed with tolerance to biotic and abiotic stresses are highly beneficial, as unfavorable environmental factors can challenge cultivation and crop productivity. Although crops tolerant to biotic and abiotic stresses have been selected by conventional breeding programs, speeding up the pace of breeding is essential for developing improved varieties.

Soil salinity has become a major limiting factor that adversely affects crop production [2]. Worldwide, it is estimated that around 800 million hectares of land are affected by salinity, with salinity levels ranging from 2 to 4 dS m^{-1} [2]. Salinity affects plant cells, causing alterations in water relations, ionic and metabolic perturbations, generation of reactive oxygen species (ROS), and tissue damage [3]. Development of salt-tolerant cultivars by conventional, mutational, and biotechnological approaches can augment the utilization of salinity-affected regions. The availability and screening of large populations for mutagenesis are prerequisites to obtaining sufficient genetic variability. In vitro culture in combination with radiation-induced mutations has become an important method to induce genetic variability and rapidly multiply the selected mutants [4–6]. Methods of chemical- and/or radiation-induced in vitro mutagenesis have been successfully used to improve agronomic traits including salinity and drought tolerance in several crop plants [7–10]. Determinations of radiosensitivity and of optimal doses of ionizing radiation are important steps for undertaking induced mutagenesis for crop improvement. Their importance has been well demonstrated in plants such as rice [11], groundnut [12], sweet potato [10], banana [13], and *Zoysia* [14].

Although studies of salt selection are available for diverse plant species, limited research has been conducted in sugarcane. Sugarcane embryogenic callus has been shown to be sensitive to sodium chloride (NaCl) [15] and gamma radiation [16]. Saif et al. [17] reported the isolation of salt-tolerant mutants from irradiated sugarcane callus. Although these studies have demonstrated the application of mutagenesis and in vitro techniques to study radiosensitivity or isolation of mutants in sugarcane, little information on characterization of salt tolerant callus and progeny is available. Studies of the application of ionizing radiation for developing novel mutant germplasm in sugarcane will accordingly be beneficial for sugarcane improvement. The objective of the present study was to apply gamma ray-induced mutagenesis to isolate sugarcane mutants with improved tolerance to salinity, followed by morphological and agronomical characterization of selected mutants.

2. Materials and methods

2.1. Plant material and culture conditions

The commercial sugarcane variety Co86032 was used as the experimental material. The tops of mature canes were harvested from field-grown plants at the Vasantdada Sugar Institute, Manjari, Pune (India). The explant material was washed first in tap water and then for 5 min in sterile distilled water at least three times. Surface decontamination was performed with 80% ethanol (v/v) for 5 min and mercury chloride (0.1% w/v) for 4–5 min, followed by three washes with sterile distilled water for 15 min each. After removal of the outer leaves, the innermost leaf segments were cut into 2–3 mm pieces and aseptically inoculated onto MS [18] medium supplemented with 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 3 mg L^{-1} 2,4-D, 10% coconut water and 2.0% sucrose. This medium is referred to as callus induction medium. Cultures were incubated in the dark at 25 ± 2 °C at relative humidity 70–80%. After 45 days of culture, the callus was subcultured onto modified MS medium with 1000 mg L^{-1} casein hydrolyzate, 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 3 mg L^{-1} 2,4-D, 5% coconut water, and 2.5% sucrose to obtain embryogenic callus. This medium is referred to as callus maintenance medium.

2.2. Gamma ray radiation treatments

Embryogenic callus cultures were irradiated with 0, 10, 20, 30, 40, 50, 60, 70, and 80 Gy gamma rays using Gamma Cell 220 (a ^{60}Co source) at a dose rate of 9.6 Gy min^{-1}. Post irradiation, callus cultures were transferred to freshly prepared MS medium supplemented with 3 mg L^{-1} 2,4-D, 1000 mg L^{-1} casein hydrolyzate, 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 5% coconut water, and 2.5% sucrose. Survival of the irradiated callus was determined using relative growth rate after four weeks of radiation treatment. The surviving callus was then subcultured for at least four passages on maintenance medium.

2.3. Salt stress and its effects on irradiated callus

Irradiated and non-irradiated callus cultures were subjected to treatments with different concentrations of salt (NaCl) stress (0, 50, 100, 150, 200, and 250 mmol L^{-1}) to study salt stress effects and identify the optimal concentration of NaCl to be used in the selection medium. After four weeks of treatment, the response was recorded using several parameters: tissue water content (TWC), relative electrolyte leakage (REL), relative growth rate (RGR), accumulation of proline and glycine betaine (GB), protein content, and Na^+ and K^+ concentration.

Membrane damage was determined in terms of relative electrolytic leakage (REL) by the method of Sullivan [19]. For REL measurement, callus was incubated for 24 h in a test-tube (25 mm × 150 mm) containing distilled water (25 °C) and the initial electrical conductivity (EC_1) was measured after the incubation period. Samples were then autoclaved for 15 min at 121 °C to release the ions from the tissue, and the final electrical conductivity (EC_2) was measured after cooling to room temperature. The REL was calculated as: $(EC_1/EC_2) \times 100$. TWC of the callus was determined as described by Lokhande et al. [20]. The percent tissue water content (TWC %) was determined using the following equation: TWC (%) = [fresh weight (FW) − dry weight (DW) / (FW)] × 100.

2.4. Free proline concentration

Proline concentration was evaluated by the method of Bates et al. [21] with minor modifications. Callus was ground in 3% sulfosalicylic acid and centrifuged at 4 °C. The filtrate was mixed with equal volumes of acid ninhydrin and glacial acetic acid, and then incubated at 100 °C in a hot water bath for 1 h. The reaction

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