Irradiation effects of MeV protons on dry and hydrated Brassica rapa seeds

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ABSTRACT

Although space radiation is a known risk for space travel and eventual colonization of Moon or Mars, relatively few data exist on radiation effects on potential crop plants. We studied Brassica rapa seeds to 1, 2 and 3 MeV proton ions of various fluences and examined the effect on germination and root growth. Modeling penetration depth with SRIM code indicated that the applied energy was insufficient to penetrate the seeds; therefore, all energy was deposited into the tissue. Subsequent germination varied based on the incident ion energy and fluence (dose). Dry and hydrated seeds germinate after ion fluence (10¹³ ions cm⁻²) irradiation, but the germination percentage decreased with increasing fluence for ions that could penetrate the seed coat (> 1 MeV). Despite their greater volume and mass, hydrated seeds were more sensitive to irradiation than dry seeds. Damage of the seed coat after irradiation led to faster germination and initial seedling growth. Our results suggest that the seed coat represents a valuable natural radiation protection and that low energy protons, the prevailing solar radiation, are suitable for studying radiation effects in seeds and plants.

Keywords: Germination; radiation effects; seeds

1. Introduction

Space travel and future settlements on other worlds require coping with space and life support systems that are exposed to radiation levels many times higher than the typical exposure on earth. Establishing a life support system for human habitation depends on plants as they provide food, oxygen, biomass and are integral for recycling efforts, making them essential for space exploration. Space Radiation (SR) is acknowledged as one of the major impediments for human space missions (Durante, 2009; Kennedy, 2014; Yatagai and Ishioka, 2014). SR consists of high energy particles that affect biological systems, but its stochastic and non-directional nature and varying ion composition are difficult to establish experimentally. Despite their vital functions for space exploration, the effect of ionizing radiation (IR) on especially crop plants, is poorly studied. IR-induced biological effects and damage depend on the conditions of the radiation exposure and the biological state of the recipient organism. There is no universal relation between linear energy transfer (LET) and relative biological effectiveness for a given biological system that is valid for all particles. Therefore, dose-response curves are needed to help understand the consequences of radiation exposure of a tissue.

Energetic particles (such as x-rays, γ-rays, ion beam) represents both, low (LET < 50 keV µm⁻¹ in water) and high (LET > 50 keV µm⁻¹ in water) energy IR (Durante, 2004). Based on the energy content, protons produce weakly IR; their range is characterized by the “Bragg peak” (Bragg and Kleeman, 1905) that describes the energy transfer as a function of penetration depth. Low energy (50 MeV/u) particles are of minimal environmental consequence primarily due to their stopping in absorbers (Kim et al., 2015). However, low energy particles result from nuclear
absorption of high energy particles in absorbers such as biological tissues (Wilson et al., 1988), which will lead to tissue exposure of these low energy but high LET particles. Solar particle event (SPE) mainly consist of protons with energy below 50 MeV (Durante, 2004). During an SPE, life and material may be exposed to significant doses of IR.

Most of the reported investigations on biological effects of irradiation on plants have focused on heavy ions because their LET is higher than protons (Hirano et al., 2015; Kazama et al., 2011; Kazama et al., 2008; Tanaka et al., 1997) and energetic ions are effective for ion beam mutagenesis (Hirano et al., 2015; Magori et al., 2010). Although, there have been studies on biological effects of low energy ion irradiation on plants such as rice (Song et al., 2007), wheat (Wu and Yu, 2001), and Arabidopsis (Qin et al., 2007; Qin et al., 2006), the underlying mechanisms of IR damage remain unclear. Therefore, studying the effect of low MeV protons on seeds is relevant for space research, especially for conditions that mimic heavy ions.

