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BIOTECHNOLOGY

Ethyl methane sulphonate (EMS) mediated changes in callus growth of clusterbean (*Cyamopsis tetragonoloba* L.) raised under saline conditions

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Abstract

Soil salinity is the major biotic stress, which restricts the distribution and productivity of the crops. Agricultural production in the future will increasingly rely on our ability to grow plants on salt affected and marginal lands using saline water. Ethyl methane sulphonate is the most commonly used chemical mutagen to increase genetic variability in crop plants and could be useful in increased plant tolerance to salinity. Clusterbean is one of the most important summer annual legume. In the present study, aseptically grown7-day old seedling explants of clusterbean *viz.* cotyledon, cotyledonary node, hypocotyl and the embryo axis (cut on radical side) were cultured on MS medium + B_5 vitamins (MSB_5 medium) supplemented with various growth regulators. Among various explant tried, cotyledonary node gave good response in terms of callus growth which was further selected for future experimentation. The best medium for callus growth was MSB_5 with 2 mgl^{-1} 2,4-D and 1 mgl^{-1} BAP. All the calli produced were compact and their color changed with increase of salt concentration from greenish to brownish green, dark brownish and finally blackish at 200 mM NaCl. Further, calli pieces dipped in sterile liquid MS medium adjuncted with aqueous filter sterilized solution of 0.5% EMS for a range of time duration (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 h) were raised on MSB_5 medium without NaCl. These calli were subcultured on the above medium with or without 200 mM NaCl. EMS treatment of 2.5, 3.0 and 3.5h duration improved callus growth on salt amended medium; growth being maximum after 3.0h EMS treatment. It seems that exposing mutagen treated calli to salinity/ salt stress, forces these to face stress doubly-ionic toxicity and toxic effects of mutagen. The survival and regeneration of the putative variant calli is suppressed under such circumstances.

Highlight

- Cotyledonary node gave the best response in terms of callus growth.
- 200 mM NaCl was found to be lethal for the growth of callus.
- Growth of callus was maximum at 3.0 h duration of EMS treatment.
- It is possible to increase the salt tolerance of callus by applying EMS.

Keywords: Clusterbean, ethyl methane sulphonate, mutagen, soil salinity, callus

Clusterbean [*Cyamopsis tetragonoloba* (L.) Taub.], commonly known as guar, is one of the most important summer annual legume that was introduced into USA from India in 1993 (Khanzanda *et al.,* 2003). It belongs to the family leguminasae and is a robust annual herb with long tap root and well

developed laterals. It is cultivated throughout India for its edible pod and as fodder crop (Sangwan *et al*., 2013). Guar is a drought hardy crop, widely grown in rainfed condition in arid and semiarid regions of tropical India during kharif season (Deepika & Dhingra, 2014; Sangwan *et al*., 2014a). It has great industrial importance due to the presence of gum content in its endosperm (25–35% of whole seeds) and is highly mucilaginous (Sangwan *et al*., 2014b). India is one of the main producers of clusterbean accounting for 80% of total production of the world. India export guar to more than 65 countries with a turnover of ₹ 2811 crore during 2011-2012 (Deepika *et al*., 2014). However, it is unable to meet the required demand (Dube *et al*., 2011). Soil salinity is one of the major abiotic stress which restricts the distribution and productivity of the crops. No climatic zone in the world is free from salinization, salinity mediated inhibition of growth and yield is ascribed to low osmotic potential of soil solution, specific ion toxicity, nutritional imbalance or combination of these.

Therefore, there has been a great incentive to increase salt tolerance of our conventional crops using genetic manipulation to allow greater yields in salt affected areas. To increase variability in relation to salt stress, *in vitro* tissue culture techniques offer promising scope. Somaclonal variations have been exploited for salt tolerance improvement. However, difficulties in plant regeneration from selected cell lines and existence of high level of abnormal regeneration has come in popularization of the somaclones (Luan *et al*., 2007). Another aptly viable option is to induce variability through *in vitro* mutagenesis that facilitates improvement in one or few traits of genotype without disturbing rest of the genome. Studies have shown that application of tissue culture technique following mutagen treatment increases mutagenesis frequency expands the spectrum of trait variation and results in more target mutants.

