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Can we predict mutagen-induced damage in plant systems mathematically? Insights from zygotic embryo and haploid mutagenesis in Indian mustard (*Brassica juncea*)

Deepak Prem[1*], Kadambari Gupta[2] and Abha Agnihotri[3]

1 Plant Development and Nuclear Organization Unit, Centro de Investigaciones Biológicas, Biological Research Centre (CIB), C.S.I.C. Ramiro de Maeztu, 9, 28040 Madrid, Spain,

2 Topworth Group of Companies, A- 21, IInd Floor, Green Park Main, New Delhi- 110016, India.

3 Amity Institute of Microbial Technology, Amity University Uttar Pradesh, Block E-3, 4th Floor, Sector 125, Noida - 201 303 India.

ABSTRACT: This study was undertaken to determine the relationship between mutagen concentration, exposure duration and survival of zygotic embryos along with evaluation of microspore totipotency of the mutant donor plants of *Brassica juncea*. Three Indian genotypes 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 exposure to EtBr resulted in 100% mortality, however, the survival data for EMS/ ENU analyzed by orthogonal contrast partitioning ANOVA revealed that concentration for both mutagens had a linear relationship with percent survival while exposure duration had a non-linear relationship. Multiple regression analysis was used to develop prediction functions for EMS/ ENU treatment survival and the LD_{50} for zygotic embryo mutagenesis for 20h duration ranged from 3.5mM for ENU to 6.8mM for EMS. This information was used to generate mutant donor plants for microspore culture and 48.8% EMS mutant donor plants produced 2.9 ± 0.4 embryos per Petri dish (total 943 embryos).

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

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INTRODUCTION

Brassica juncea (L.) Czern. is a major oilseed *Brassica* species grown in South East Asia and has gained rapid acreage in Canada and Australia (Small 1999; Oram *et al.* 1999). Although microspore embryogenesis and doubled haploids are now considered standard breeding tools for several *Brassica* species (Ferrie & Caswell 2011), haploid mutagenesis has not been exploited for crop improvement in Indian mustard (*B. juncea*) as yet. The main two reasons for this are, a) lack of an efficient protocol for microspore embryogenesis followed by successful conversion of microspore derived embryos to plantlets and b) information regarding lethality of chemical mutagens on *B. juncea* system *per se* is scarce. In this direction, we have developed an efficient system for microspore embryogenesis and conversion of embryos to doubled haploid plants (Prem *et al.* 2005; 2008). However, for using microspore embryogenesis in conjunction with haploid mutagenesis, a meta-analysis of the present literature reveals that although chemical mutagenesis has been used for creation of genetic variation in *B. juncea*, no consensus can be drawn for the concentration and duration of mutagen exposure. Therefore, this vital information

*correspondence: deepakandrewprem@gmail.com

is missing for utilization of haploid mutagenesis in this species. The 50% lethal dose (LD_{50}) is considered the most important determinant 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 also valuable from the perspective of understanding and predicting the lethality and efficiency of induced mutations following chemical mutagenesis on *B. juncea*. In this report we present mathematical models for explaining mutagen induced lethality for *B. juncea* zygotic embryos and a novel strategy for exploiting haploid mutagenesis in *B. juncea* using mutant plants as microspore donors.

MATERIALS 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/ zygotic embryos for three *B. juncea* genotypes, namely Pusa Bold, Varuna and BIO-902. The zygotic embryo mutagenesis experiments were planned in a three replicate 3 x 5 x 4 factorial completely randomised design wherein for each mutagen, the three genotypes were tested for four mutagen concentrations (5.0, 10.0, 20.0 and 50.0 mM prepared in 7.2 pH phosphate 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 10s followed by 0.05% mercuric chloride (w/v) for 10 min and were then soaked in sterile distilled water for 16 h in the 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 (Murashige & Skoog 1962) containing 2% sucrose (w/v), gelled with 0.7 % (w/v) agar. These cultures were incubated at 25 ± 2°C 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 normal radical and plumule growth after 15 to 20 days of culture. The proportion data were transformed using arcsine transformation and subjected to three factor ANOVA (Gomez & Gomez 1984a).