Plant seeds, due to their cryptobiotic state have high resistance to environmental conditions, including radiation (Kumagai et al., 2000; Real et al., 2004). Therefore, seeds are robust vectors for life that propagate biological information (Tepfer and Leach, 2006). For example, seeds of Arabidopsis thaliana and Raphanus sativus germinated after exposure to 1000 Gy of $\gamma$-irradiation (Kumagai et al., 2000) whereas mammalian cells (golden hamster embryos) were killed by 10 Gy of $\gamma$-irradiation (Watanabe et al., 1990). Although the effect of IR on A. thaliana (Davies et al., 1994; Tanaka et al., 1997) serves as a valuable model and reference (Hase et al., 2017; Kozeko et al., 2015; Mei et al., 2011); there are no reports on the effect of IR on most crop plants such as B. rapa. Investigations on A. thaliana concentrated on ion-energy combinations that allowed ions to pass through the seeds. In contrast, B. rapa seeds are some 50 times larger than A. thaliana. B. rapa has been used for microgravity studies (Allen et al., 2009; Berkovich et al., 2016) and hypoxic conditions (Park and Hasenstein, 2016) but no data on IR sensitivity exist. Studying plants and seeds of different size and mass characteristics but closely related to Arabidopsis takes advantage of the wealth of genetic information available for Brassicaceae and therefore can help elucidate radiation effects on plants in general. In addition, Brassica comprises many economically important cultivars and therefore is a likely candidate for cultivation on future outposts. Its diverse secondary metabolites and short generation times provide desirable nutraceuticals that can alleviate space flight effects.

The present study investigates effects of protons on the germination of dry and hydrated B. rapa seeds with exposure to proton radiation with LET values between 10 to 30 keV $\mu$m$^{-1}$ as a function of ion fluence. The results indicate that dry B. rapa seeds have high resistance towards IR that diminishes upon hydration.

2. Methodology
2.1 Plant material
Experiments were performed on dry and hydrated B. rapa seeds (diameter $\approx$ 2 mm). Hydrated seeds were obtained by soaking seeds in deionized water for 12 hours. Germination was determined from about eight seeds in three replicates (20 – 24 seeds total).

2.2 Seed germination
The effect of irradiation was assessed by germination, which was evaluated by radicle protrusion through the seed coat and subsequent growth. Control and irradiated seeds (immediately post-irradiation) were sown on water-saturated paper in Petri dishes and germinated under ambient condition (25 °C, continuous cool fluorescent light, ca. 400 lux). Some seeds were exposed to
45 °C to examine the heating effect of irradiation. Temperature of dry seeds during the longest irradiation interval was measured using K-type thermocouple connected to Cen-Tech P37772 digital multi-meter and rose to 39±3 °C. To determine the effect of heat on germination, dry B. rapa seeds were kept at 45 °C in Thermolyne Type 48000 oven for 10 min exceeding the longest irradiation interval (5 min). Germination experiments after heat treatment were based on ≥35 seeds. Germination and root length were examined three days after sowing and evaluated using ImageJ (v. 1.45 NIH, USA).

2.3 Microscopy
Seeds were sputter coated (~ 15Å) with gold using Electron Microscopy Sciences sputter coater (EMS 550X) and examined with a scanning electron microscope (Jeol JSM-6460 LV).

2.4 Irradiation
We used a proton beam of a cross sectional area of approximately 6×6.4 mm² with energies of 1, 2 and 3 MeV. Negative ions with energy of ≈ 25 keV were produced using a National Electrostatics Corporation’s (NEC) Duoplasmatron ion source. The ions were injected into a NEC’s 5SDH-2 Tandem Pelletron accelerator. Upon exiting the accelerator, protons were focused by a magnetic quadrupole doublet lens system and directed to the experimental chamber (Fig. 1). The beam exited the vacuum through a 12.7 µm thick titanium window and reached a monolayer of 7-8 seeds that were secured in a holder about 5 mm from the Ti window. To ensure uniform irradiation, the beam profile was monitored using NEC’s BPM-80 beam scanner located about 150 cm before the Ti window. The proton beam and profile was stable during irradiation. The delivered ion fluence (protons cm⁻²) was calculated using ion counts measured with a high precision Ortec 439 Digital Current Integrator and Ortec 871 Timer/Counter.