Among chemical mutagens, the alkylating agent like EMS is a potent inducer of specific and predictable mutations (Talebi *et al*., 2012). It can induce high frequency of gene mutation and low frequency of chromosomal aberrations. Moreover *in vitro* culture is an ideal system for screening of salt tolerant mutants as it is carried under controlled conditions with limited space and time.Thus, the reproducible selection protocol can be employed in NaCl tolerant plants. Moreover, unlike whole plant, a very large number of callus can be screened at one time for a desired trait. To the best of our knowledge, there are no reports on deployment of EMS or other chemical mutagen for improvement of salt tolerance and cell line selection. The present investigation therefore was undertaken on clusterbean, HG 2-20, characterized by profuse branching, early maturing (85-100 days) and high grain yield (21-22 quintal/ha).

Materials and Methods

Sterilization of glassware/plasticware

Glasswares and plasticwares were thoroughly washed with teepol (liquid detergent). The glasswares were dried in oven at 160°C for 2 h and plasticwares at 60°C for 2 h and rinsed with double distilled water before use. The pipettes were washed with chromic acid followed by washing in running tap water.

Preparation and storage of stock solutions

Stock solutions of major and minor salts, chelating agent (Fe-EDTA), vitamins, growth regulators were prepared (mg/ml) according to their solubility separately and stored in refrigerator at 4°C. Wherever required amber coloured bottles were used for storage to eliminate the light effect. All the stock solutions were used within one month of their preparation.

Preparation, sterilization and storage of culture media

Murashige and Skoog's (MS, 1962) medium was used as basal medium with B_5 vitamins, 3% sucrose, myo-inositol (100 mg, *w/v*) and 0.8% agar throughout the course of present investigation. Various growth regulators viz. 2, 4-dichlorophenoxy acetic acid (2, 4-D), α-indole 3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2–ip [2-(6-isopentenyl) adenine] cytokinins *viz*. 6-benzyl amino purine (BAP) and kinetin (Kn), thidiazuran (TDZ) were used alone or in various combinations with basal media. All the components of the medium were mixed and final volume was made by adding double distilled water and then pH was adjusted to 5.8 (before adding agar) with the help of 1 N

NaOH/1 N HCl followed by addition of agar. After melting the agar, medium was dispensed into culture flasks/tubes. These were plugged with non-absorbent cotton. The medium was sterilized in an autoclave at a pressure of 1.08 kg/cm^2 (Temp. 121°C) for 15 min. The autoclaved medium was stored at 26 ± 2°C and used within 3-4 days of its preparation.

Explants and their preparation

Seeds of *Cyamopsis tetragonoloba* HG 2-20, were washed thoroughly with tap water containing a drop of teepol for 5-10 min. Subsequently the seeds were surface sterilized with 70% alcohol for 1 min and then with 0.1% mercuric chloride solution for 5 min. The seeds were then rinsed thoroughly three to four times in sterile distilled water on the hood of laminar flow to remove all traces of mercury. These sterilized seeds were germinated on germination medium containing 3% sucrose, 0.8% agar under aseptic conditions initially under dark condition until germination and then shifted to light conditions. For the present study, 7-10 day old seedling explants like cotyledon, cotyledonary node, embryo axis and hypocotyl were used.

Inoculation of explants and culture conditions

All the operations were carried out in a laminar clean air flow. The laminar floor was cleaned with rectified spirit and then sterilized by UV radiation for about half an hour before use. Different explants measuring 4-5mm obtained from aseptically grown seedlings were inoculated on the surface of culture medium. Embryo axis explants were excised from surface sterilized seeds and three explants per flask were cultured. Inoculated flasks were kept in culture room at 25±1ºC temperature, under photoperiod of 16h light and 8h darkness.

