Development of an empirical relationship for chemical mutagen induced lethality in zygotic embryos and microspore embryogenesis from mutant donor plants of Indian mustard (*B. juncea*)

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Abstract

The haploid mutagenesis has not been exploited for crop improvement in Indian mustard (*Brassica juncea*) as yet and information for LD_{50} of chemical mutagens is not available. The present study was thus undertaken to understand the relationship between mutagen concentration, exposure duration and survival of zygotic embryos along with evaluation of microspore totipotency of the mutant donor plants. Three Indian *B. juncea* genotypes, Pusa Bold, Varuna and BIO-902, were tested for varying mutagen concentrations (5-50 mM) and exposure durations (10-50 h) of three chemical mutagens, Ethyl methanesulfonate (EMS), Ethyl nitrosourea (ENU) and Ethidium bromide (EtBr), to study the effect of mutagen exposure on zygotic embryos. The seeds, after mutagen treatment, were rinsed with sterile water, cultured on Murashige and Skoog basal medium, incubated at 25 ± 2 °C / 16 hours photoperiod, and scored for percent survival after 15 to 20 days. Exposure to EtBr resulted in 100% mortality. The survival data from ENU and EMS treatments was subjected to orthogonal contrast partitioning ANOVA for determining the relationship between the dependent (survival) and the independent variables (concentration, linear to quartic and duration, linear to cubic orthogonal polynomial). Both genotypes showed a similar response for percent survival after EMS/ENU treatment, however, significant variation was caused individually and by interaction of the concentration and exposure duration for both the mutagens. The mutagen concentration for both mutagens had a linear relationship with percent survival while the exposure duration had a non-linear relationship with the latter. Multiple regression analysis was carried out to develop prediction functions for EMS/ ENU treatment survival and this revealed that log transformation of the percent survival data resulted in linearizable non-linear regression equations that adequately explained the relationship between the dependent and the independent variables. Using these equations the LD_{50} for zygotic embryo mutagenesis for 20h exposure duration ranged from 3.5mM for ENU to 6.8mM for EMS. This information was used to generate mutant donor plants for microspore culture. The microspores from ENU treated donor plants, irrespective of the genotype, were recalcitrant to embryogenesis, while 48.8% EMS mutant donor plants produced 2.9 ± 0.36 embryos per Petri dish (total 943 embryos). Genotype comparison revealed that mutant donor plants of the three genotypes had similar microspore embryogenic response.

Key words: Indian mustard, *Brassica juncea*, LD_{50}, multiple regression, haploid mutagenesis

Introduction

B. juncea is the major oilseed *Brassica* species grown in South East Asia and has gained rapid acreage in Canada and Australia. While chemical mutagenesis has been used for creation of genetic variation in *B. juncea*, from the present literature no consensus can be drawn for the concentration and duration of mutagen exposure for determining 50% lethality (LD_{50}), that are the most important determinants of the extent of genetic variation that can be created by chemical or physical mutagenesis (Konzak et al., 1965). Therefore, an empirical relationship between mutagen concentration and exposure duration is valuable from the perspective of understanding and predicting the lethality of induced mutations following chemical mutagenesis on *B. juncea* system. An efficient doubled haploid production protocol has been developed in our laboratory for *B. juncea* (Prem et al., 2004; 2005) and in this report we present a novel strategy for exploiting haploid mutagenesis in *B. juncea* using mutant plants as microspore donors.