2.5 Proton stopping power and dose calculations
When an energetic particle impinges on matter of thickness x, it decelerates because of its interaction with nuclei (nuclear stopping) or electrons (electronic stopping) and its remaining energy E decreases. The mass stopping power of protons in dry B. rapa was obtained using SRIM (Stopping and Range of Ions in Matter) code (Ziegler et al., 2010). Calculations were based on seed composition of carbon (33%), nitrogen (5%), oxygen (40%), hydrogen (15%), and minerals (7%). SRIM is a Monte Carlo simulation based on binary collision approximation (Möller et al., 1975; Robinson and Torrens, 1974), i.e., the interaction between incident ion and target atoms, and recoil cascades are simulated as binary collision sequences governed by universal two body potential (Ziegler et al., 1985). The program calculates the distribution and trajectory of ions and provides information associated with the energy loss, target damage, ionization, and phonon production (Ziegler et al., 1985). Nuclear stopping at the chosen energy levels is negligible for protons and the main contribution to the energy loss originated from collisions with electrons (Fig. 2). The mass stopping power (S/ρ) obtained from SRIM was used to convert ion fluence (θ) into ion dose. The ion dosage D can be expressed as mass stopping power and ion fluence using equation 1 (Osinga et al., 2014):

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D(\text{Gy}) = \frac{S}{\rho} (\text{J cm}^2 \text{ kg}^{-1}) \times \theta \text{ (ions cm}^{-2})
\] (1)

3. Results
3.1 LET
The energy loss through the Ti window was about 28 keV μm⁻¹ for 3.5 MeV protons. The LET of energetic protons in dry and hydrated seeds is a function on target depth and varies with the beam energy. The calculated penetration depth varied from 30 μm in dry B. rapa seeds for 1 MeV protons to 190 μm in hydrated B. rapa seeds for 3 MeV protons (Fig. 3). Importantly, these penetration dimensions confine IR effects to the outer tissue, mostly the seed coat, of the seed. The effect on the seed coat was demonstrated by SEM. Severe damage consisting of cracks and fissures was seen after irradiation (Fig. 4). Control experiments confirmed that this effect was not the result of mechanical damage as fissures were not observed in control seeds which went through similar handling. Neither exposure to high vacuum nor exposure to heat (45 °C) produced fissures as those shown in Fig. 4. Non-irradiated but heat-treated seeds (10 min at 45 °C) showed no fissures on seed coat and germination was 97%.

3.2 Effect of irradiation energy on B. rapa seeds
Dry seeds and 12 h hydrated seeds contained 5.95±0.2% and 32.6±0.5% water, respectively. The density of the dry and hydrated seeds amounted to 1.1 and 1.03 g cm⁻³, respectively. Seed germination of non-irradiated seeds was >98%.

Germination of dry B. rapa seeds after 2 and 3 MeV exposure decreased with increasing ion fluence and dose (Fig. 5). While all control seeds germinated, irradiated seeds showed reduced germination as the beam energy and fluence increased. However, one MeV protons had no detrimental effect regardless of fluence up to 7×10¹⁴ ions cm⁻². Irradiation with 2 MeV protons reduced germination of dry B. rapa to 65%. Three MeV irradiation inhibited germination more severely than lower energy protons; germination declined sharply to 63%, followed by a plateau (fluence ~3×10¹⁴ proton cm⁻²), and declined to 21% at 7×10¹⁴ proton cm⁻². The 50% lethal dose (LD₅₀) was 8 MGY for 3 MeV protons (about 5×10¹⁴ protons cm⁻², equation 1). Measurable germination inhibition required fluences of 1×10¹⁴ and 1×10¹³ proton cm⁻² for 2 and 3 MeV protons, respectively.

