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Development of pre-breeding diploid potato germplasm displaying wide phenotypic variations as induced by Ethyl Methane Sulfonate Mutagenesis

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Abstract

Mutations are the key drivers for the evolution and diversification in plants. In varietal selection, sources for variation are always sought as starting breeding materials. Thus, in the absence of desired natural variations in breeding populations, targeted or random mutagenesis is applied to induce variations. Cultivated potato (*Solanum tuberosum* L.) is autotetraploid crop species with a narrow and highly heterozygous genetic base, and the complexity of its genome makes its genetic studies more difficult. In the current study, induced mutagenesis was performed in diploid potato using ethyl methane sulfonate (EMS) to enlarge the genetic variability for its use as pre-breeding materials in both polyploid and diploid potato breeding. As starting materials, true potato seeds were treated with 1.2% EMS for 4-6 h along with non-treated seeds as controls. A large variation in terms of germination rate, plant, flower and tuber phenotype was observed in EMS-treated plants compared with their non-treated counterparts. Of particular, abnormal phenotypes including twisted stem, partial and/or completely chlorotic leaves and stems, variations in stem color and weak-stemmed plants with lateral growth habit as well as plants with determinate growth habit were observed along with normal plant characteristics. Moreover, variations in flower color, and tuber color, shape and size as well as yield potential were observed in EMS-treated lines. The reported phenotypic characterization of EMS mutagenized diploid potato collection is to our knowledge the first in its kind and represents a premium genetic resource for potato breeding programs and plant biologists for genes functional characterization in potato.

**Keywords:** Potato, *Solanum tuberosum*, diploid, EMS, mutations, phenotypic variations
Résumé

Les mutations sont la clé de l’évolution et de la diversification des plantes. En sélection variétale, les sources de variation sont très recherchées au sein du matériel d’amélioration. En absence de variations naturelles désirées dans les populations de sélection, la mutagenèse ciblée ou aléatoire est réalisée pour induire des variations. La pomme de terre cultivée (*Solanum tuberosum* L.) est une espèce autotétraploïde ayant une base génétique réduite et une forte hétérozygotie, et la complexité de son génome rend ses études génétiques difficiles. Dans cette étude, la mutagenèse induite a été faite sur la pomme de terre diploïde avec l’éthyle méthane sulfonate (EMS) pour élargir la base génétique et pour son usage tant en amélioration des pommes de terre tétraploïdes que diploïdes. Au départ, de vraies graines ont été traitées avec 1.2% d’EMS pendant 4-6 h et comparées avec des témoins non traités. Une forte variation du taux de germination, d’aspects des plantes, des fleurs et tubercules a été observée chez les plantes traitées comparativement aux plantes non traitées. En particulier, des aspects anormaux incluant des tiges déformées, des feuilles et tiges partiellement ou totalement chlorotiques, des variations de couleur des tiges, des plantes à tiges affaissées et habitudes de croissance latérale, et des plantes à croissance déterminée ont été observées en marge de plantes totalement normales. De plus, des variations de couleur des fleurs et tubercules, de la forme, la taille et du rendement des tubercules ont été notées chez les plantes traitées à l’EMS. Ce rapport sur la caractérisation morphologique d’une collection de pomme de terre diploïde mutagenisée est, à notre connaissance, le premier en son genre et représente des ressources phylogénétiques de premier choix pour les programmes d’amélioration et pour les biologistes en caractérisation fonctionnelle de gènes chez la pomme de terre.
Mots-clés: Pomme de terre, Solanum tuberosum, diploïde, EMS, mutations, variations morphologiques

1. Introduction

Potato (Solanum tuberosum L.) is a starchy tuberous crop originated from the Andean regions of South America (Hardigan et al. 2017), and it ranks fourth after maize, rice and wheat as one of the most important food crop species (Food and Agriculture Organization 2014). The global potato production was 376 million tons in 2016, with China being the world’s largest producer (Food and Agriculture Organization 2016). Cultivated potato is autotetraploid (2n=4x=48) with a narrow and highly heterozygous genetic base (Srivastava et al. 2016). The genetic base of the cultivated potato can be expanded by crossing with wild Solanum species which are rich source for disease and pest resistance along with yet untapped quality traits (Yang et al. 2017). However, interspecific and interploidy crosses between S. tuberosum and other Solanum species are proven to be difficult due to sexual incompatibility and difference in endosperm balance numbers (EBNs) (Watanabe 2015). Also, because of the tetraploid nature of cultivated potatoes it is difficult to breed for specific traits such as disease resistance and quality attributes without dragging undesirable traits hidden in the wild species (Lindhout et al. 2011). In contrast to tetraploid cultivated potatoes (2n = 4x = 48), diploid potato species (2n = 2x = 24) have small and less complex genomes making their genetic studies more attractive. Thus, although self-incompatibility and inbreeding depression are common issues encountered in diploids (Peterson et al. 2016), the use of diploids in potato breeding has become a promising alternative and undesirable traits, if present, can be easily removed through self-pollination of self-compatible
germplasm, unlike tetraploids (Li et al. 2013). Therefore, the development and maintenance of a
diverse pre-breeding diploid potato germplasm is seen as vital resource for a fast and effective
potato breeding.

