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Engvild, K.C.

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Mutagenesis of the Model Grass *Brachypodium Distachyon* with Sodium Azide

Kjeld C. Engvild

Risø National Laboratory
Roskilde
Denmark
March 2005

Risø-R Report

Author: Kjeld C. Engvild
Title: Mutagenesis of the Model Grass *Brachypodium distachyon* with Sodium Azide
Department: Biosystems Department

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Abstract (max. 2000 char.):

The model plant *Brachypodium distachyon* (L) Beauv. can be difficult to mutate by chemical mutagens. Three diploid (2n=10) accessions of this model of cereals and temperate grasses varied widely in their sensitivity to mutagenesis by acidified sodium azide and ethyl methanesulfonate (EMS). Azide was an effective mutagen at high doses. EMS was ineffective. One accession, BDR018, showed essentially no chlorophyll mutants in the M_2 generation; another, BDR001, gave only albinos, while BDR037 showed the common types of chlorophyll mutants of whites, yellows, light greens and striped in the M_2 generation. The highest rate was chlorophyll mutants in 45 per cent of the progenies of the M_1 plants, corresponding to 5 percent of the M_2 plants.

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Risø National Laboratory
Information Service Department
P.O.Box 49
DK-4000 Roskilde
Denmark
Telephone +45 46774004
bibl@risoe.dk
Fax +45 46774013
www.risoe.dk

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Introduction

The grass *Brachypodium distachyon* (L.) Beauv, purple false brome, has been proposed as a model plant for forage grasses and cereals (Draper et al., 2001; Vogel, 2003, Hasterok et al., 2004; Mur et al., 2004; Routledge et al., 2004; Christiansen et al., 2005, Garvin and Stockinger, 2005). Diploid *Brachypodium distachyon* has a genome size of about 150 Mega base pairs. This is comparable to the genome size of Arabidopsis and about one third of the size of the rice genome. The plant is a self-fertile, 20-50 cm tall, mediterranean weed with a generation cycle of 11-18 weeks, depending on the requirement for vernalization. Diploid, tetraploid and hexaploid types are found (Hasterok et al., 2004). The seed weight is 2-5 mg. Atrazine-resistant biotypes of *B. distachyon* covered roadsides in Israel for several years, but have now disappeared (Gressel and Kleifeld, 1994).

The mutagen of choice in many cultivated plants is HN_3, formed from sodium azide buffered at pH 3 (Kleinhofs et al., 1978; Awan et al., 1980). Azide is only marginally mutagenic in humans and animals (Sadiq and Owais, 2000) and Arabidopsis (Gichner and Veleminsky, 1977); it is therefore quite safe to use. Ethyl methanesulfonate has been a standard mutagen in many organisms for many years (Sega, 1984). With the final aim of finding a reproducible protocol for inducing mutants in *Brachypodium distachyon* for markers, mapping and tilling (Henicoff and Comai, 2003) the present preliminary work was undertaken to see if standard barley and rice treatment methods (Awan et al., 1980; Manual on Mutation Breeding, 1977; Vizir et al., 1996) were applicable to *Brachypodium distachyon* where mutagenesis so far has been attempted by irradiation (Draper et al., 2001).

Material and Methods

Three accessions of *Brachypodium distachyon* (L.) Beauv. were used in the present work. BDR001 (= ***B****rachypodium* ***d****istachyon* **R**isø 001 = PI 170218), diploid (2n=10, Christiansen et al., 2005) from Turkey, requiring 6 weeks of vernalization, BDR018 = PI 245730, diploid (2n=10, Christiansen et al., 2005) from Turkey, responsive to 2 weeks of vernalization, and BDR037, diploid (2n=10, Christiansen et al., unpubl.) from Israel, no vernalization requirement. The protocol described is the most successful, used for BDR037. The treatments for BDR001 and BDR018 are mentioned briefly in the tables.

Sodium azide treatment (CAS 26628-22-8, from Sigma): 2 g of seed of *Brachypodium distachyon* (L) Beauv., BDR037 were pre-soaked in a few ml tap water for 2 hours. Portions of 75 seeds were then suspended in 50 ml solutions of 1, 3, 10 and 30 mMol l^{-1} sodium azide in 0.1 Mol l^{-1} KH_2PO_4 buffer adjusted to pH 3 with phosphoric acid for 2 hours in a fume hood with intermittent shaking. The seeds were rinsed by decantation in 4 changes of tap water for one hour. The mutagenic solutions were inactivated by neutralization with sodium carbonate.

Ethyl methanesulfonate treatment (EMS, CAS 62-50-0, from Sigma): 2 g of seed of BDR037 were pre-soaked in a few ml of tap water for 2 hours. Portions of 75 seeds were treated in 10 ml solutions of 30, 100, and 300 mMol l^{-1} EMS for two hours in a fume hood with intermittent shaking. The seeds were rinsed by decantation for 1 hour in 5 changes of tap water. The EMS solutions was inactivated in the hood by an excess of ammonia overnight.