The survival data from ENU and EMS treatments for zygotic embryo survival were further analysed by

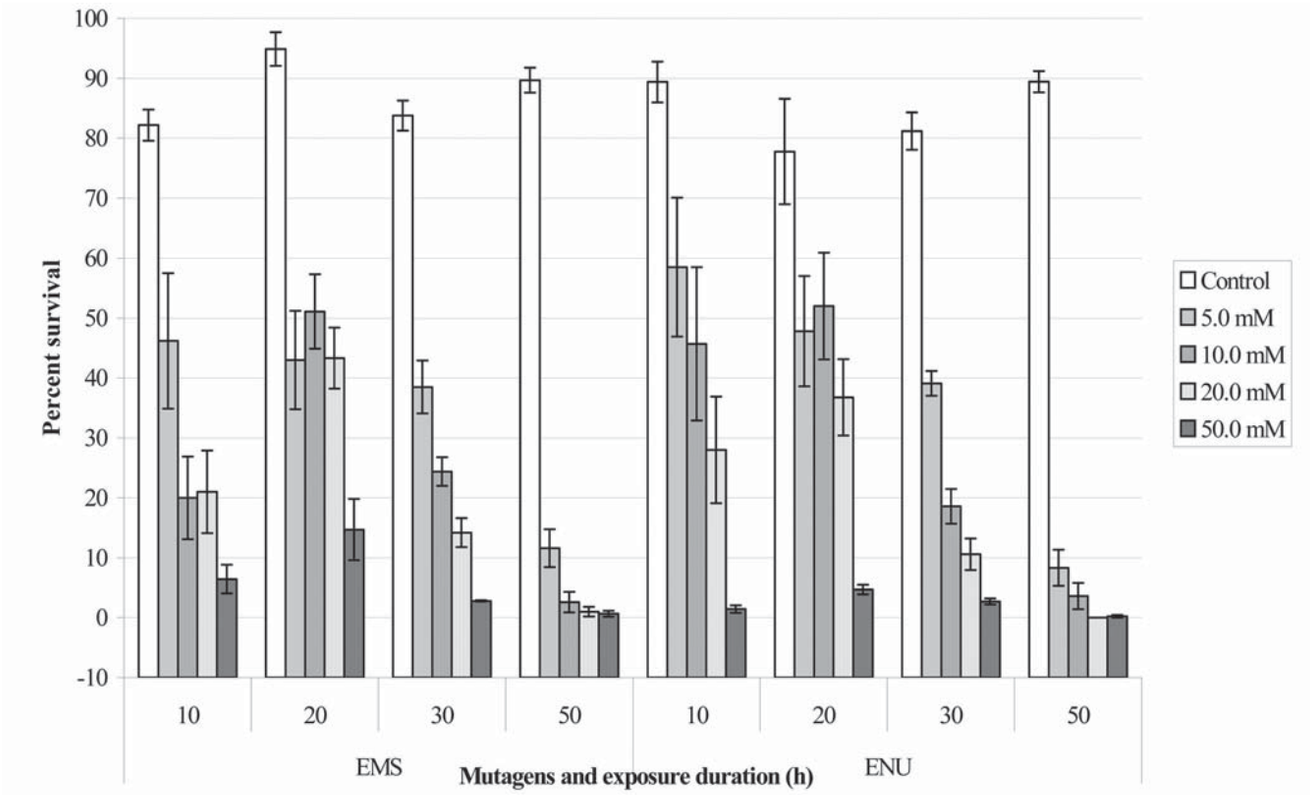

Fig. 1. The interaction effect of concentrations and exposure durations for EMS and ENU treatment on *B. juncea* zygotic embryos
Each histogram represents average survival proportion in terms of normal radical and plumule development after mutagen treatment for the three *B. juncea* genotypes (100 seeds 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

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 a = 0.05; ^{ns} non-significant

orthogonal contrast partitioning (Gomez & Gomez 1984b), for determining the relationship between the dependent (survival) and the independent variables (concentration, linear to quartic and duration, linear to cubic orthogonal polynomial). For developing mathematical models 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. CoStat statistical software (CoHort Software, 798 Lighthouse Ave. PMB 320, Monterey, CA 93940, U.S.A) was used for multiple regression analysis and the coefficients of the regression equations were tested for their statistical significance by ANOVA method (Sokal & Rohlf 1981).