Different breeding methods have been reported and used to diversify potato germplasm
(Machida-Hirano 2015). Among these methods, conventional breeding methods are reported to
be time consuming (Ashkani et al. 2015) and the displeasure expressed by consumers towards
the genetically modified crops and their products is alarming and indisputable (Telem et al.
2013). Induced mutagenesis has been regarded as a valuable alternative to genetically modified
organisms (GMO’s) because it is a flexible, workable, unregulated, non-hazardous low-cost
procedure (Jain 2010) and has been used in multiple crops including potato (Shu and Lagoda
2007; Muth et al. 2008; Uitdewilligen, 2012). In nature, spontaneous mutations occur as a result
of cell replication errors or exposure to environmental factors such as radiations (ultraviolet) and
chemical agents such as aflatoxin B1 (Maki 2002; Smela et al. 2001). Mutations that escape
DNA repair mechanisms are inherited and passed on to progenies (Oladosu et al. 2016). On top
of natural mutations, mutations can be induced artificially into plants using physical (Ion beam,
gamma-rays, x-rays etc.), chemical (Ethyl methanesulfonate, sodium azide, hydroxylamine etc.)
agents (Jain 2010), or the emerging gene editing enzymatic tools such as TALEN (Malzahn et al.
2017) and CRISPR-Cas9 (Braatz et al. 2017; Yang et al. 2017). Gamma rays and ethyl-methane
sulfonate (EMS) are so far the most widely used physical and chemical mutagens for induced
mutations (Jain 2010). Barley was the first crop to be mutagenized using x-rays (Stadler 1928;
Oladosu et al. 2016). During the past 60 years, more than 3,000 varieties were developed in
about 180 plant species worldwide using induced mutations (Shu and Lagoda 2007). Gamma-ray
induced mutants showing reduced glycoalkaloid content have been reported in Russet Burbank
potato tubers (Love et al. 1996) whereas EMS mutagenesis has been used to characterize the \textit{waxy} gene in diploid potatoes (Muth et al. 2008). The molecular feature of induced mutations is primarily determined by the type and extent of DNA damage caused by various mutagens (Till et al. 2007). Of all the artificial mutagens, chemical mutagens are the widely used since they induce mostly SNPs leading to missense and nonsense mutations at a high mutation rate and are ideal for producing gain-or loss- of function (Bhat et al. 2007). The benefit of using chemical mutagens over physical mutagens lies in its high mutation rate and in its predominant tendency to produce point mutations (Jain 2005). Majority of the chemical mutagens are classified as alkylating agents which include ethyl methane sulphonate (EMS), diethyl sulphate (dES), ethyleneimine (EI), ethyl nitroso urethane (ENU), ethyl nitroso urea (ENH), methyl nitroso urea (MNH) and azides (Joint F.A.O. 1977). Whereas many potato breeding programs exist around the world, less data and genetic resources have been reported and made available on diploid potato germplasm development. With an exception to Muth et al. (2008) study focused on \textit{waxy} gene, the use of EMS mutagenesis for developing mutagenized diploid potato germplasm and characterizing for multiple phenotypic traits is sparse and none has yet been reported to our knowledge to date. The objective of this study is to 1) generate a large mutagenized diploid potato population using EMS mutagenesis, and 2) screen and characterize the extent of genetic variation in the population for plant and tuber morphological traits with an ultimate goal of generating pre-breeding germplasm for both diploid and tetraploid breeding.

\textbf{Materials and Methods}

\textbf{Plant material}
The botanical true potato seeds (TPS) used in this study were obtained from nine crosses involving eleven diploid potato germplasm (Table 1). The crosses were performed at the Fredericton Research and Development Centre (FRDC), New Brunswick and the Lethbridge research and development Centre, Alberta, between 1994 and 1996. Seeds were collected and stored at 4°C before shipping and use in Charlottetown. Prior to the EMS mutagenesis test, a pilot germination test was performed using 50 seeds from each cross to ensure the viability of the seed lots during summer, 2013. The germination test was carried out on pre-wet filter paper placed in Petri dishes and incubated in an oven set at 22°C in the dark. Seeds from two crosses (A373 x BLR07 and A262 x BLR07) did not germinate and were discarded from the rest of the study. Seeds from the remaining seven crosses were used for EMS treatment experiments.