Callus induction and growth

Various explants viz. cotyledon, cotyledonary node, embryo axis and hypocotyl were cultured on MS basal medium alone and with various concentrations of different auxins (2, 4-D, IAA, IBA, NAA) and cytokinins (BAP, Kn) alone and in combinations. Visual observations like number of days required for induction of callus, percent callus induction, colour and texture of callus were recorded periodically and used to select best callus induction medium.

For each treatment, 10 flasks were used.For good callus growth, 28 days old callus pieces were subcultured on fresh MSB_5 medium supplemented with same and different concentrations and combinations of growth regulators. It was also harvested after 28 days and growth was measured in terms of fresh and dry weights. Dry weight was determined after drying in the oven at 60°C till constant weight was attained.

Callus induction and growth on NaCl amended medium

Callus obtained from cotyledonray node segments on MS medium with 2,4-D (2mg/l) and BAP (1 mg/l). After 4 weeks of culture, callus was cut into pieces of approximately 5mm size under aseptic conditions and recultured on the above medium supplemented with 10 mg/l of ascorbic acid and different concentrations of NaCl (0, 50, 100, 150, 175 and 200 mM) for 30 days to find out the lethal dose of NaCl concentration. Fresh and dry weights of these calli were recorded. In another set of experiment, callus pieces were treated with filter sterilized solution of 0.5% EMS for different duration. These treated calli were rinsed 5 times in sterile distilled water and finally cultured on MS medium Corresponding untreated control and EMS treated callus clumps raised on MSB_5 medium without NaCl serves as control. These calli were subcultured thrice at an interval of 20 days and their fresh and dry weights were recorded.

Results and Discussion

Callus induction and growth response

The basal medium without growth regulators failed to support callus induction as well as axillary bud break from different explants used. This lack of callus formation might result from the insufficieny of endogenous level of phytohormones in explants to trigger callus formation.

Therefore, synthetic auxins were added to medium to promote callus induction and proliferation (Konate *et al.*, 2013). Supplementation of growth regulators to MSB_5 medium induced callus induction, the frequency however, varied with the type and concentration of growth regulator and explant used.

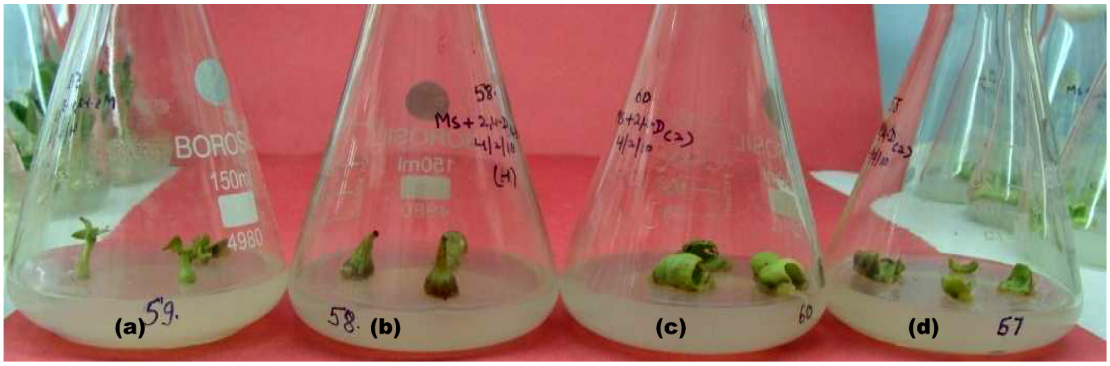

Fig. 1: Depicting callus formation from [cotyledonary node (a), hypocotyl (b), cotyledon (adaxial, c) and cotyledon (abaxial, d) on MSB_5 medium + 2mgl^{-1} 2,4-D in clusterbean

