

Ion beam irradiation mutagenesis in rye (*Secale cereale* L.), linseed (*Linum usitatissimum* L.) and faba bean (*Vicia faba* L.)

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Ion beam irradiation is a potential tool for inducing novel mutations in plants. We chose three crop species (rye, linseed, and faba bean) to determine the potential of nitrogen ion beam irradiation for inducing mutations. We tested ion beam irradiation with nitrogen ions at six different fluencies (5×10^5 , 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 , and 1×10^8 N-ion cm^{-2}) on dry grains. The three studied crop species had different sensitivities to the irradiation. Increased doses of ion irradiation had more effect on survival than on germination. Rye seedlings had the lowest survival rate at high doses of irradiation and significantly higher off-type plant phenotypes than the other two species. In M_1 seedlings, stunted growth, failure to complete the plant life cycle and chlorophyll mutants were observed in all three species. Terminal-inflorescence mutations and sectional chimeras in faba bean were observed in the M_2 generation. We conclude that ion beam irradiation is an effective tool for mutation breeding of diverse crop species when the appropriate dose is defined.

Key words: irradiation, mutation, ion beam, survival rate, field crops

Introduction

Plant breeding depends on the existence of variation in useful traits, and where the amount of natural variation is not adequate in available germplasm, novel mutations may be induced by irradiation or chemical mutagenesis. Favourable phenotypes are then selected for subsequent breeding objectives, during which the desirable characteristics are generally stabilized in subsequent generations. Physical and chemical mutagens have demonstrated their usefulness in plant mutation induction (Oladosu et al. 2016). The favoured forms of irradiation for mutation have been fast neutron bombardment (Le et al. 2001), X-, and gamma-ray irradiation (Beyaz and Yildiz 2017). Key problems with mutagenesis include the shortage of physical and chemical mutagen sources, especially in Finland, and the low viability of seeds or other plant parts after neutron and gamma-ray irradiation. A further problem is that suitable radiation sources are becoming less readily available to plant breeders and replacements need to be found. Chemical mutagens such as ethyl methane sulphonate (EMS) are considered objectionable from an occupational health viewpoint, environmental issues and post treatment handling (Neel 1970, Predieri and Di Virgilio 2007, Oladosu et al. 2016).

During the last two decades, the use of ion beam irradiation has emerged as an effective and unique technique for inducing mutations in plants (Tanaka et al. 2010). Ion beams provide high linear energy transfer (LET) compared to low LET radiation such as gamma-ray and X-ray, which simply means applying a large amount of energy in a small area, when aligned along the direction of the beam. The higher LET radiation has greater biological effects on plants than lower LET radiation. The remarkable advantages of ion beams for mutation plant breeding over the other mutagens include high mutation rates, high survival rates, wide phenotypic variation, and targeted-trait specificity (Tanaka et al. 2010, Abe et al. 2012). The application of ion beams in plant mutation breeding was introduced in the 1990s in Japan. Ion beam irradiation has since been used on several plant species mainly in Japan (reviewed in Abe et al. 2012, 2015) and China (reviewed in Dong et al. 2016). The effectiveness of ion beam mutation has been studied in several food crops (e.g. Maekawa et al. 2003, Yamaguchi et al. 2009a, Ishikawa et al. 2012), ornamentals (e.g. Yamaguchi et al. 2003, Yamaguchi et al. 2009b, Yamaguchi 2018), and *Arabidopsis thaliana* as a model plant species (e.g. Hirano et al. 2015, Du et al. 2018). The method has not been tested on arable crops grown in the cool-temperate zone.

To be effective, the ion beam has to penetrate the seed coat and reach meristematic tissue in the embryo. Seed anatomy is important in this regard. In the small-grain cereals such as rye (*Secale cereale* L.), the embryo is presented at the proximal end of the grain, and is easily irradiated through the outer layers of the caryopsis. In dicotyledonous crops, the embryo is partly protected by the cotyledons as well as the seed coat. In linseed (*Linum usitatissimum* L.), an example oilseed, the seeds are small and the seed coat is relatively thin, whereas in faba bean (*Vicia faba* L.), an example grain legume, the dimensions are much larger.

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Hence we chose these three species as suitable test cases in a study to determine the potential of nitrogen ion beam irradiation for inducing mutation in crop species that had not yet been investigated with this mutation technique.