Optimization of EMS treatment conditions

A preliminary EMS mutagenesis test was conducted using 20–30 TPS treated with 0.2, 0.5, 0.8, 1.0, 1.2, or 1.5% (v/v) EMS (Sigma Aldrich, Oakville, ON, Canada) diluted in water and incubated for 3, 4, 5, 6, 8, or 16 hours at room temperature. After incubation in the EMS solution, the seeds were washed and rinsed five times with water and dried on a filter paper at room temperature. The diluted EMS wash solution was deactivated using sodium thiosulfate and discarded following the standard dangerous chemical management procedures in place at our facility. The dry seeds were recovered in Falcon tubes. For the germination test, the treated seeds were counted and placed onto a single layer of filter paper pre-wetted with sterile water in 90 mm x 15 mm Petri dishes and incubated in dark at 22°C. Germination rate was determined by counting the number of seeds that germinated within 4 weeks. The combination of EMS
concentration and exposure time that showed a germination rate ranging from 50 to 60% was selected for the final EMS mutagenesis experiment.

**EMS mutagenesis experiment**

A total of 2,947 TPS from the 7 crosses that passed the pilot germination test were treated with 1.2% EMS. The seeds from each cross were incubated in the EMS solution as described in Table 2 and washed five times and air-dried. The treated seeds from each cross were recovered separately, counted and planted in 20-row germination trays filled with Promix soil (PREMIER TECH, Rivière-du-Loup, Quebec, Canada). Ten to fifteen seeds were planted in each row of the 20-row trays to ease the counting of germinated seedlings. A total of 40 non-treated seeds from each cross were planted in separate trays and used as control. The trays were watered and covered with dark tray covers and maintained in a greenhouse at 22°C until germination. After the first sign of germination (~7 days after planting), the covers were removed and plants were exposed to 16/8h light regime at 22°C in a greenhouse environment, and manually watered as required and the germinated seedlings were counted daily for four weeks to determine the germination rate. After 4 weeks, each germinated seedling was transplanted in individual mini pots (8.25 x 8.25 cm), assigned a unique ID number for traceability, and arranged in 18 cell trays (25.4 W x 50.3 L x 8.89 H cm) for mini tuber production. Trays were properly fertilized three times using 0.4% (v/v) of 20:20:20 All purpose™ fertilizer (Plant-Prod ULTIMATE™). The normal daily watering (25 mL/day/pot) was performed using an automatic watering system (Senninger Irrigation Inc.).
Assessment of phenotypic variants in greenhouse

The transplanted seedlings that survived were monitored for variations in leaf morphology (normal/abnormal), stem colour, presence/absence of flower, and flower colour. After 120 days of growth, water supply was withheld to allow the plants to senesce and mini tubers were harvested. Tuber’s characteristics such as total number of tubers, total weight and colour of tubers for each plant were recorded.

Tuber production and phenotypic characterization in the field

For a first screening, the lines that died during the regeneration as well as the non-tuber forming lines were dropped. A genetic screening using next-generation sequencing targeting 4 target genes (Asparagine synthase 1 and 2, Solanidine glycosyltransferase 1 and 2) was then performed (not reported in the current study) to further reduce the population size to 836 EMS and 70 CTL lines before more detailed plant morphological variation assessments were performed in the field condition for increasing the tuber production (Table 3). Based on the tuber availability, 1–4 tubers per line were planted in the field and the germination percentage was recorded for each line. Germinated and survived plants were screened for variations in leaf morphology, stem color, growth habit, flower color, presence or absence of fruit. After harvesting, tuber characteristics such as total number of tubers, total weight of tubers and tuber color in each plant across 7 crosses were evaluated.

Statistical analysis
The phenotypic data were analysed using GenStat 64-bit Release 16.1. A one way ANOVA was used to test the effect of EMS treatment on seed germination and to test difference between EMS-treated and control plants for tuber traits in each cross. An analysis of unbalanced design using GenStat regression was also performed to test differences between tuber traits in EMS-treated and control plants in each cross. SAS® OnDemand for Academics (SAS, SAS Institute Inc., Raleigh, North Carolina) was further used to define categories of phenotypic traits such as tuber number, weight, and color for comparison.