Germination of hydrated B. rapa seeds was more strongly affected by IR than that of dry seeds. Two MeV proton irradiation produced a projected LD₅₀ at 9×10¹⁴ ions cm⁻² (15 MGY). Inhibition of hydrated seeds after 3 MeV irradiation resulted in an estimated LD₅₀ of 2×10¹⁴ ions cm⁻² (1.7 MGY). Interestingly, irradiation with 2 or 3 MeV protons inhibited germination at the same fluency (1×10¹⁴ proton cm⁻²).

Surprisingly, seed germination was stimulated after exposing seeds to low doses of IR (Fig. 7). Dry B. rapa seeds exposed to 3 MeV protons fluences (1×10¹³ to 1×10¹⁴ ions cm⁻² (0.13 to 0.84 MGY) resulted in faster germination in comparison with non-irradiated controls (Fig. 7). Although, the root length for non-irradiated control seeds after 72 hours was greater than that of irradiated seeds (3 MeV proton, 3×10¹³ ions cm⁻² or 0.15 MGY and 1×10¹⁴ ions cm⁻² or 0.84 MGY fluences). Radiation induced damage to the seed coat (Fig. 4) and perforation of the outer tissue layers may have contributed to faster germination and initial seedling growth (Fig. 7).

4. Discussion
Quantitative modeling showed the dominance of electronic stopping power of protons in B. rapa seeds, which is characteristic of low MeV ions and common for biological materials. The total
electronic stopping power decreases with increasing ion energy (Fig. 2). At 3 MeV, stopping power decreases to half its value at 1 MeV incident ion energy. These observations indicate two important arguments for using low MeV ions; first: higher energy is deposited at shallow depth as opposed to e.g., 100 MeV ions; second: the ions deposit their entire energy in the tissue towards the end of their range. However, for low MeV protons (< 5 MeV) beam deformation due to energy transfer to the target of the vacuum window becomes a major source of uncertainty (Correa et al., 2012; Shimizu et al., 2009; Siiskonen et al., 2011). To compensate for angular deviations and considering the size of the beam, thick material (approx. 12.7 µm) was used as exit window as opposed to thin or ultra-thin (Calligaro et al., 2000) material available for external irradiation. The interaction between low energy ions and biological systems is more complex than penetrating IR as the implanted ions not only deposit energy in the target, but also result in mass accretion.

Biological damage produced by charged particles in tissue depends on the absorbed dose delivered and on the linear energy transfer (Takatsuji et al., 1999). The effectiveness of ion beams can range from surface effects (see Fig. 4) to mutagens (Shirasawa et al., 2016); however, these effects are not determined by the ion species but by the LET of the ions (Kazama et al., 2008). Ion beams with LET of around 10-500 keV µm\(^{-1}\) are suggested to be suitable for ion beam mutagenesis (Magori et al., 2010; Tanaka et al., 2010). Therefore, examination of low energy proton beams address non-mutagenic effects. Modification of DNA inhibits biological systems and manifests itself as reduction in plant growth (expressed as increase of mass over time). Evaluating generic responses to IR is important to identify the sensitivity of vectors (seeds, seedlings, or non-differentiated callus tissue) as robust material for future space exploration. The challenge of assessing radiation damage is complex because radiation damage depends on various parameters that are difficult to assess in a laboratory. In addition to the ion energy, ion species, location, frequency and other environmental parameters (pressure, atmosphere, storage conditions, and temperature) affect radiation damage. For example, carbon ions with LET of 30 keV µm\(^{-1}\) were most effective for mutagenesis in the second generation i.e., progeny derived from the original generation that was treated with IR (Kazama et al., 2008). Thus, potential damage in germinating seeds may not be completely assessed unless recombination in second generation crosses can be examined.

The LET of the protons in our study varied from 10-30 keV µm\(^{-1}\) (Fig. 3), falls within the lower end of LET values (10-500 keV µm\(^{-1}\)) that can be used for mutagenesis (Magori et al., 2010). The dose at the end of the survival curve or less than these doses is more efficient for ion beams as a mutagen (Magori et al., 2010), suggesting that doses of 3 MGy and 1 MGy for 2 and 3 MeV protons respectively, may be most efficient for mutagenesis and suitable for assessing space radiation effects.