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. For 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. Untreated donor plants of the three genotypes were used as controls. Microspores from these plants were cultured as per established protocol (Prem *et al.* 2008) and the microspore embryogenesis response was quantified in terms of mean number of embryos produced per Petri dish. Average genotypic response of mutant donor plants for microspore embryogenesis was compared with the two-tailed t-test assuming unequal variance (Sokal & Rohlf 1981).

RESULTS AND DISCUSSION

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 (Fig. 1). 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 (Table 1). In addition to this, the interaction effect of these two factors was also highly significant. The effect of the genotype either singly or as one of the interacting factors, was observed to be

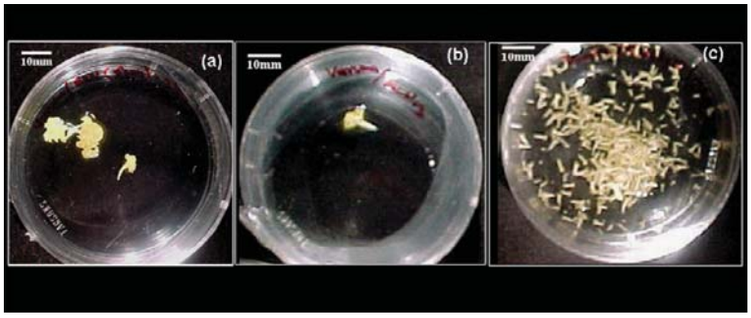

Fig. 2. Microspore embryogenesis from mutant and control donor plants of *B. juncea*
(a) Microspore embryogenesis response from EMS (5mM, 20h) treated donor plants of genotype Pusa Bold.
(b) Microspore embryogenesis response from EMS (5mM, 20h) treated donor plants of genotype BIO- 902.
(c) Microspore embryogenesis response from control non-mutant donor plants of genotype Pusa Bold
Bars indicate size reference.

a non-significant contributor to the overall variation (Table 1). The zygotic embryos of Pusa Bold treated with ENU showed 38.5 ± 4.3% (mean ± SEm) survival, irrespective of concentration and exposure duration, followed by Varuna (33.3 ± 4.2%) and BIO-902 (32.1 ± 4.5%). 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 36.9 ± 4.7% survival, irrespective of EMS concentration and exposure duration, followed by Varuna (35.4 ± 4.6%) and BIO-902 (31.7 ± 4.4%). 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 indicated that the functional relationship between these variables is rather complex since the genotype does 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 mutagen concentration and duration were observed to be similar indicating that instead of a polynomial relationship there may also be a non-linear relationship between zygotic embryo survival vis-à-vis mutagen concentration and exposure duration variables.

The results of polynomial partitioning (Table 1) indicate that the mutagen concentration had a significant linear relationship while the higher polynomial orders were non-significant contributors to the percent survival. The exposure duration, on the other hand, had a non-linear relationship with the percent survival as the polynomial order partitioning coefficients for exposure duration were non-significant contributors to the overall variation. The results of the multiple regression analysis further validated the salient findings of orthogonal contrast partitioning mentioned above (Table 2). The coefficient of determination (R^2) for the regression equations developed using dataset–I for ENU and EMS indicated that these are not suitable as prediction functions. Contrary to this, the regression equations developed from dataset–II for ENU and EMS treatments were adequate models for predicting the percent survival response for a given exposure duration and concentration of mutagens. The statistical significance of the exposure duration when log-transformed data for the percent survival were used, shows that the two variables had a linearisable non-linear relationship.