Results

Effect of EMS treatment on TPS germination

To determine the optimal time and suitable EMS concentration, a pilot germination test was conducted. Seed treatment with 1.2% EMS for 4 to 6 h showed a germination rate slightly higher than 50% and these conditions were used to generate the mutagenized population (Table 2). In the final mutagenesis test, non-treated seeds showed a high germination rate (82.5–100%) across the 7 crosses (Table 4), with an average germination percentage of 91%. Control plants in cross 1 (CTL1) showed the highest germination rate with 100% germination whereas CTL3 showed the lowest (82.5%) germination rate (Table 4). Of the 2,967 EMS-treated seeds planted, 1,965 (66%) germinated, with germination rates ranging from 55 to 75 % (Table 4). Overall, EMS treatment significantly (P<0.001) affected the seed germination in EMS-treated seeds (66%) when compared to non-treated controls (91%). Whereas no significant difference could be observed between seed exposed to EMS for 4 and 5 h (70% and 69% germination, respectively), seed germination was reduced after seed incubation in EMS for 6 h (62%) (Fig 1). A significant
difference ($P<0.001$) was also observed between crosses for their reaction to EMS treatment. Although exposed to EMS treatment for a long time, cross 6 showed a higher germination rate (71.7%), a rate close to that observed in cross 2 (74.7%) and cross 3 (75.3%) both exposed to EMS for a shorter time. Cross 6 also showed a lower seedling death rate. Moreover, EMS treatment had a significant ($P<0.05$) impact on seedling death after germination (Table 4).

**Effect of EMS treatment on plant morphological characteristics**

*Plant phenotypic variations observed in greenhouse*

Among the surviving 1,776 EMS seedlings grown in the greenhouse, 111 (6.25%) showed abnormal phenotypes such as twisted stem, crinkle leaf, purple/burnt leaf tip, complete/partial chlorotic leaves, bushy growth habit, and vine-like stem growth habit. Stem color variation was observed in all the 7 crosses (Table 5). In general, a higher stem color variation was observed in the EMS-treated lines compared with the control lines. The stem color variations in EMS-treated population ranged from 0.1% (yellow/chlorotic) to 25.5% (green/purple) whereas it ranged between 0 % and 24 % in the non-treated control lines (Table 5). Green stem color was the dominant stem phenotype in the population. No difference was observed between EMS-treated lines (54%) and non-treated lines (55.45) for the green stem color. In contrast, yellow/chlorotic stem color was observed only in the EMS-treated lines, with 0.1 % of the EMS lines displaying this phenotype. A slightly higher percentage of EMS lines (13%) were found to produce flowers compared to 8.3% in non-treated lines (Table 5).
Effect of EMS treatment on tuber yield characteristics in greenhouse

Effect of EMS on number of tubers

To assess whether EMS treatment affected yield potential, tuber yield characteristic were evaluated. Whereas no significant differences were observed in the average number of tubers produced by non-treated and EMS-treated lines in each cross, EMS-treated lines tend to produce more tubers per plant (Table 6). In particular, non-treated lines in cross 4 showed a lower maximum of tubers per plant (8 tubers/plant) compared to their counterpart EMS-treated lines in the same cross (21 tubers/plant) (Table 6). To further discriminate the changes caused by EMS treatment, plants were categorized by class of tuber number produced. EMS-treated lines in cross 4, 5, and 7 were found to produce more tubers in higher range compared to their respective controls (Fig 2).

Effect of EMS treatment on tuber weight

To determine if the EMS treatment affected starch structure and tuber biomass, tuber weight was recorded in non-treated and EMS-treated lines in each cross. Whereas no significant differences were observed in the average weight of tubers produced by non-treated and EMS-treated lines in each cross, the extent of tuber weight variation per plant was found to be higher in the EMS-treated lines (Δ=37) compared to non-treated lines (Δ=12) (data not shown). Moreover, some plants in EMS-treated group were found to produce very small and single fluffy tubers falling under the balance detection limit of 0.01g. None of such fluffy tubers were seen in non-treated lines. To further assess the diversity caused by EMS treatment, non-treated and EMS-treated
lines were categorized by class of tuber weight (Fig 3). EMS-treated lines in cross 1, 2, and cross 5 produced bigger tubers with higher weight ranges compared to their respective controls (Fig 3).

**Effect of EMS treatment on tuber color**

Tuber color was evaluated to determine if EMS treatment induced tuber color variations in the population. Except for cross 1 and cross 7, EMS treatment induced in each other crosses at least one new color in comparison with the respective non-treated lines (Fig 4). Cross 2 and 3 showed the most EMS-induced color variations, with two new colors not observed in their respective controls. White color was the most predominant color observed in the EMS-treated (72%) and non-treated lines (69%). EMS-treated lines in cross 2 (EMS2) produced tubers with a unique black skin color, not observed in rest of the EMS-treated and non-treated control lines. Tubers with black, dark purple, red and variegate of purple and pink skin color was only found in the EMS-treated lines but not in non-treated lines.

