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In vitro MUTAGEGENESIS IN OIL SEED BRASSICA

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ABSTRACT: Two Concentrations of EMS mutagens were used to induce the genetic variations in microspore culture of two genotypes of oilseed rape (*Brassica napus*). Embryos derived from mutated microspores were placed in liquid medium NLN. Within a few days only surviving embryos were transferred to solid medium B5 for further development. The effects of various core of EMS concentrations on the production and survival of embryos and plants in both genotypes has been discussed and it was concluded that the lower concentration had the desirable mutagenic effects for a practical mutation breeding programme of genetic improvement of oilseed brassicas.

INTRODUCTION: Induced mutations are well-established methodologies for enhancement of genetic variability in field crops [8]. Since the recovery of recessive mutants through classical breeding methods is difficult in higher plants because of diploid nature of sprophyte tissue. Mutagenesis of haploids seems very attractive alternative method. Microspore (immature haploid pollen grains) mutagenesis coupled with in vitro selection can provide an extremely powerful system for modifying and selecting of the traits in many economic important crops, such as oilseed brassica, because this single cell culture system exhibits rapid embryo develop-ment and allows easy manipulation of extremely large population of individuals with minimum labor or cost [4,6,10,13,14] without difficulty, several times per week, hundred million microspores can be isolated, and mutagenized through physical as well as chemical mutagens [1, 2,7,9,11,12,15,]. In this studies, in-vitro mutagenesis through isolated microspore culture in oilseed brassica (Brassica napus L.) for the development of doubly haploid mutant plants is discussed and found that the in vitro mutagensis at the single cell level has a very promising potential for the rapid genetic gain percycle within a very short period of time for the sustainable breeding of the brassica oilseed in Pakistan.

MATERIAL AND METHODS

Donor Plants: Two stable strains of Brassica napus LLA-1 and LLA-2 with high oleic acid were used as donor plants for these studies.

Donor Plant Conditions: Seeds were planted in 6 pots tilled with Redi-earth soil mix. Two seeds per pot were planted and thinned when the plants are at the 2-3 leaf stage. Slow-release fertilizer was added to each pot. These pots were put in a growth chamber at 20/15 °C. 16 hr photoperiod.

Microspore Culture:

A) Selection and sterilization: Approximately 50-75 buds were selected based on size (usually 2-3 mm). These buds were placed Lipshaw baskets and surface sterilized in 6% sodium hypochlorite for 15 minutes on a shaker followed by three to five minute washes with sterile water.

B) Isolation of microspores: The buds were macerated in 5ml of half strength B5-13 medium (B5 medium supplemented with 13% sucrose) (Gamborg 1968) with a glass rod then filtered through 44 uM nylon screen cloth into a 50 ml tube. The filter and breaker was rinsed three times with 5 ml of half strength B5-13 for a total of 20 ml. The crude microspore suspension was centrifuged at 130-150 g for 3 minutes, the supernatant was decanted and 5 ml B5-13 was added to the pellet. This procedure was repeated.

C) Mutagen treatment: The supernatant was removed and the required amount of NLN-17 and ethylemethanesulphonate (EMS) was added to the centrifuge tube. Two concentrations of 0.25 and 0.5% of EMS with a control treatment (0% EMS) were applied. The microspores in EMS and NLN-17 were incubated at room temperature for 1.5 hours. They were gently mixed periodically. After incubation, the microspores were centrifuged (3 min, 130-150 g).The supernatant was discarded appropriately and the microspores were washed 3 times with half strength B5-13

D. Culture media: The number of microspores was determined using a hemacytometer, and then the required amount of modified Lichter (1982) medium was added to achieve a density of 10 microspores ml. This media (NLN) was supplemented with 17% sucrose, 0.83 mg/l potassium iodide, and 0.1% benzyladenine, but without potato extract and adjusted to pH 5.8. Ten mililitre of microspore suspension was dispensed into 100 x 15 mm sterile petri plates. The plates were cultured at 32°C in the dark.

E. Media change: The plates were removed from the 32°C incubator after 48 hours. The media was removed from the plates by pipette and put into a 50 ml centrifuge tube. The tubes were centrifuged as in the microspore culture protocol (3 min, 130-150 g). The supernatant was removed and the same amount of NLN-10 media was added. The plates were resealed and put into a 25.4°C incubator for the remainder of three weeks. Formation of embryos visible to the naked eye was started taking place about ten days after induction.

F) Chromosome doubling: Microspores were cultured as outlined above. NLN-17 media with colchicine (10M) was substituted for regular NLN-17 culture initiation media. After 48 hours, media was changed from NLN-17 with colchicine to NLN-10 without colchicine.

G) Embryo Culture: Embryos were counted three weeks after induction. They were then placed on a rotatory shaker in a tissue culture room (22°C, 16 h light). After a week on the shaker the embryos were plated on 60 mm x 15 mm petri plates containing 0-B5 media (1% agar, 1% sucrose, pH 5.8).

H) Plantlet Culture: Plantlets developed after three weeks. The normal plantlets were transferred to 150 mm x 25 mm petri plates containing solid media (0-B5 with 0.8% agar, l2% sucrose, pH 5.8). These were placed in a tissue culture room, 12hr photoperiod, 22°C for three weeks

I) Plantlet transfer to Soil: The plantlets were put in flats containing a soil-less mix. The flats were maintained in the walk-in

growth cabinet (20/15°C, 12 hr photoperiod) on the bottom shelf with reduced light for two weeks. After two weeks, the flats were raised to the shelf, with increased light. After another week, the lids were tipped for two days and then removed for the remainder of the week.