Germination of *B. rapa* declined as the ion fluence and energy increased. While this relationship is expected, the greater sensitivity of hydrated seeds was unexpected as secondary events are typically reduced by shielding or spacing (expansion) that is provided by the absorbed water. The main cause of lethality after exposure to IR is a result of DNA damage or related cellular responses (Hada and Georgakilas, 2008). Prior work on *Arabidopsis thaliana* using gamma rays (Culligan et al., 2006; Naito et al., 2005; Ricaud et al., 2007; Shirasawa et al., 2016), x-rays (Furner and Pumfrey, 1992; Kurimoto et al., 2010; Wang et al., 2016) and various ions at high energy (Hirano et al., 2015; Kazama et al., 2008; Qin et al., 2006; Tanaka et al., 1997) showed similar results as our low MeV ions in *B. rapa*. Although high MeV ions pass through
e.g., dry seeds of *A. thaliana* (Tanaka et al., 1997), these authors also focused on germination and survival of dry *A. thaliana* after heavy ion (He, C, Ne, Ar) and electron irradiation with LET values of 17-549 keV µm⁻¹. *A. thaliana* germination after irradiation with 48 MeV He ions decreased to 6% for doses of about 11,000 Gy (Tanaka et al., 1997) confirming our similarly high, and unexpected Gy values (Fig. 6). These values indicate that seeds are much more resilient to radiation than typical animal cells or systems. When the incident ion energy is high enough to penetrate the whole seed as for *A. thaliana* (seed diameter is about 0.2 mm), meristematic tissue will be affected. The larger size of *B. rapa* (1.9 and 2.2 mm diameter for dry and hydrated seeds, respectively) provided more shielding to meristematic tissue. Despite the increase in volume, the deeper penetration depth in hydrated seeds (Fig. 3), resulted in greater sensitivity to radiation (LD₅₀ at 3 MeV protons was 8 and 3 MGy for dry and hydrated seeds, respectively). The energy range used in this study was chosen so that all energy is deposited within the seeds. The ion doses (≈ 12 MGy) in the present study (Fig. 5) as compared to Tanaka et al. (≈ 11 kGy) could be attributed to lower energy and penetration depth of protons in *B. rapa* than for 48 MeV He ions that passed through dry *A. thaliana* seeds. The ion fluence used in the present study is comparable to fluences (1×10¹⁴ proton cm⁻²) reported Qin et al. (2006) who used lower energies (1.1, 2.6 and 6.5 MeV) to investigate germination of hydrated *A. thaliana* seeds. Strong inhibition in germination rate of hydrated *A. thaliana* seeds after 2.6 and 6.5 MeV H⁺ irradiation was reported; however, for 1.1 MeV H⁺ irradiation at highest fluence (1×10¹⁴ proton cm⁻²), seed germination was reduced to 85% (Qin et al., 2006). LD₅₀ for hydrated *B. rapa* after 2 MeV H irradiation is higher (700×10¹² proton cm⁻², Fig. 5) than LD₅₀ for hydrated *A. thaliana* after 2.6 MeV H irradiation ca. 50×10¹² proton cm⁻² (Qin et al., 2006). The variation in ion dose required to inhibit germination to the same extent could be related to difference in size of the seed volume of *B. rapa*, which is approximately 50 times bigger than that of *Arabidopsis*. This observation suggests that radiation resistance increases with seed size. Therefore, seed size should be considered as selection criterion for ‘space crops’.