**Effect of EMS treatment on plant morphological characters in the field**

**Effect of EMS treatment on leaf morphology**

To confirm the morphological variations observed in the greenhouse, plant morphology was further assessed in the field. Numerous variations in the leaf, stem morphology, and plant growth habit were observed in the field in the EMS-treated plants (Fig 5). Of the 836 EMS lines planted, 5, 3, and 8 lines showed purple leaf tip (Fig 5c) partially chlorotic (Fig 5e and f), and completely chlorotic (Fig 5g) phenotypes (Fig 5). A line (line#594) from EMS5 showed deformed and
incomplete leaf structure (d) with fully grown midrib and burnt leaf tips, and some of which had a needle shape (Fig 5). A line (line#72) from EMS1 showed a multiple leaf variations on the same plant, and included normal, round, and half-leaf (midrib and half of the leaf) (b).

**Effect of EMS treatment on plant growth habit**

Growth habit variation was observed in EMS-treated lines from all the 7 crosses (Fig 6). EMS-induced growth habit variants ranged from plants growing laterally with determinate growth habit (Fig 6a) to bushy plants with numerous erect stems and leaves (Fig 6f). Plants with rosette growth habit showing short main stem, compact internodes and numerous leaves (b), very small plants (c), although rare, plants with weak stem, lodging on ground, with numerous leaves (d), as well as plants with large internodes, small leaf size and reduced leaf number (e) were observed in EMS-treated lines (Fig 6a-e).

**EMS induced floral color variations as observed in the field**

Flower color was evaluated to determine if EMS treatment induced floral variations. Of the 906 potato lines planted in the field, only 251 lines produced flowers, of which 44 were non-treated and 207 was EMS-treated lines. Non-treated lines produced white (a), blue (b) and violet (c) flowers (Fig 7a-c), whereas the EMS-treated lines produced flowers with light and dark variants of white (d), violet (e), variants of blue (f and g), and purple (h) colors (Fig 7d-h) along with the three wild type colors observed in the non-treated lines (Fig 7a-c). White color was the predominant flower color in both non-treated and EMS-treated lines, followed by the blue and
violet colors. Variants of white flower observed in EMS-treated lines include white petals with yellow (a) and green (d) anthers. None of the non-treated control lines produced purple flowers as opposed to some EMS lines in from cross 2, cross 3, and cross 4 that showed purple (h) flowers.

**Effect of EMS treatment on tuber characteristics in the field**

**Effect of EMS treatment on tuber color in the field**

To confirm the tuber color variation observed in the greenhouse, plants in the field were assessed for tuber color (Fig 8). Tubers from non-treated lines were found to be yellow (a), red (b), purple (c), and brown (e) colors, whereas tubers from EMS-treated lines showed white (d), light red (f), light purple (g) and dark purple (h) along with the colors observed in the non-treated control lines (Fig 8). White tuber color was the major tuber color observed in non-treated (76.2%) and EMS-treated lines (80.6%).

**Effect of EMS treatment on tuber number in the field**

The tuber number was assessed in EMS-treated and non-treated plants in the field. Although, a significant difference was observed in most crosses between the EMS-treated and non-treated plants for the minimum and maximum number of tubers produced per plant, no significant difference was found between the two treatments in terms of average number of tubers (Table 7).
Effect of EMS treatment on tuber weight in the field

Plants in the field were assessed for tuber weight. A large difference in the average weight of tubers was observed between EMS-treated and non-treated lines for each cross (Table 8). Due to high standard deviation within each group (cross), the observed difference was deemed statistically insignificant. Significant variation was however observed in the minimum and maximum weight of tubers per plant in each cross (Table 8). Among the non-treated controls, lines from cross 6 produced the highest maximum weight of tubers (2,777 g) and cross 7 the lowest maximum weight (1,093 g). In the EMS-treated lines, the highest maximum weight of tubers was observed in cross 2 (3,394 g) and the lowest in cross 5 (1,676 g) (Table 8). To further characterize the EMS induced variations in tuber weight, the plants were categorized by class of tuber weight (Fig 9). A significant difference was observed between non-treated and EMS-treated lines in cross 2, 3, and 7 with occurrence of larger tuber classes. There was no significant difference observed between treatments in cross 1, 4, 5 and 6 (Fig 9).

Discussion

Developing potato germplasm with genetic variation is of interest as raw materials to breeders (Machida-Hirano and Niino 2017), and induced mutagenesis has been proven as a powerful tool for such purpose (McCallum et al. 2000; Slade and Knauf 2005; Uchida et al. 2011; Chantreau et al. 2013, Fofana et al. 2017). As such, EMS mutagenesis has been successfully used in crops including Arabidopsis (Kim et al. 2006), eggplant (Xi-ou et al. 2017), tomato (Shikata et al. 2015), pepper (Arisha et al. 2015), wheat (Dhaliwal et al. 2015), rice (Serrat et al. 2014) and flax (Chantreau et al. 2013; Fofana et al. 2017) to generate plant mutant...
resources and to screen for improved traits. In the current study, an EMS mutagenized diploid potato population was generated and characterized for induced genetic variations in plant and tuber morphological traits. The study reports on the development of a mutant diploid potato germplasm collection and documents for the first time a wide range of EMS-induced phenotypic variations in a mutagenized diploid potato germplasm collection.