Materials and methods

Seed was obtained of spring rye (cv. Juuso), linseed (cv. Helmi), and faba bean (cv. Hedin/2). The germination rate of all three batches was about 98%.

Dry seeds were carefully placed into plastic petri dishes (35 mm \varnothing \times 10 mm, Fig. S1) using forceps and held in place with Blu Tack mounting putty (Bostik Smart Adhesives, <https://www.bostik.com/>) with the embryo on the top surface, presented to the ion beam, ensuring high and uniform irradiation. Irradiation was conducted at the JYFL cyclotron beam facility (Department of Physics, University of Jyväskylä, Finland) and the chosen ions were $^{14}\text{N}^{6+}$ (Gorelick et al. 2007). For the range of irradiation, fluencies were 5×10^5 , 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 , and 1×10^8 ions cm^{-2} accelerated to 310 MeV. The controls for the three studied species (non-irradiated seeds) were prepared the same way as other treatments. The window of the instrument was 40 mm in diameter, limiting the number of seeds that could be irradiated. This was not a problem for the small seeds of both rye and linseed, but it was for faba bean since its seeds are larger than those of rye and linseed, so several dishes had to be prepared. The sample size was 100 seeds of rye and linseed and 50 of faba bean. Each dose was replicated three times for all three species used in this study, giving seven treatments \times three replicates \times three species.

After irradiation, seedlings were transplanted into a standard peat-soil mixture (White 420 W; Kekkilä Oy, Vantaa, Finland) in plastic 1020 Trays (54 \times 28 \times 6 cm), and grown in a climate-controlled glasshouse of the Department of Agricultural Sciences, University of Helsinki, Finland. The photoperiod was set to 16h day / 8h night, and the temperature was maintained at 21 °C day / 18 °C night. The minimum photosynthetic photon flux density was 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The relative humidity was maintained at 60%. Survival rate (%) was calculated as the number of seedlings from the irradiated seeds divided by number of seedlings from the non-irradiated control.

M_1 plants were scored for visible phenotypic variations (mutants) from control plants and after 6 weeks each individual mutant was transferred into a 1 l plastic pot filled with the same peat-soil medium for secure seed harvest. Only faba bean plants (treatments 5×10^7 ions cm^{-2} and 1×10^8 ions cm^{-2}) were taken to the M_2 generation. Two-hundred faba bean M_2 plants were grown in individual 4 l pots in insect-proof cages, and the frequency of chlorophyll deficient and terminal-inflorescence mutants were recorded.

Results and discussion

The germination rate was not affected by irradiation at the doses tested for faba bean and linseed, but rye grains exposed to the highest dose of ion irradiation (1×10^8 ions cm^{-2}) had 17% poorer germination (Table 1). The germination rate of linseed in all treatments including control was lower than initially, which might be due to physical damage of the seeds by the forceps. Similarly, in soybean (*Glycine max* [L.] Merr., Im et al. 2017) and tobacco (*Nicotiana tabacum* L., Kazama et al. 2008), germination rate remained unchanged when the irradiation dose on dry seeds was increased. In rice (*Oryza sativa* L., Maekawa et al. 2003) and einkorn wheat (*Triticum monococcum* L., Murai et al. 2013), with similar grain shapes to rye, the rate of germination dropped when the irradiation doses were increased.

Table 1. Germination rate and frequencies of mutant plants at M1 on the three studied crop species

N ion dose (cm^{-2})	Germination rate (%)			Number of abnormal plants		
	rye	linseed	faba bean	rye	linseed	faba bean
Control (0)	100	85.0	100	0	0	0
5×10^5	100	79.0	100	1	0	0
1×10^6	100	76.7	100	5	0	0
5×10^6	100	76.3	100	10	0	0
1×10^7	100	85.7	100	25	0	0
5×10^7	95.3	79.3	100	192	15	21
1×10^8	82.7	75.7	100	169	35	34

Number of seeds sown were: 300 rye, 300 linseed and 150 faba bean for each treatment (over three replicates)