Material and Methods

Three chemical mutagens namely Ethyl methanesulfonate (EMS), Ethyl nitrosourea (ENU) and Ethidium bromide (EtBr) were used for experiments carried out to study the effect of mutagen exposure on zygotic embryos. The response was studied in terms of germination of mutagenized seeds for three *B. juncea* genotypes namely Pusa Bold, Varuna and BIO-902. The zygotic embryo mutagenesis experiment were planned in a three replicate 3 × 5 × 4 factorial completely randomised designs wherein for each mutagen, the three genotypes were tested for four mutagen concentration (5.0, 10.0, 20.0 and 50.0 mM prepared in 7.0 pH buffer) along with controls and four exposure durations (10, 20, 30 and 50 h). For each replicate, approximately 100 seeds of each genotype were surface sterilized in 70% ethanol (v/v) for 10 s followed by 0.05% mercuric chloride (w/v) for 10 min and were then soaked in sterile distilled water for 16 h in dark. Thereafter these were washed twice with sterile water and subjected to mutagen treatment. Following this the seeds were washed 6 to 7 times with sterile distilled water and cultured on half salt concentration MS basal medium containing 2% sucrose (w/v), gelled with 0.7 % (w/v) agar. These cultures were incubated at 25 ± 2 °C temperature and 16 h photoperiod with 175 µmol/ m^2/ s light intensity. The cultures were scored for total number of seeds incubated/ treated and total number of seeds that showed healthy plantlet

growth after 15 to 20 days of culture. The proportion data was transformed using arcsine transformation and subjected to three factors ANOVA (Gomez and Gomez, 1984). The data for factors and their interaction effects recognised for significant effect on zygotic embryo survival and development of healthy plants was subjected to polynomial partitioning using orthogonal contrast procedure (Gomez and Gomez, 1984). For developing equations that explained the empirical relationship between the concentration of mutagen, exposure duration and percent survival of zygotic embryos, multiple regression analysis was done using two datasets; dataset–I constituted the linear mutagen concentrations and exposure durations along with the linear interaction effect of the two variables as independent variables and percent survival as the dependent variable and dataset–II constituted the linear mutagen concentration and the linear exposure duration along with the linear interaction effect of the two variables as independent variables and log transformed percent survival as the dependent variable. The coefficients of the regression equations were tested for their statistical significance by ANOVA method (Sokal and Rohlf, 1981). For the donor plant mutagenesis, 50 seeds of each genotype were treated with 5.0 mM EMS and ENU for 20 h and the resulting mutant plants were used as microspore donors. Microspores from these plants were cultured as per established protocol (Prem et al., 2005) and the microsprore embryogenesis response was quantified in terms of number of embryos produced per Petri dish.

Results

The exposure of zygotic embryos to the minimum concentration and exposure duration of EtBr resulted in 100% mortality while EMS/ ENU exposure caused a linear decrease in survival with increasing concentration/exposure duration. The three factor ANOVA for the survival of ENU and EMS treated zygotic embryos revealed that the concentration and duration of mutagen exposure are highly significant determinants of zygotic embryo survival after mutagen treatment. In addition to this, the interaction effect of these two factors was also found to be highly significant. The effect of the genotype either singly or as one of the interacting factors, was observed to be non-significant contributor to the overall variation (Table 1). The zygotic embryos of Pusa Bold treated with ENU showed 38.54 ± 4.34% (mean ± SEm) survival, irrespective of concentration and exposure duration, followed by Varuna (33.27 ± 4.21%) and BIO-902 (32.09 ± 4.51%). The effect of 10 mM and 20 mM concentration exposure of the zygotic embryos was equivalent irrespective of the exposure duration. Similarly the 10 h and 30 h exposure duration resulted in an equivalent percent survival irrespective of the mutagen concentration for all genotypes (Fig. 1). Similarly the zygotic embryos of Pusa Bold treated with EMS showed 36.87 ± 4.71% survival, irrespective of EMS concentration and exposure duration, followed by Varuna (35.39 ± 4.61%) and BIO-902 (31.74 ± 4.35%). While all mutagen concentrations resulted in significantly different mean percent survival, the 10 h and 20 h exposure durations resulted in similar survival irrespective of genotype and EMS concentration (Fig. 1). The results of the three factor ANOVA indicate that the functional relationship between these variables is rather complex since the genotype do not contribute significantly to the overall variation indicating that the relationship between the variables is independent of the genotype (Table 1). In addition to this, at least two treatments of the independent variables were observed to be similar indicating that instead of a polynomial relationship there may also be a non-linear relationship between the independent and dependent variables. The results of polynomial partitioning (Table 1) indicate that the mutagen concentration has a significant linear relationship while the higher polynomial orders are non-significant contributors to the percent survival. The exposure duration, on the other hand, has a non-linear relationship with the percent survival since the polynomial order partitioning coefficients for exposure duration are non-significant contributors to the overall variation. The multiple regression analysis further validated these findings (Table 2).