In the current study, a germplasm collection was developed. A 1.2% rate of EMS was found to be optimal for generating mutagenized potato seedlings from true potato seeds. In previous studies, 0.6 - 1% (v/v) EMS was used to induce variations in *Solanum melongena* L and diploid potato (Xi-ou et al. 2017, Muth et al. 2008; Uitdewilligen, 2012) while Li et al. (2017) applied 1.5% EMS for mutagenesis in hexaploid wheat. The data from these studies suggest that there is a need for optimizing EMS concentration for each crop. EMS treatment reduced the germination rate in the EMS-treated seed (55-75%) compared to controls (82 - 100%). This observation was expected and is in agreement with previous studies reporting that the absorption of EMS mutagen by the plant tissue (Serrat et al. 2014) leads to the alteration of some enzymatic activities by either delaying or completely inhibiting several biological processes (Arisha et al. 2015; Talebi et al. 2012). Our data showed that seed exposure to 1.2% for 6 h resulted in very low seed germination, supporting the detrimental effect of EMS on seed biology following a dose and exposure duration relationship. EMS treatment also affected seedling survival compared with the controls, probably due to cellular and metabolic damage caused to germ cells and other cell constituents (Serrat et al. 2014).

After mutagenesis and plant regeneration, extensive phenotypic variations were observed in EMS-treated plants compared to non-treated controls as expected. The extent of morphological variations ranged from variation in growth habit, leaf structure, flower color,
tuber color, number of tubers and tuber weight. As reported in previous induced mutagenesis studies in other crops (Arisha et al. 2015; Kolar et al. 2011), variegate and chlorotic leaves were the most visible variants observed both in the greenhouse and in the field trials. Partial and completely chlorotic leaves were more expressed in field trial compared to greenhouse trial. One may expect that alteration of chloroplast biogenesis genes in the leaves and stems may also be extended to the tuber and, hence, impacting on tuber greening when exposed to light. Since the field-grown plants were obtained from the minitubers collected from the greenhouse but not from M2 true seed, this observation suggests that the induced M1 mutations in the seed and seedling were passed on, clonally conserved in the minitubers, and phenotypically expressed in the adult plants regenerated in the field. Arisha et al. (2015) reported residual effect of EMS on germination in different generations of pepper seed, highlighting the segregation behavior of heterozygous induced mutations in the true seeds. Variations observed in the plant growth habit and plant architecture were more manifested in the field conditions and are evidence that cell wall building processes including lignification may have been altered in the lines showing such phenotypes. Of interest were also the induced variations in the flower and tuber color, tuber number and size which are of agronomic interests. Variability in the tuber shape, size and weight in EMS-treated potatoes may be due to induced mutations in granule bound starch synthesis genes such as SSI known as Waxy (Muth et al. 2008), SSII, SSIII, SSIV and genes encoding starch branching enzymes such as SBEI, SBEII (Zeeman et al. 2010; Brummell et al. 2015). More tuber color variations were observed in EMS-treated plants than in non-treated plants. This observation indicates that EMS treatment altered color biosynthesis genes including flavonoid and anthocyanins biosynthesis genes in some lines.
Changes in the plant phenology as well as in the plant canopy were also some interesting traits observed in the population. Some lines within the same treatment were rendered early or late maturing whereas others had developed large canopy and abundant root system, but unable to produce any tubers. Genetic variation in the maturity phenology is of high agronomic interest, especially in regions with short growing season (Blackman 2017). The observed inability for some lines to tuberize is an evident sign of mutation in one or multiple genes involved in starch synthesis (Muth et al. 2008) and mobilization into the tubers (Van Harsselaar et al. 2017) as well as in the tuber inducing biomolecules such as gibberellins (Xu et al. 1998; Dutt et al. 2017), auxins (Roumeliotis et al. 2012) phytochrome B (Jackson et al. 1998), and sucrose transporter genes namely SUT1, SUT2 and SUT4 (Chincinska et al. 2008). The use of forward genetics by targeting genes involved in important agronomic traits such as starch content and structure, critical photosynthetic genes, or anti-nutritional factors will help understanding the genetic, molecular and physiological mechanisms involved in some of the phenotypic variations. As such, the mutant diploid potato genetic resources is currently under further investigation by using a targeted NGS approach coupled with the phytochemical profiling of tuber tissue to identify EMS-induced mutations causing loss-of-function in a panel of 11 genes associated with the production of anti-nutritional factors such as steroidal glycoalkaloids and acrylamide, and ultimately leading to identifying mutant potato lines low in one or both of these toxicants.