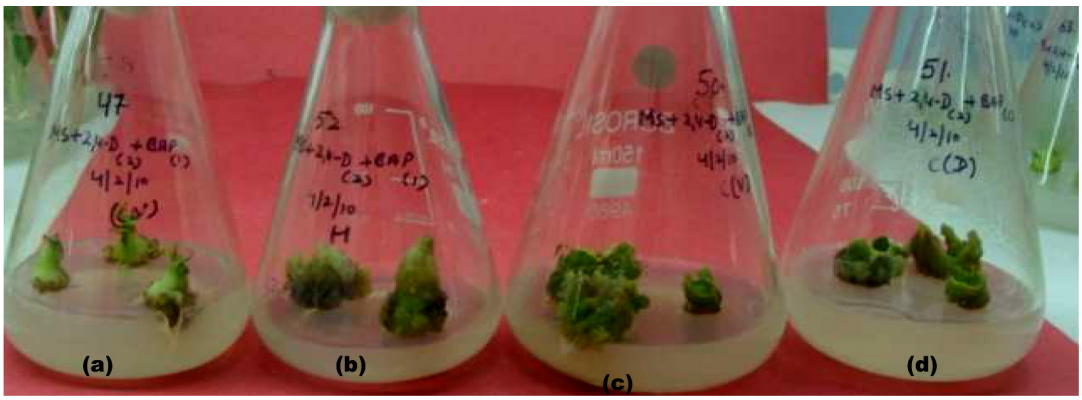

Fig. 2: Depicting callus formation from [cotyledonary node (a), hypocotyl (b), cotyledon (adaxial, c) and cotyledon (abaxial, d) on MSB_5 medium + 2mgl^{-1} 2,4-D + 1 mgl^{-1} BAP in clusterbean

Culture medium and explants

MSB_5 medium supplemented with 2 mgl^{-1} 2,4-D alone, supported very poor callus growth from all explants except embryo axis explants (Fig. 1). The MSB_5 medium without growth regulators served as control. Furthermore, callus initiation was delayed until 45-50 days of inoculation in all cases. Supplementation of different concentrations of BAP to the above medium revealed cotyledonary node to be the best responding explants. The medium comprising of 2mgl^{-1} 2,4-D and 1mgl^{-1} BAP, was the best, Callus obtained from cotyledonary node were compact, while those from other explants were fragile (Fig. 2 and 3). Various permutation and combinations of 2,4-D and BAP failed to support organogenesis from callus of different explants of guar. Anju *et al.* (2013) found that cotyledonary node explant was the most responsive explant for plant regeneration in all the three species of *Cyamopsis*. Induction and growth of callus from cotyledon explants using different type and concentrations of growth regulators have already been reported in legume species including *Vigna radiate* (L.) (Rao *et al.*, 2005) and *Cicer arietinum* (L.) (Khan *et al.*, 2011). A study revealed that 0.5 mg/l 2,4- D alone is the best plant growth regulator for callus induction in *Vigna subterranean* (L.) as explants source (Konate *et al.*, 2013*).*

Data presented in Table 2 clearly evinces that BAP (1 and 2 mgl^{-1}) alone supported 100% callusing from cotyledonary node while 2.0 mgl^{-1} BAP in combination with 1.5 mgl^{-1} NAA supported minimum callusing. Good callusing along with good callus growth was evident in the medium supplemented with 3.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. In general, single shoot emerged from cotyledonary node, but multiple shoot formation was observed on medium supplemented with 2.0 mgl^{-1} BAP alone. Interestingly, medium supplemented with 1.0 mgl^{-1} NAA + 1.0 mgl^{-1} BAP+ 3.0 gl^{-1} activated charcoal supported direct organogenesis without intervening callus formation (Figure 4). Among different concentrations of BAP, 2.0mgl^{-1} supported best shoot formation coupled with callusing (Figure 5) and also multiple shoot formation was observed. Prem

Fig. 3: Depicting callus formation from embryo axis on MSB_5 medium supplemented with 2mgl^{-1} 2,4-D +0.5 mgl^{-1} BAP (a), 2mgl^{-1} 2,4-D+1.0 mgl^{-1} BAP (b) and 2mgl^{-1} 2,4-D (c) in clusterbean

Fig. 4: Depicting shoot formation from cotyledonary node on MSB_5 medium supplemented with 1.0 mgl^{-1} NAA +2mgl^{-1} BAP +3.0 gl^{-1}charcoal (a) and 1.0 mgl^{-1} NAA +1.0mgl^{-1} BAP +3.0 gl^{-1} charcoal (b) in clusterbean.

et al. (2003) developed a rapid shoot morphogenesis system in four Indian guar cultivars (HG 365, HG 75, RGC 936 and CH_{4-2}) by using cotyledonary nodes.