The coefficient of determination (R^2) for the regression equations developed using dataset–I for ENU and EMS indicates that these are not suitable as prediction function. Contrary to this regression equation developed from dataset–II for ENU and EMS treatment were adequate models for predicting the percent survival response for given exposure duration and concentration of mutagens. The statistical significance of the exposure duration when the log transformed data for the percent survival is used explains that the two variables have a linearisable non-linear relationship. Based upon the above analysis for zygotic embryo/ seed mutagenesis a mid value average range of 5.15 ≈ 5.0 mM was used for EMS and ENU seed treatment for generating mutant donor plants.

The microspores cultured from ENU treated donor plants did not produce any embryos for any of the three genotypes whereas those obtained from EMS treated donor plants produced on an average 2.9 ± 0.36 embryos per Petri dish (total 943 embryos) irrespective of the genotype. Out of the 15 Pusa Bold EMS mutated donor plants, buds collected from 10 plants produced microspore embryos. Similarly for Varuna and BIO-902, five out of 12 and six out of 16 EMS mutated plants, respectively, produced microspore embryos.

The various mutant donor plants were found to be equally responsive for microspore embryogenesis indicating absence of any within plant differences for microspore embryogenic response. Genotype comparison revealed that mutant donor plants of the three genotypes had similar microspore embryogenesis response wherein Pusa Bold produced maximum microspore derived embryos (504) with an average production of 3.36 ± 0.29 embryos produced per Petri dish followed by BIO-902 with a total of 312 embryos produced with an average 3.46 ± 0.31 embryos per Petri dish and Varuna with a total of 127 embryos produced with an average 1.69 ± 0.19 embryos per Petri dish.

Table 1: Three factor ANOVA with polynomial partitioning of main effects for *B. juncea* zygotic embryos treated with ENU and EMS

Source due to	Degree of Freedom	ENU	EMS
		Mean Square	Mean Square
Genotype (G)	2	680.57^{ns}	232.30^{ns}
Mutagen concentration (C)	4	19610.79*	18889.11*
$SS_{(Linear)}$	1	603.00*	643.0*
$SS_{(Quadratic)}$	1	78.03^{ns}	24.67^{ns}
$SS_{(Cubic)}$	1	44.94^{ns}	28.65^{ns}
$SS_{(Quartic)}$	1	0.44^{ns}	3.06^{ns}
Exposure duration (D)	3	4875.08*	4798.77*
$SS_{(Linear)}$	1	99.12^{ns}	96.32^{ns}
$SS_{(Quadratic)}$	1	0.57^{ns}	10.17^{ns}
$SS_{(Cubic)}$	1	9.15^{ns}	0.06^{ns}
G × C	8	194.98^{ns}	223.34^{ns}
G × D	6	208.56^{ns}	212.57^{ns}
C × D	12	491.56*	813.92*
G × C × D	24	121.77^{ns}	236.98^{ns}
Error	120	142.85	197.19