The highest doses of irradiation ($5 \times 10^7 \text{ cm}^{-2}$ and $1 \times 10^8 \text{ ions cm}^{-2}$) induced more mutant phenotypes in linseed and faba bean M_1 seedlings, although the same trend followed at lower dose rates in rye with considerably more off-type phenotypes (Table 1). In all three species the survival rates significantly decreased at $5 \times 10^7 \text{ ions cm}^{-2}$ dose of irradiation (Fig. 1), as noted in several other crop species (see Abe et al. 2012). Rye seedlings had the lowest and linseed seedlings had the highest survival rates (Fig. 1). The higher doses of ion irradiation also resulted in slower growth of all three crops particularly on faba beans (Figs. 2A, B and C). The slow growth rate of faba beans at doses of $5 \times 10^7 \text{ cm}^{-2}$ and $1 \times 10^8 \text{ ions cm}^{-2}$ most likely is due to radiation injury on seeds in the M_1 generation. For example, in mung bean (*Vigna radiata* Wilcz.), seedling elongation / growth rate was negatively correlated with irradiation dose (Grasso et al. 2016).

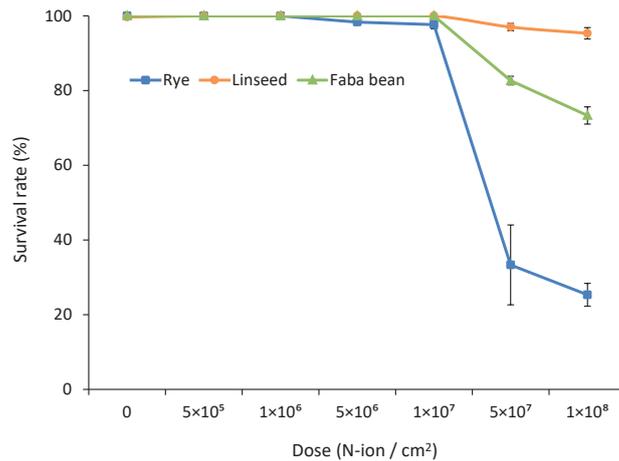


Fig. 1. Effect of ion beam irradiation on the survival rate of rye, linseed and faba bean. The error bars show ± 1 standard error of the mean.

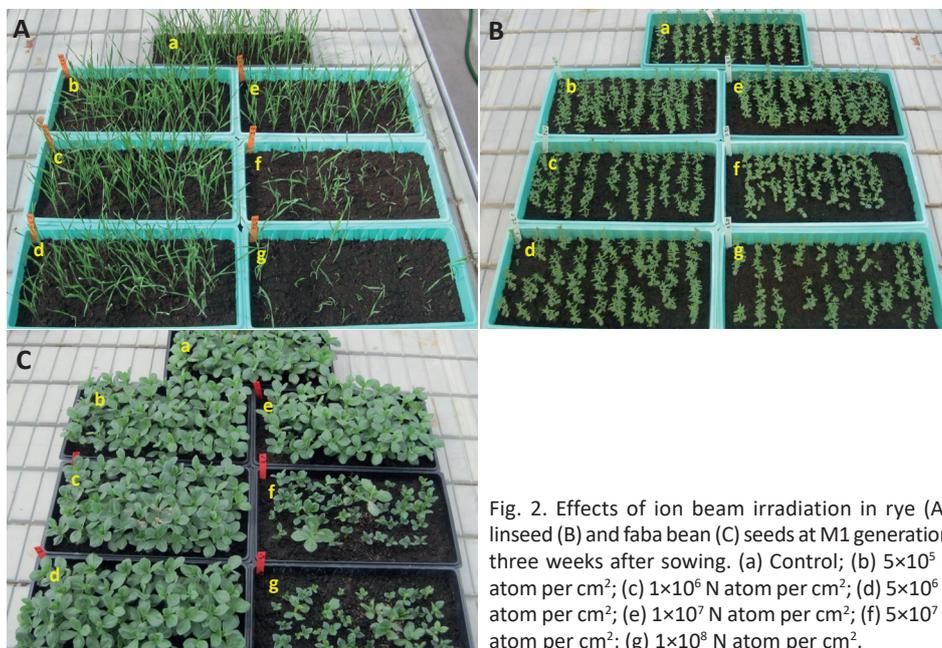


Fig. 2. Effects of ion beam irradiation in rye (A), linseed (B) and faba bean (C) seeds at M_1 generation, three weeks after sowing. (a) Control; (b) $5 \times 10^5 \text{ N atom per cm}^2$; (c) $1 \times 10^6 \text{ N atom per cm}^2$; (d) $5 \times 10^6 \text{ N atom per cm}^2$; (e) $1 \times 10^7 \text{ N atom per cm}^2$; (f) $5 \times 10^7 \text{ N atom per cm}^2$; (g) $1 \times 10^8 \text{ N atom per cm}^2$.