The estimated penetration depth of 1 MeV protons in dry (6% water) and hydrated (≈ 33% water) *B. rapa* was approximately 27 and 34 µm, respectively. Since 1 MeV ions were not able to penetrate the seed coat (≈ 40±5 µm thickness), they did not affect germination. Irradiation with 3 MeV protons penetrated seeds to a depth of 145 and 190 µm in dry and hydrated seeds, respectively. This penetration depth is unlikely to affect more than a small portion of the –close to the surface positioned- root meristem but likely had no effect on the shoot apical meristem. Despite the strong influence of IR on penetration depth, no significant reduction in germination rate was observed for ions penetrating less than 80 µm deep, which is twice the thickness of seed coat (Fig. 4). Lower LD₅₀ doses are likely related to reduced fluence and lower stopping of ions for same energy.

Surprisingly, irradiated seeds germinated faster than controls. While damaged seed coat could facilitate water uptake, the effect of low level IR is likely to involve other mechanisms such as hormetic effects, i.e., advantageous response to low levels of stressors, as has been reported for gamma irradiation or barley roots (Okamoto and Tatara, 1995) and pepper (Kim et al., 2005). Low dose IR is regarded as priming treatment for seeds (Araujo et al., 2016) and may correspond to our observation. Enhanced germination and potentially stress-induced production of reactive oxygen species (ROS) (Roldan-Arjona and Ariza, 2009) that originates from radiolysis of water surrounding the incident tracks (Schlathölter, 2012) may contribute to a beneficial biological response. IR produces ROS (Cobut et al., 1998; Laverne and Pimblott, 1995) and its role in biological system is known as generic stressor (Gligorovski et al., 2015).
Higher concentrations of radiolytic ROS accumulation is considered a major factor in radiation damage (Hwang et al., 2016; Mei et al., 2011; Qin et al., 2007). The initial hormetric effect could explain the observed shoulder in the dose response curve (at about 3×10^{14} ions cm^{-2}) for dry B. rapa seeds. A similar observation was reported after low energy nitrogen ion implantation in tyrosine (Zengliang and Chunlin, 1994) and proton irradiation of water-imbibed A. thaliana seeds (Qin et al., 2007).

The current study presents the first findings for biological effects of MeV protons on B. rapa seeds. Estimated LD_{50} values for 2 and 3 MeV protons in hydrated and dry seeds were higher than those reported for smaller Arabidopsis seeds. The most important factors for IR-induced damage are penetration depth, and hydration status of seeds. Because germination was not affected by the IR that could not penetrate the seed coat (Fig. 4), the presence of a thick and dense seed coat, in addition to the seed size, provides protection for seeds. These features are important for IR resilience and should be considered for biological vectors for future space exploration.

Acknowledgements

This research was supported by NASA grant NNX13AN05A.

References


Instruments & Methods in Physics Research Section B-Beam Interactions with Materials and Atoms 265, 495-500.


Figures:

Fig. 1. A photograph of the irradiation setup. The sample holder was positioned about 5 mm from the Ti window (inset = oblique view of Ti foil) during irradiation.

Fig. 2. Stopping power of proton in dry *B. rapa* seeds calculated using SRIM code.
Fig. 3. The specific energy loss along the track of protons of different energies in dry (solid symbols) and hydrated (open symbols) *B. rapa* seeds.

Fig. 4. SEM image of the dry *B. rapa* (control, a), and after irradiation with 3 MeV H⁺ for 2 min (b), 3 MeV H⁺ for 4 min (c).

Fig. 5. Germination of dry (solid symbols) and hydrated (12 h in DI water, open symbols) *B. rapa* seeds after irradiation with proton ions at different ion fluences at 1 MeV (diamonds), 2 MeV (circles) and 3 MeV (squares). Error bars indicate SD for three replicates of 7-8 seeds each.
Fig. 6. Germination of hydrated *B. rapa* seeds three days after irradiation with 3 MeV H\(^+\) at the indicated fluences. Bar indicates 10 mm.

Fig. 7. Germination of dry *B. rapa* seeds after irradiation with 3 MeV H\(^+\) (squares) show faster germination than in non-irradiated controls (circles). Data (±SE) are the average of 5–15 plants.