Shoot formation was observed on medium supplemented with 1.0 mgl^{-1} IBA and with 0.5 mgl^{-1}Kn +1.0 mgl^{-1} BAP but the callus growth was very poor (Table 3,4). Prem *et al*. (2005) developed an efficient system for callus induction and *de novo* regeneration from callus. They found that MS medium containing 10.0 µM 2,4-D in combination of 5.0 µm BAP is more suitable for induction of green and friable morphogenic callus from cotyledon or embryo explants and achieved efficient *de novo* shoot regeneration by culturing the callus on MS medium supplemented with 13.0 µM NAA and 5.0 µM BAP subsequently. Ramulu and Rao (1993) found kinetin to be an essential for callus induction and maintenance of callus growth in guar. Bajaj and Gosal (1988) reported maximum callus induction frequency from hypocotyl and young embryo explant on MS medium with 2 mgl^{-1} 2, 4-D in combination with 0.5 mgl^{-1} BAP.

Salt resistance in *in vitro* studies has generally been determined by measuring the increment of fresh and dry weights of callus and suspension cultures. Present study evinced that calli survived on all salt concentrations used except 200 mM NaCl and callus growth decreased with the progressive increase in salt concentration in the culture medium. All the above calli were compact and their color changed with increase of salt concentration from greenish to brownish green, dark brownish and finally blackish at 200 mM NaCl. Thus, 200 mM NaCl was found to be the lethal concentration of salt (Fig. 6). Rahman and Kaul (1989) reported that incorporation of 10 mg l^{-1} ascorbic acid to the medium was essential for continued growth of callus over several subcultures.

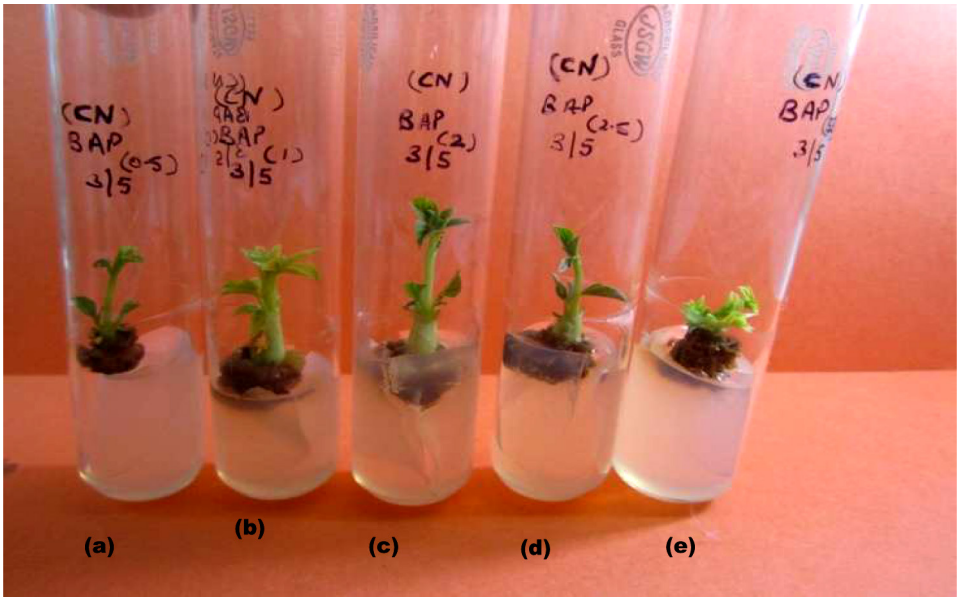

Fig. 5: Depicting shoot formation as well as callus formation from the cut end of cotyledonary node on MSB_5 medium supplemented with different concentration of BAP [0.5 (a), 1.0 (b), 2.0 (c), 2.5 (d) and 3.0 mgl^{-1} (e) in clusterbean