In conclusion, we developed a mutagenized diploid potato germplasm collection with a wide induced genetic and phenotypic variation. This potato genetic resource is valuable not only as a pre-breeding material but also for understanding physiological phenomena such as potato greening, self-incompatibility in potato, and the potential for selecting diploid lines with reduced anti-nutritional factors.
References


Table 1. Pedigree and total number of true potato seeds used as starting material in each cross

<table>
<thead>
<tr>
<th>Cross</th>
<th>Pedigree</th>
<th>Female</th>
<th>Ploidy</th>
<th>Male</th>
<th>Ploidy</th>
<th>Cross</th>
<th>Number of seeds</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
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<td>1</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>10301-07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2x</td>
<td>1994</td>
<td>504</td>
<td>Fredericton</td>
<td>aDziewońska and Was, 1994</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>11059-01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2x</td>
<td>1994</td>
<td>550</td>
<td>Fredericton</td>
<td>cWang-Pruski et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>11065-01</td>
<td>2x</td>
<td>1996</td>
<td>500</td>
<td>Fredericton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>F20.1 I&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2x</td>
<td>1997</td>
<td>460</td>
<td>Fredericton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>10875-04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2x</td>
<td>1997</td>
<td>500</td>
<td>Fredericton</td>
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<tr>
<td>6</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>10612-03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2x</td>
<td>1997</td>
<td>480</td>
<td>Fredericton</td>
<td>dShaterian et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>DW84-1457&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2x</td>
<td>12120-03</td>
<td>2x</td>
<td>1998</td>
<td>586</td>
<td>Fredericton</td>
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<tr>
<td>8</td>
<td>A373&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1x</td>
<td>BLR07&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>1996</td>
<td>200</td>
<td>Lethbridge</td>
<td>bLynch et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>A262&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>BLR07&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2x</td>
<td>1996</td>
<td>420</td>
<td>Lethbridge</td>
<td>cZimnoch-Guzowska et al. (1999)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a, b, c, d, e</sup>Source references for the indicated plant material; All true potato seeds derived from the nine crosses were a courtesy gift by Dr. Benoit Bizimungu, Fredericton Research and Development Centre, Agriculture and Agri-Food Canada.
Table 2. Conditions used for treating individual true potato seed lots with EMS

<table>
<thead>
<tr>
<th>Cross</th>
<th>EMS (%)</th>
<th>Incubation Time (h)</th>
<th>Number of seeds planted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTL</td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
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<td>40</td>
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<tr>
<td>4</td>
<td>1.2</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>1.2</td>
<td>6</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 3. Detailed representation of the number of lines planted in the field for each cross

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of tubers planted</th>
<th>Ratio per cross (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTI</td>
<td>EMS</td>
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<td>1</td>
<td>10</td>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>10</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>104</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>129</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>79</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>166</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>836</td>
</tr>
</tbody>
</table>
Table 4. Effects of EMS treatment on seed germination and seedling survival in greenhouse. The number of seed planted, % germination, and number of dead seedlings are shown.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of seeds planted</th>
<th>Number of seeds germinated</th>
<th>Germination %</th>
<th>Number of seedlings transplanted</th>
<th>Dead seedlings</th>
<th>Seedlings survived</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>EMS</td>
<td>CTL</td>
<td>EMS</td>
<td>CTL</td>
<td>EMS</td>
<td>CTL</td>
</tr>
<tr>
<td>1</td>
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<td>420</td>
<td>40</td>
<td>274</td>
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<td>65.2</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>430</td>
<td>38</td>
<td>321</td>
<td>95</td>
<td>74.6</td>
<td>36</td>
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<tr>
<td>3</td>
<td>40</td>
<td>442</td>
<td>33</td>
<td>333</td>
<td>82.5</td>
<td>75.3</td>
<td>31</td>
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<td>40</td>
<td>361</td>
<td>34</td>
<td>225</td>
<td>85</td>
<td>62.3</td>
<td>31</td>
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<tr>
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<td>397</td>
<td>39</td>
<td>231</td>
<td>97.5</td>
<td>58.2</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>460</td>
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<td>330</td>
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<td>71.7</td>
<td>34</td>
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<tr>
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<td>251</td>
<td>90</td>
<td>54.9</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>280</td>
<td>2,967</td>
<td>256</td>
<td>1,965</td>
<td>91.1</td>
<td>66.2</td>
<td>246</td>
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</table>
Table 5. Plant morphological variations in non-treated and EMS-treated lines in greenhouse

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of observed plants</th>
<th>Plants with defined stem color type</th>
<th>Plants with flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
<td>Purple</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTL</td>
<td>EMS</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>39</td>
<td>273</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>36</td>
<td>306</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>30</td>
<td>329</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>28</td>
<td>139</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>39</td>
<td>180</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>34</td>
<td>322</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>36</td>
<td>227</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>242</td>
<td>1776</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 6. Variations between non-treated and EMS-treated lines for tuber number as collected from the greenhouse

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of plants observed</th>
<th>Number of tubers per plant</th>
<th>Mean ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>EMS</td>
<td>CTL*</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Minimum</td>
</tr>
<tr>
<td>1</td>
<td>39</td>
<td>273</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>306</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>329</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>139</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>180</td>
<td>2</td>
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<tr>
<td>6</td>
<td>34</td>
<td>322</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>227</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation

* In the non-treated control lines a total of 4 and 1 plants did not produce any tubers in crosses 1 and 2, respectively.