* Significant at $\alpha = 0.05$; ^{ns} non-significant

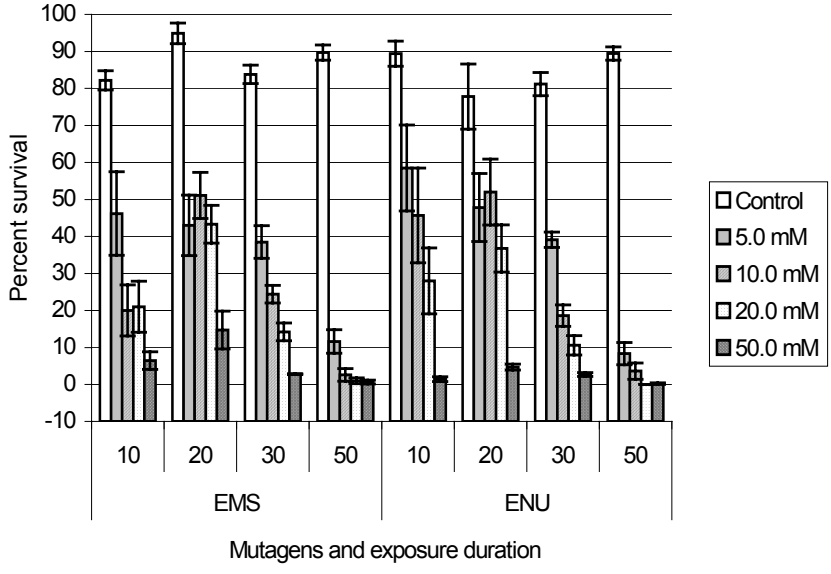

Fig. 1: The interaction effect of concentrations and exposure durations for EMS and ENU treatment.

Each histogram represents average survival proportion in terms of normal radical and plumule development after mutagen treatment for the three *B. juncea* genotypes (100 seeds per replication × 3 replications × 3 genotypes = 900 seeds). Bars represent standard error of mean. Lack of error bar indicates no survival of zygotic embryos. $LSD_{0.05}$ for comparison of any two means for ENU treatments = 11.04 and for EMS = 11.68

Discussion

The exposure of zygotic embryos to EtBr probably resulted in lethal mutations that caused high mortality of the zygotic embryos. In the present study genotypic variation for zygotic embryo germination after ENU/ EMS exposure was not observed probably because of the non-linear response to exposure duration and random alkylation caused by these mutagens. The available literature on seed mutagenesis in *B. juncea* reveals that 8.0 to 120.9 mM EMS and 6 to 12 h exposure duration has been most exploited for seed mutagenesis (Prasad and Singh, 1986; Mahla et al., 1991; Jha and Sinha, 1992; Kumar and Chaudhary, 1996; Bhatt et al., 2001). However, this concentration and exposure duration range is very varied and no general consensus can be drawn on the effect of chemical mutagens on *B. juncea* system *per se*. However, an increase in mutagen induced lethality with increasing concentration/ exposure duration has not been proven unequivocally for *B. juncea* seed mutagenesis. Contrary to our findings reports cited above highlight genotypic specificity for mutagen action. The present study for the first time elaborates the extent of biological damage resulting from varying the concentration and exposure duration for two chemical mutagens on the zygotic embryos of *B. Juncea*. Novel empirical relationships that explain the dependence of seed germination on the exposure duration and mutagen concentration is presented and multiple regression

based model have been developed for prediction of seed survival after mutagenesis. The non totipotent response of microspores from ENU mutant donor plants can not be conclusively explained. This may be because of haplontic selection that resulted in survival of only 'fit' microspores that do not have lethal macromutations may limit the availability of viable pollens in the M_1 plants. However, its predominance in ENU treated plants is not explainable as of now. Similar non-totipotent response for microspores obtained from some of the EMS donor plants was also observed, however, the frequency of microspore embryogenesis for individual EMS donor plants was similar. This difference in microspore embryogenesis ability for EMS donor plants can be attributed to macromutations that altered the genes responsible for microspore totipotency. The donor plant mutagenesis approach is based on the philosophical premise of harnessing mutation induced gametic variations for fixing the desired mutant genotypes using androgenesis. Although this approach offers a theoretical possibility of using haploid mutagenesis, it has not been used for *Brassica* haploid mutagenesis.

Table 2: Multiple regression equation developed for dataset–I and II; testing of regression coefficients and predicted LD_{50} for ENU and EMS concentrations for 20 h exposure duration