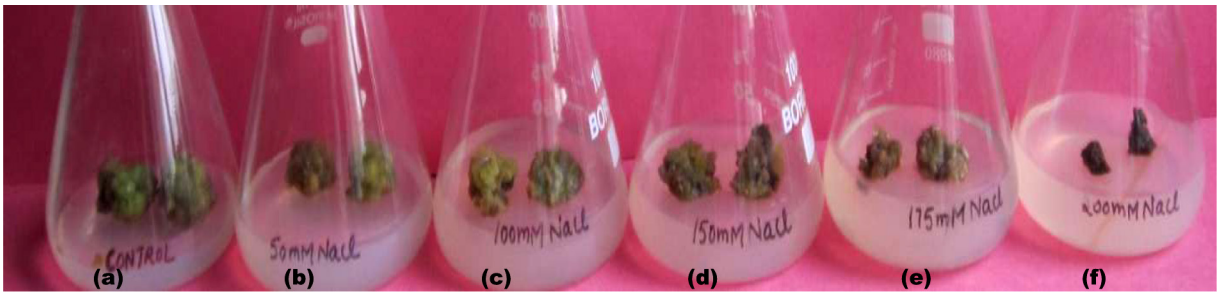

Fig. 6: Depicting callus growth on MSB_5 medium + 2 mgl^{-1} 2,4-D+ 1 mgl^{-1} BAP + 10 mgl^{-1} ascorbic acid with control (a), 50 mM NaCl (b), 100 mM NaCl (c), 150 mM NaCl (d), 175 mM NaCl (e) and 200 mM NaCl (f) in clusterbean

Fig.7: Depicting callus growth after third subculture on MSB_5 medium + 2 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP + 10 mgl^{-1} ascorbic acid + 200 mM NaCl following EMS treatment of different durations [control (a), 0.5 (b), 2.5 (c), 3.0 (d) and 3.5h (e)] in clusterbean

Addition of 10 mgl^{-1} ascorbic acid to the medium that improved callus growth in the present study. Belal *et al.* (1998) reported that the growth rate of callus in *Acacia saligns* decreased with increasing concentration of NaCl (0.5 to 2.0%) which was ascribed to the inhibition of cell multiplication caused by partial blocking DNA synthesis. Slowing down of growth as reported in a number of systems, not help the plant to save the energy for defence purpose but also limits the risk of heritable damage. Abd-Elrahem *et al.* (1983) developed NaCl resistant lines from suspension cultured tobacco cells by stepwise increase of NaCl concentrations in the medium and resistance was stable through

24 generation in the absence of added NaCl. Lutts *et al.* (1997) reported a decrease in agronomic performance of salt selected plants probably being first generation plants coming from *in vitro* cultures.

Mutation breeding is a powerful tool for enriching variation for specific characters in a crop especially leguminuous ones where exploitable and useful genetic variability is very meager. In mutation breeding, the possibility exists to change a single gene or only a few genes without altering the total genetic makeup of specific (outstanding) genotype. Efforts have been made to create more and purposeful variability in guar by various scientists through induced mutations (Arora and Pahuja, 2008). India has been the first to make successful attempt for inducing mutations in guar through physical mutagens. Gohal (1970) reported five plants with profuse vegetative growth in M1 plants raised from guar seeds treated with EMS.