** In the EMS-treated lines a total of 5, 2, 2, 17, 2, 7, 3 plants did not produce any tubers in crosses 1, 2, 3, 4, 5, 6 and 7, respectively.
Table 7. Variations between non-treated and EMS-treated lines for tuber number as collected from the field

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of plants observed</th>
<th>Number of tubers per plant</th>
<th>Mean ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>EMS</td>
<td>CTL</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Minimum</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>74</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
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<td>14</td>
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<tr>
<td>4</td>
<td>9</td>
<td>47</td>
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</tr>
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<td>5</td>
<td>10</td>
<td>105</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>65</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>105</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation
Table 8. Variations between non-treated and EMS-treated lines for tuber weight as collected from the field

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of plants observed</th>
<th>Weight of tubers per plant (g)</th>
<th>Mean ± SDa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>EMS</td>
<td>CTL</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Minimum</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>127</td>
<td>141</td>
</tr>
<tr>
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<td>10</td>
<td>85</td>
<td>565</td>
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<tr>
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<td>47</td>
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<td>10</td>
<td>105</td>
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<tr>
<td>7</td>
<td>10</td>
<td>105</td>
<td>16</td>
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</table>

aStandard deviation
**Figure Legends**

**Fig 1.** Effect of EMS treatment and incubation time on germination percentage

**Fig 2.** Distribution of potato lines by number of tubers produced in each cross. The number of tubers in individual lines within each cross was organized by classes in non-treated controls (a) and EMS-treated lines (b). Note that EMS4, EMS5, and EMS7 have 5, 4, and 4 categories in the higher number of tubers compared to their respective CTLs.

**Fig 3.** Tuber weight variation between non-treated (CTL) and EMS-treated potato lines as observed in the greenhouse. Tuber weight in individual lines within each cross was categorized by classes for comparison. Note that EMS1, EMS2 and EMS5 have 4, 5, and 5 categories, respectively in the higher tuber weight compared to their respective CTLs.

**Fig 4.** Tuber color variation between non-treated (CTL) and EMS-treated potato lines as observed in the greenhouse. The percentage of lines showing a specific color is shown. Note that EMS2, EMS3, EMS4, EMS5, and EMS6 have 4, 2, 1, 2, 1 more tuber color categories compared to their respective CTLs.

**Fig 5.** Variations in leaf shape and color as observed in the EMS-treated lines as observed in the field. (a) normal leaf; (b) incomplete (half) leaf; (c) purple leaf tip with needle shape; (d) deformed leaf with only midrib; (e, f) partially chlorotic mutants; (g) completely chlorotic mutant.

**Fig 6.** Variations in the growth habit of EMS treated lines in the field. (a) Plant growing laterally with determinate growth habit; (b) rosette plant with compact internodes; (c) small plant with
single main stem and few leaves; (d) weak stem with lodging growth habit; (e) large plant, multiple main stems with small and few leaves; (f) large bushy plant.

**Fig 7.** Floral color variations observed between non-EMS treated controls (a, b, c) and EMS-treated potato lines (a-h) in the field.

**Fig 8.** Variations in tuber color observed between non-EMS treated controls (a, b, c) and EMS treated lines (a, b, c, d, e, f, g, h) in the field.

**Fig 9.** Variations observed between non-treated and EMS-treated lines in the field for tuber weight. The percentage of lines showing a specific range of tuber weight is shown. Note that EMS2, EMS3 and EMS7 have 4, 3, and 3 categories, respectively in the higher tuber weight compared to their respective CTLs showing only 2 categories.
Fig. 1

Germination (%)

Incubation Time

4h  5h  6h

56  66  62

68  70  64

72

127x76mm (150 x 150 DPI)
Fig. 2

171x62mm (150 x 150 DPI)
Fig. 3

162x84mm (143 x 143 DPI)
Fig. 4

160x102mm (150 x 150 DPI)
Fig. 5

160x77mm (150 x 150 DPI)
Fig. 6

157x94mm (150 x 150 DPI)
Fig. 7

150x72mm (150 x 150 DPI)
Fig. 8

158x68mm (150 x 150 DPI)
Fig. 9

154x88mm (150 x 143 DPI)