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 have been most exploited for seed mutagenesis (Prasad & Singh 1986; Mahla *et al.* 1991; Jha & Sinha 1992; Kumar & Chaudhary 1996; Bhatt *et al.* 2001). However, these concentrations and exposure duration range are 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 and contrary to our findings, reports cited above highlight genotypic specificity for mutagen action. The present study therefore elaborates the extent of lethality resulting from varying the concentration and exposure duration for two chemical mutagens on the zygotic embryos of *B. juncea*. The empirical relationship presented by us explains the lethality of induced mutations, though these do not indicate directly the efficiency of inducing useful macro or micro mutations for crop improvement in this species.

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 average 2.9 ± 0.4 embryos per Petri dish (total 943 embryos) irrespective of the genotype. 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 individual mutant donor plants were found to be equally responsive for microspore embryogenesis (data not presented). Genotype comparison revealed that mutant donor plants of the

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 a= 0.05; ns non-significant

three genotypes had similar microspore embryogenesis, wherein Pusa Bold produced the maximum microspore-derived embryos (504) with an average production of 3.4 ± 0.3 embryos produced per Petri dish followed by BIO-902 with a total of 312 embryos produced with an average of 3.5 ± 0.3 embryos per Petri dish and Varuna with a total of 127 embryos produced with an average of 1.7 ± 0.2 embryos per Petri dish (Fig 2 a, b). However, the embryogenesis response from mutant donor plants was significantly lower than the untreated control donor plants (251.0±2.5 embryos/Petri dish, Fig. 2c). The non-totipotent response of microspores from ENU mutant donor plants cannot be conclusively explained. A possible explanation for this may be haplontic selection that results in survival of only 'fit' microspores that do not have lethal macromutations (Van Harten 1998). In our case the ENU treatment perhaps resulted in non-viable pollens in the M_1 plants owing to haplontic selection. However, we have not tested this hypothesis experimentally and its predominance in ENU-treated plants is intriguing. A similar non-totipotent response for microspores obtained from some of the EMS donor plants is also not explainable as of now. 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 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 to the best of our knowledge.

CONCLUSION

The regression models developed in the present study for prediction of survival following chemical mutagenesis should be 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 mutant embryos produced from the haploid embryogenesis strategy presented in this paper are being tested for conversion to plantlets and subsequent field evaluation of the generated variability.

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REZIME

Može li se oštećenje mutagenima kod biljaka predvideti matematički? Slučaj zigotičnog embriona i haploidne mutageneze kod slačice (*Brassica juncea*)

Deepak Prem, Kadambari Gupta i Abha Agnihotri

U ovom radu analiziran je odnos koncentracije mutagena, vremena ekspozicije i preživljavanja zigotičnog embriona putem procene totipotentnosti mikrospore mutanta donora biljke *Brassica juncea.* Tri genotopa slačice su ispitivani u uslovima različitih koncentracija mutagena (5-50 mM) i različitih vremena ekspozicije u njima (10-50 h). Kao mutageni su korišćeni su etil-metan-sulfonat (EMS), etil-nitrozourea (ENU) i etidijum bromide (EtBr). Izlaganje EtBr uzrokuje 100% smrtnost, dok je preživljavanje u druga dva mutagena EMS/ENU omogućilo analizu ortogonalnim kontrastom u ANOVA. Oba mutagena imaju linearnu zavisnost sa procentom preživljavanja dok je vreme ekspozicije pokazalo nelinearan odnos. Multipla regresiona analiza je korišćena za razvoj funkcije predikcije za preživljavanje u tretmanima sa EMS/ENU, a LD_{50} (50% preživljavanja) za mutagenezu zigotičnog embriona tokom 20 h u koncentracijama 3.5mM za ENU do 6.8mM za EMS. Ovako dobijeni podaci korišćeni su za odredjivanje biljke donora mutanta za culture mikrospora. 48.8% EMS biljke donora mutanta produkovalo je 2.9 ± 0.4 embriona po Petri kutiji (total 943 embriona).

Ključne reči: slačica, *Brassica juncea*, LD_{50}, multipla regresija, haploididna mutageneza