In the present study, calli pieces dipped in sterile liquid MS medium adjuncted with aqueous filter sterilized solution of 0.5% EMS for a range of time duration (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 h) were raised on MSB_5 medium without NaCl. These calli were subcultured on the above medium with or without 200 mM NaCl. EMS treatment of 2.5, 3.0 and 3.5h duration improved callus growth on salt amended medium; growth being maximum after 3.0h EMS treatment (Fig. 7). Kenganal *et al. (*2008) also observed that calli treated with 0.5% EMS for 2.5h could only gain higher weight and 15 healthy sugarcane plants were successfully regenerated. Further increment in the duration of EMS treatment resulted in poor growth. Salt tolerant mutant of sweet potato (*Ipomoea batatas*) has also been obtained by 0.5% EMS treatment of calli for 2.0 and 2.5h followed by plant regeneration through somatic embryogenesis (Luan *et al.,* 2007). Lu and Jia (1994) succeeded in regenerating NaCl resistant plants by treating embryogenic calli of millet (*Setariaitalica*) with 0.5% EMS for 2.5h.

Conclusion

Guar is a recalcitrant crop. Various growth regulators applied to medium shows differences in callus growth. Ethylmethane sulphonate, a chemical mutagen affect the growth of callus at different salt level. Changes in dry weight and colour of callus was observed. Duration of EMS treatment also played important role in affecting callus growth. Growth of callus was maximum at 3.0 h duration of EMS treatment. The study reveals that it is possible to increase the salt tolerance of callus by applying EMS.

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References

Abd-elrahem A.W., Reinhold, L. and Lerne, H.R. 1983. Comparison between a Stable NaCI-Selected Nicotiana Cell Line and the Wild Type' K+, Na+, and proline pools as a function of salinity. *Plant Physiol.* **73**: 624-629.

Anonymous. 2012. APEDA. India Export of Agro food Products. DGCIS Annual Export.http:// agriexchange. apeda.gov.in/indexp/Product_description_32 head. aspx?gcode= 0502

Arora, R.N. and S.K. Pahuja. 2008. Mutagenesis in guar [*Cyamopsis tetragonoloba* (L.) Taub.]. *Plant mutation reports* **2**(1): 7-9.

Bajaj, Y.P.S. and Gosal, S.S. 1988. Establishment of tissue cultures and the regeneration of plants in guar. *Sabrao Journal* **20**: 145-149.

Belal, A.H., Moustaga, Y. and Sabbah, M. 1998. Image analysis to detect genetic variation of *Acacia saligna* cells under salt stress *in vitro.* Bull. *Faculty Agric. University of Cairo.* **49(**3): 389-402**.**

Deepika., Dhingra, H.R. and Goyal, S.C. 2014. Callus induction and regeneration from various explants of clusterbean [*Cyamopsis tetragonoloba* (L.) Taub.]. *Ind. J. Plant Physiol*. **19**(4): 388-392.

Deepika, and Dhingra, H.R. 2014. Effcet of salinity stress on morphophysiological, biochemical and yield characters of clusterbean [*Cyamopsis tetragonoloba* (L.) Taub.]. *Ind. J. Plant Physiol.* **19**(4): 393-398.

Dube K.G., Baja, K.G. and Gawanda, A.M. 2011. Effect of gamma rays and ethyl methane sulphonate in alone and combination treatments on mitotic chromosomal aberrations in *Cyamopsis tetragonoloba* (L.) Var. Sharada. *Asiatic J. Biotechnol. Resources* **2:** 577-581.

Gohal, M.S. 1970. Effect of ethylmethanesulphonate on the mutation spectrum in guar. *Indian J. Heredity* **2**: 51-54.

Henry, A. and Mathur, B.K. 2007. Genetic diversity in clusterbean germplasm. *J. Arid Legumes.* **4:** 134-136.

Kenganal, M., Habchinal, R.R. and Nadaf, H.L. 2008. Ethylmethanesulphonate (EMS) induced mutation and

selection for salt tolerance in sugarcane *in vitro. Indian J. Plant Physiol.*, **13**(4): 405-410.