The wet seeds were sown with forceps in a Pindstrup Nr. 2 gardeners potting soil in small peat "jiffy-pots" in plastic trays, 25 x 60 cm, 60 seeds per tray and placed in a standard greenhouse, in winter kept at 18°C and 16 hours of supplementary light. Flowering started in 5 weeks; the plants were allowed to dry out after 11 weeks. After 13 weeks the straws of individual plants were harvested in separate envelopes. Un-threshed spikes of individual tillers were sown in soil, covered with sand and scored for chlorophyll mutations "viridis = light green", "xantha = yellow", "albina = white" and "other" which included striped and combination types.

The accessions BDR018 and BDR001 were vernalized for 2 and 6 weeks respectively, two-three weeks after germination, at 5 °C, 8 hours of low intensity fluorescent light.

Results and Discussion

The results on mutant induction (Vizir et al., 1996) in BDR037 at different concentrations of azide and EMS are shown in table 1. The number of mutants after azide treatment varied from a few per cent of the M_1 plant progenies to 45 per cent of the progenies at the 10 mMol l^{-1} concentration. Only 3 mutants were recovered from all of the EMS treatments. There was only slight variation in seed germination in the azide treatments, but the germination was reduced at the highest EMS concentration. In some experiments in the summer on M_2 seeds there were difficulties with low or very slow germination when scoring for mutations; this was perhaps caused by heat induced dormancy analogous to water sensitivity in barley.

Table 1. Chlorophyll mutants observed after 2 hours of mutagenesis of Brachypodium distachyon *BDR037. Number of M_1 plants 51-58. Number of M_2 plants about 1000.*

Azide Concentration	Mutants, % of M_1 progenies	Mutants, % of M_2 plants
1 mMol l^{-1}	17	2.4
3 mMol l^{-1}	34	3.1
10 mMol l^{-1}	45	5.0
30 mMol l^{-1}	17	3.1
EMS concentration		
30 mMol l^{-1}	0	0
100 mMol l^{-1}	0	0
300 mMol l^{-1}	6	0.4

An experiment with mutagenesis of BDR001 with azide gave 16 mutants among 381 M_2 progeny; all were albinos (table 2). Another experiment with mutagenesis of BDR018

Table 2. Chlorophyll mutants, all albino, observed after mutagenesis of Brachypodium distachyon *BDR001. Treatment: seeds pre-soaked overnight at 7 ^{o}C. Number of M_1 plants 19 and 17. Number of M_2 plants 281 and 100.*

Hours in 1 mMol l^{-1} azide	Mutants, % of M_1 progenies	Mutants, % of M_2 plants
2	16	3.6
4	34	6.0

with azide and EMS yielded only one albino after azide among 7300 M_2 plants (table 3). The results show that standard azide and EMS mutagenesis protocols cannot always be expected to work in *B. distachyon*. Some accessions may not be responsive at all. High mutagen doses may be necessary. Preferably wide concentration ranges should be tested. The wide variation in the response of different *B. distachyon* makes preliminary experimentation desirable when mutagenesis of unknown types is attempted. Such preliminary experimentation has always been recommended (Manual on Mutation Breeding, 1977) but the differences in *Brachypodium* are much larger than those usually observed in barley, rice, and other diploid inbreeding crops.

Table 3. One albino chlorophyll mutant observed after mutagenesis of Brachypodium distachyon *BDR018. Treatment: seeds pre-soaked overnight at 7 ^{o}C; azide treatment 3 hours; EMS treatment 1 hour. Number of M_1 plants 41-57. Number of M_2 plants about 1000.*

Azide Concentration	Mutants, % of M_1 progenies	Mutants, % of M_2 plants
0.5 mMol l^{-1}	0	0
1 mMol l^{-1}	1.8	0.1
1.5 mMol l^{-1}	0	0
EMS concentration		
25 mMol l^{-1}	0	0
50 mMol l^{-1}	0	0
100 mMol l^{-1}	0	0

Germination tests alone are not always sufficient. In the BDR001 type seed dormancy interfered with germination viability tests. In the BDR037 there was reduced viability at 300 mMol l^{-1} EMS, but the number of mutants obtained was quite low.

EMS has not been an effective mutagen in B. *distachyon*. To my knowledge this has no parallel in plants. EMS mutagenesis in rice seed sometimes requires special measures such as dehulling, high pressure or well controlled pre-soaking, but EMS mutagenesis is possible (Sega, 1984). The general outcome of the azide mutagenesis is comparable to the results obtained with barley and rice (Kleinhofs et al., 1978; Awan et al., 1980; Manual on Mutation Breeding, 1977) but 5-10 times higher concentrations give the highest mutation rate. Plants of small nuclear volume and genome size generally have high radiation resistance (Sparrow et al., 1963). A similar generalisation seems not to have been done with chemical mutagens. Even so it is perhaps not surprising that *B. distachyon* with a genome several magnitudes smaller than that of barley requires higher mutagen doses. What is surprising is the large difference in the reaction of different accessions of *B. distachyon*. They must be very different genetically, although they are all diploid (2n=10) as shown by flow cytometry and chromosome counts (Christiansen et al., 2005 and unpubl.). This is consistent with the large genetic differences between diploid and hexaploid *B. distachyon* (Hasterok et al., 2004) where the hexaploid seems to be an allopolyploid and therefore a result of a hybridization between two "different" species.

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