A wide range of abnormal phenotypes was observed in M_1 plants (Fig. 3A–N). In general, aberrant phenotypes observed in M_1 generation can be due to a dominant mutation, a homozygous recessive mutation, or non-genetic radiation injury. In most mutagenesis, visible phenotypes in the M_1 generation are attributed to dominant mutations (see Abe et al. 2012). Some of the present mutations, however, were more typical of homozygous recessive changes. For example, the radiation-induced terminal-inflorescence mutation in faba bean (Fig. 3J) is generally controlled by a single recessive gene (Sjödén 1971, Benlloch et al. 2015). Ion beam irradiation has been shown to induce homozygous recessive mutations that can be detected already in the M_1 generation (Abe et al. 2012, Lee et al. 2015, Du et al. 2017).



Fig. 3. Variation in phenotypes of M_1 and M_2 seedlings produced by ion beam irradiation in studied species. (A) albino plant of rye; (B) curly leaf in rye; (C) coleoptile mutant in rye; (D) first leaf stunt growth in rye; (E) control (left) vs mutant (right), naked spike in rye; (F) mutant spike of rye; (G) rusty leaves in faba bean; (H) sectional chimera leaf in faba bean at M_2 ; (I) Maculate leaflet in faba bean at M_2 ; (J) terminal inflorescence mutation in faba bean which was observed at M_1 and M_2 ; (K) multi-branching linseed seedling; (L) foliage leaves showing early damage in linseed; (M) early damage in linseed; (N) pollen diversity in linseed; bar = 1 cm

The molecular nature of mutations induced by ion beam mutagenesis has been extensively studied in mammals, where it generally has produced double-stranded DNA breaks. In Arabidopsis, it has been shown that ion beams led to clustered DNA damage in the chromosomes, and that they have great potential to induce complicated intra-chromosomal reshuffles (Shikazono et al. 2005, Hirano et al. 2015). The point-like mutations and intergenic rearrangements such as deletions and insertions are reported as being more frequent in mutants induced by ion beams than in those induced by ionizing radiations such as gamma rays (Abe et al. 2012, Yamaguchi 2018).

In this study faba bean only was taken to the M_2 generation. Sectional chimera (Fig. 3H), maculate leaf shape (Fig. 3I) and terminal-inflorescence (Fig. 3J) phenotypes were observed in these seedlings. The chlorophyll-deficient mutants (albino and sectional chimera) had the highest frequency (4%), following by terminal-inflorescence phenotype (2%). Considering the small sample size in this study, the above numbers confirm a relatively high mutation rate and a broad spectrum of phenotypes. For example, the percentage of albino mutants in a large population of einkorn wheat was lower than ours (Murai et al. 2013). Our results indicated the potential of ion beam irradiation as an ideal mutation method for faba bean breeding. The high mutation rate in small mutation populations would reduce the cost and labour intensiveness for phenotyping in mutation breeding programs.

Since Stadler's (1928) report that mutation was induced in barley (*Hordeum vulgare* L.) by X-rays, radiation mutagenesis has been considered as an important tool for generating new plant phenotypes for plant breeding and genetic studies. Ion beam irradiation has now been shown to be a valuable tool for mutation breeding of cool-temperate crops. The ion beam induced numerous mutations in rye at relatively low doses, with a slight effect on germination. In faba bean, the mutagenesis did not affect germination, but induced some mutations. The seed coat of faba bean is thicker than those of the other two species (Rowland 1977, Abbo et al. 2014, Smýkal et al. 2014), which could shield the embryo from some of the effects of the radiation. Due to the high mutation frequency observed in this study at M_1 , we suggest that this technique is useful to increase the genetic diversity in target plant germplasm in an early mutation generation to produce mutant crops with agronomically important traits for particular purposes.

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Fig. S1. Dry seed preparation for ion beam irradiation. (a) overview on all three species, (b) view from above and (c) view from side in rye