Khan, S., Ahmad, F., Ali, F., Khan, H., Khan, A. and Swati, Z.A. 2011. Callus induction via different growth regulators from cotyledon explants of indigenous chick pea (*Cicerarietinum* L.) cultivars KK-1 and Hassan-2K. *Afr. J. Biotechnol.* **10**(40): 7825-7830.

Khanzada, B., Ashraf, M.Y., Shirazi, M.U., Alam, S.M., Samo, K.B. and Mujtaba, S.M. 2003. Study of photosynthetic efficiency of some guar (*Cyamopsis tetragonoloba* L.Taub) genotypes grown under different water regimes. *Asian J. Plant Sci*., **2**: 127-131.

Konate, S., Kone, M., Kouakou, H.T., Koudio, J.Y. and Zouzou, M. 2013. Callus induction and proliferation from cotyledon explants in Bambara groundnut. *Afr. J. Biotechnol*., **21**(3): 255-263.

Lu, W. and Jia, J.F. 1994. Selection of NaCl-tolerant cell line from embyogenic calli of millet and studies on its physiological and biochemical characterstics. *Acta Agronomica Sinica.* **20:** 241-247.

Luan, Y.S., Zhang, J. and Gao, X.R. 2007. Mutation induced by ethyl methane sulphonate (EMS), *in vitro* screening for salt tolerance and plant regeneration of sweet potato (*Ipomoea batatas* L.). *Plant Cell Tissue Organ Culture* **88:** 77-81.

Lutts, S., Bouharmont, J. and Kinet, J.M. 1997. **Physiological Characterisation of Salt-resistant Rice (***Oryza sativa***) Somaclones.** *Australian Journal of Botany*. **47**(6): 835 – 849.

Prem, D., Subhadra, S., Gupta, P.P., Singh, J.V. and Kadyan, S.P.S. 2005. Callus induction and *de novo* regeneration from callus in guar (*Cyamopsis tetragonoloba*). *Plant Cell Tiss. Org. Cult.* **80:** 209-214.

Prem, D., Subhadra, S., Gupta, P.P., Singh, J.V. and Yadav, G. 2003. High frequency multiple shoot regeneration from cotyledonary nodes of guar. *In vitro* cellular and developmental. *Biol. Plant.* **39:** 384-387.

Rahman, M.M. and Kaul, K. 1989. Differentiation of Sodium Chloride Tolerant Cell Lines of Tomato (*Lycopersicon esculentum* Mill.) cv. Jet Star. *J. Plant Physiol.*, **133**: 710-712.

Ramulu, C.A. and Rao, D. 1993. In *vitro* effect of phytohormones on tissue culture of clusterbean [*Cyamopsis tetragonoloba* (L.)Taub.]. *J. Physiol. Res*. **20:** 7-9.

Rao, S., Patil, P. and Kaviraj, C.P. 2005. Callus induction and organogenesis from various explants in *Vigna radiata* (L.) Wilczek. *Indian Journal of Biotechnology* **4**: 556-560.

Sangwan, P., Kumar, V., Khatri, R.S. and Joshi, U.N. 2013. Chromium (VI) induced biochemical changes and gum content in cluster bean (*Cyamopsis tetragonoloba* L.) at different developmental stages. *Journal of Botany* **1-8**: 578627.

Sangwan, P., Kumar, V. and Joshi, U.N. 2014a. Chromium (VI) Affected Nutritive Value of Forage Clusterbean (*Cyamopsis tetragonoloba* L.). *International Journal of Agriculture, Environment and Biotechnology* 7(1): 17-23.

Sangwan, P., Kumar, V., Joshi, U.N. 2014b. Effect of chromium (VI) toxicity on enzymes of nitrogen metabolism in clusterbean (*Cyamopsis tetragonoloba* L.). *Enzyme Research.* **1–8**: 784036.

Talebi, B.A., Talebi, A.B. and Shahokhifar, B. 2012. Ethyl Methane Sulphonate (EMS) Induced Mutagenesis in Malaysian Rice (cv. MR219) for Lethal Dose. *American Journal of Plant Sciences* **3**: 1661-1665.