Regression equation[#]					
ENU					
Data set I: % Survival = 68.90 - 0.536(EXP) – 1.224(CONC) +0.0027 (EXP)(CONC) $R^2 = 0.499$					
Data set II: Log (% Survival) = 2.02 – 0.013(EXP) – 0.011(CONC) + 0.00045 (EXP)(CONC) $R^2 = 0.756$					
EMS					
Data set I: % Survival = 78.56 – 0.827(EXP) – 1.535(CONC) +0.0106 (EXP)(CONC) $R^2 = 0.623$					
Data set II: Log (% Survival) = 2.71 – 0.014(EXP) – 0.024(CONC) - 0.00026 (EXP)(CONC) $R^2 = 0.861$					
Testing of regression coefficients by ANOVA					
Source due to	Degree of freedom	Mean Squares			
		Data set I		Data set II	
		ENU	EMS	ENU	EMS
Regression (R^2)	3	3141.05*	3912.97*	1.97*	2.68*
EXP	1	1051.31^{ns}	1829.27^{ns}	2.02*	1.36*
CONC	1	8361.69*	9752.33*	3.60*	6.57*
EXP × CONC	1	10.15^{ns}	157.31^{ns}	0.29^{ns}	0.09^{ns}
Error	16	591.14	443.73	0.12	0.08
Prediction of LD_{50} concentration for 20h duration					
ENU					
Log (50) = 2.02 – (0.013 × 20) – 0.011 × (CONC) + 0.00045 × 20(CONC) = LD_{50} CONC = 3.5 mM					
EMS					
Log (50) = 2.71 – (0.014 × 20) – 0.024 × (CONC) - 0.00026 × 20(CONC) = LD_{50} CONC = 6.84 mM					

[#] EXP, exposure duration in hours; CONC, concentration in mM; *Significant at $\alpha = 0.05$; ^{ns} non-significant

Conclusions

The regression models developed in the present study for prediction of survival following chemical mutagenesis are valuable for future researchers since these could be used as prediction functions for determining mutagen concentration or exposure duration required for achieving a desired level of plant survival after chemical mutagenesis. The use of mutant donor plants as microspore donors circumvents mutagen exposure of isolated microspores or microspore derived embryos and therefore potentially limits the loss of embryogenesis/ embryo regeneration following mutagen exposure. The present results indicate that this technique could be used for genetic manipulation of *Brassicas* for economically important traits.

References

Bhatt S.R., Haque A., Chopra V.L. (2001) Induction of mutations for cytoplasmic male sterility and some rare phenotypes in Indian mustard. Indian Journal of Genetics and Plant Breeding **61,** 335-340

Buraas T., Skinnes H. (1984). Genetic investigations on seed dormancy in barley. Hereditas **101**, 235–244.

Gomez K., Gomez A.A. (1984). Statistical Procedures for Agriculture Research (2nd edition). IRRI publication, New York: John Willey and Sons.

Jha A.M., Sinha A.K. (1992). EMS and sodium azide induced biological damage in *Brassica juncea* Var. Krishna. Acta Botanica Indica. **20**, 152-155.

Konzak C.F., Nilan R.A., Wagner J., Faster R.J. (1965). Efficient chemical mutagenesis. The use of induced mutations in plant breeding. Radiation Botany **5,** 49-70.

Kumar S., Chaudhary B.D. (1996). Effect of doses of physical and chemical mutagens on the germination and mortality/ survivability of induced mutations in Indian mustard (*Brassica juncea* (L.) Czern and Coss). Agricultural Science Digest **16**, 17-20

Mahla S.V.S., Mor B.R., Yadava J.S. (1991). Mutagenic effectiveness and efficiency in mustard (*Brassica juncea* L. Czern and Coss). Cruciferae Newsletter **14/15**, 46-47.

Prasad R., Singh B. (1986). Mutagenic effectiveness and efficiency of gamma rays and ethyl methanesulphonate in Indian mustard. Journal of Oilseed Research **3**, 102-106.

Prem D., Gupta K., Agnihotri A. (2004). Development of an efficient high frequency microspore embryo induction and doubled haploid generation system for Indian mustard (*Brassica juncea*). In Proceedings: 4th International Crop Science Congress, Sept. 26-Oct. 1, 2004, Queensland, Australia.

Prem D., Gupta K., Agnihotri A. (2005). The effect of various exogenous and endogenous factors on microspore embryogenesis in Indian Mustard (*B. juncea*). *In Vitro* cellular and development biology – PLANT **41**, *266-273.*

Sokal R.R., Rohlf F.J. (1981). Box 17.6, Test of independence (using G-statistic)- pp 736. Biometry: The principles and practices of statistics in biological research 2nd ed. W. H. Freeman and Company, New York.