RESULTS AND DISCUSSION

The microspore mutagenesis and selection system can produce a plant population with genetically fixed novel traits [1,2,7,9,11,12,15]. The advantage of a large-scale microspore selection system over traditional somatic tissue selection systems is evident. This technique involves applying mutagenic treatment of powerful chemical muta-genethylmethanesulphonate (EMS), to embryogenic microspores. Embryos developing from mutated microspores can be regenerated into plants. The chemical mutagen can be incorporated directly into the culture medium and potential and the efficiency of the mutagen concentrations can be analyzed very easily. Several oilseed brassicas cultivars with economic importance characteristics such as resistant to herbicides: imidazolines, chlorsulfuron and glyphosate have been developed using in vitro mutagenesis [7,1,6].

This in vitro mutagenesis system has been investigated on microspores treated with EMS immediately after isolation. Reduction of embryogenesis at the 50% rate, (LD50) for microspores of line # LLA-1 after EMS treatment is achieved with 0.25% (Fig. 1). But to obtain higher mutation ratio in this experiment the microspores were subjected to EMS 0.5% also.

In the case of genotype # LLA-2 the same concentrations (0.25 & 0.5 %) of the EMS were examined. The concentration 0.25% gave just a little more than 50% reduction of microspore embryogenesis (Fig. 2). Young embryos seemed inhibited by higher concentration. The survival of the embryos was observed on B5 semisolid medium. The higher concentrations of EMS in both genotypes sharply reduced the embryo survival Figure 3.

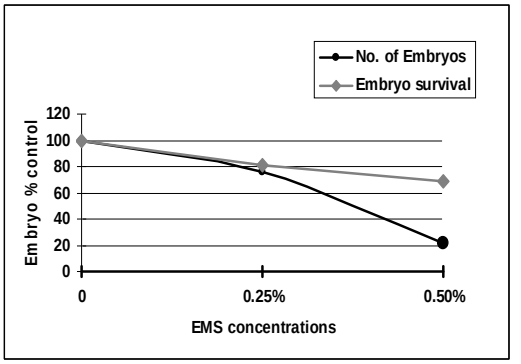


Figure – 1: Embryos production and survival following mutagenic treatment of microspore line # LLA-2 of *B. napus* with EMS

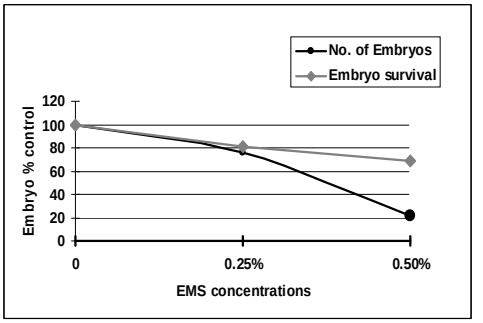


Figure – 2: Embryos production and survival following mutagenic treatment of microspore of line # LLA-2 of *B. napus* with EMS

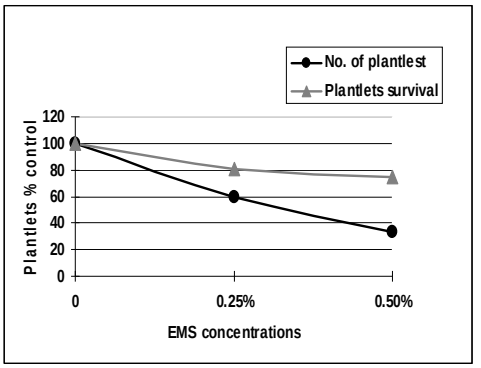
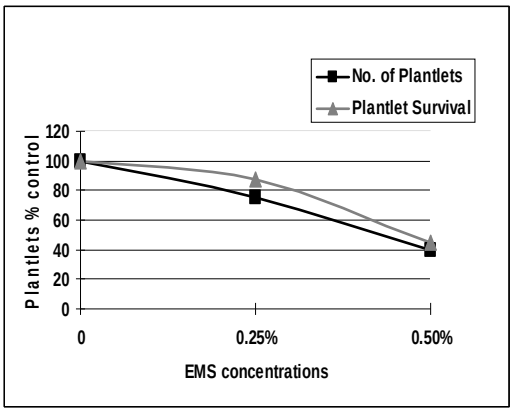


Fig. – 3: Plant regeneration and survival following mutagenic treatment of microspore of line # LLA-1 of *B. napus* with EMS

The results obtained in under these studies agreed with that of Ahmad, Swanson, Ferrie and Turner [1,7,9,12]. Plants were regenerated from the survived mutant embryos, which were subcultured once or twice until normal leaves and roots were formed. The higher concentration of EMS (0.5%) signifycantly in both genotypes decreased the regeneration of the young plants (77 & 60%). The lower concentration of 0.25% also produced 40 and 25 % less plants as compared with the untreated experiment (Fig. 3 & 4). The regenerated plants survived on solid B5 medium with similar pattern (Fig.3 & 4).

The results of the study indicate that embryos at *in vitro* stage and the progeny of plants developed from survived embryos demonstrated different level of sensitivity to the concentrations of chemical mutagen EMS. The variation in embryo production and in plant regeneration with the application of physical and chemical mutagens such as UV, /*gamma rays and MNU in microspore culture of oilseed brassicas is also reported by other researchers [1,2,7,9,11,12].



Figur –4: Plant regeneration and survival following mutagenic treatmentof microspore of line # LLA-2 of *B. napus* with EMS

The system in vitro mutagenesis on microspore-embryos level is very convenient for generating and discovering a novel genetic variation in *Brassica napus* L. especially when microspores from stable and homozygous donor plants were used. Then it can supposed, that every novel trait of doubled haploid mutant lines regenerated from treated microspores will be mutagenic change.

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