Mutagenesis in Gynonomoecious Spinach (Spinacia oleracea L.) Plants and Selection of Low Oxalate Variants

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This study was conducted to evaluate mutagenesis in gynonomoecious spinach (Spinacia oleracea L.) plants for inducing low oxalate variants. Gamma-ray and ion beams of 220 MeV $^{12}$C$^{+}$ and 50 MeV $^{4}$He$^{2+}$ were used as mutagen in seed irradiation. Optimum dosages for irradiation were determined to be about 100 Gy, 15-20 Gy and 150-200 Gy in gamma-ray, $^{12}$C$^{+}$ and $^{4}$He$^{2+}$, respectively. In $M_2$ generation, there was one line segregating albino seedlings, one line segregating xantha seedlings and two lines segregating viridis seedlings, which indicates that recessive mutation could be expressed in $M_2$ generation due to the capacity for self-fertilization of gynonomoecious plants in an otherwise dioecious spinach. To save on labor and time for analysis, selection of low oxalate variants in $M_2$ generation was conducted by a two-step selection which consisted of the first analysis of bulked leaves from 2 plants as one specimen followed by the second analysis of selected individual plants. In the first analysis of 813 specimens, we selected 13 specimens as low and 9 specimens as high in oxalate content. In the second analysis, there was consistency in the distribution of low and high oxalate content corresponding to the first screening, indicating that selection of low oxalate variants could be achieved by this two-step selection with half the labor and time for analysis as compared to non-bulked method. There were no clear differences in distribution of oxalate content between $M_3$ progenies of plants selected as low or high oxalate content, suggesting that the low oxalate content in plants isolated in $M_2$ generation was not of a genetic origin. From these results, it seems to be necessary to explore a variant with obvious deviation from the continuous variation of oxalate content in the $M_2$ generation.

Key words: gamma-ray, ion beam, low oxalate, mutation breeding, screening.

Introduction

Spinach (Spinacia oleracea L.) is one of the common vegetables grown worldwide for its high nutritious value. However, it accumulates large amounts of oxalate, which causes inhibition of calcium absorption and contributes to the formation of urinary stones. Thus, reduction of leaf oxalate content is one of the most important issues in spinach production. Several attempts to reduce oxalate content in spinach leaves have been made by cultivation techniques, including nitrogen management, water stress or biotic stress. However, such reduction of oxalate content was accompanied by reduction in plant growth. To date, no effective method capable of substantially reducing the oxalate content without concomitant reduction in plant growth has been reported. There is a potential for reducing oxalate content genetically through breeding and selection strategies. Recently, Kawazu et al. investigated the oxalate content of 213 spinach varieties, with the result that none of these had significantly low oxalate content. Mutation breeding could be a potential strategy for producing novel variant with low oxalate content.

In mutation breeding of self-fertilized species, recessive mutant genes are induced in $M_1$ plants, and identified in homozygous status in $M_2$ progeny. In cross fertilized species, however, mutant genes in-

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duced in M₁ plants remain heterozygous in M₂ progeny, making phenotypic detection of mutants difficult. Due to this, mutation breeding programs in cross fertilized species have been limited²⁹.

Spinach is a typical dioecious species, but there are spontaneously a few gynomonoecious plants with the capacity for self-fertilization, which can be applicable to mutation breeding programs. Handke⁶ reported that a mutant with a very long lasting vegetative phase in spinach ‘Fruremona’ was selected from EMS-treated M₁ plants. The mutant was initially gynoecious, but selfed-M₂ seeds could be obtained by subsequent formation of 5–10% male flowers in this M₁ plant.

A line with high frequency of gynomonoecious plants and a stable occurrence of hermaphroditic flowers is potentially useful in mutation breeding programs. The expression of gynoecomy was affected by genetic and/or environmental factors. Occurrence of hermaphroditic flowers in gynomonoecious plants was promoted by high temperature and long photoperiod²⁹. A line segregating gynomonoecious plants with high frequency can be established by successive self-fertilization of gynomonoecious plants⁶,¹⁷,²⁹. Production of self-fertilized seeds in gynomonoecious spinach plants can be performed effectively by cultivation at around 25°C after bolting induction at 20°C or cultivation during short photoperiodic season in Japan by extending the photoperiod artificially to around 16h⁹.

Gamma-ray has been used as a common mutagen. Recently, ion beams have been found to be very effective mutagens. These have much higher linear energy transfer (LET) and relative biological effectiveness (RBE) than those of gamma-ray²⁹. Komai et al.⁵⁰ reported the effect of ion beam irradiation as some kind of stress to spinach seeds on its sex expression, but the effects of ion beams as a mutagen were not investigated fully.

In this study, the potential for induction and selection of low oxalate variants in gynomonoecious spinach plants irradiated with gamma-ray and ion beams was investigated.

**Materials and Methods**

**Plant material**

In the present study, *Spinacia oleracea* L. ‘Shin-Nippon’ (Atariya Seed, Chiba) was employed. Self-fertilized seeds of gynomonoecious plants found in a commercial seed population were used. Seeds were soaked in tap water for 1.5h. After removing pericarps, the seeds were dried back and stored.

**M₁ plant culture**

Lots of 100 de-coated seeds, divided randomly, were irradiated with gamma-ray at 100–300 Gy and 220 MeV ³²Si ions at 5–30 Gy or 50 MeV ⁴He²⁺ ions at 50–300 Gy, at JAEA, Takasaki, Japan. The seeds were sandwiched between Kapton films (7.5 µm thickness; Toray-Dupont, Japan) in order to make a monolayer, which enabled uniform irradiation.

These M₁ seeds were sown in cell flat (288 cells per tray, 30 × 60 cm) filled with vermiculite on 7 February, 2003. Seedlings were irrigated with a complete nutrient solution (Ohtsuka A-Solution, Ohtsuka Chemical, Osaka) from 10 days after sowing. On 18 March, surviving seedlings were transplanted to plastic pots (10 cm diam.), containing a 1:1 mixture of vermiculite and commercial soil. In mid-April, these plants were planted out in an open-sided plastic house, in which N, P₂O₅, and K₂O had been applied to the soil at 15 kg · 10 a⁻¹, with spacing of 20 cm between and 15 cm within rows. Bolted plants were covered with transparent bags before anthesis to prevent cross-pollination. Self-fertilized M₂ seeds were harvested from 337 M₁ plants individually in July.

**M₂ plant culture**

On 15 September, 2003, 16 seeds per M₂ line, total 5392 seeds in 337 M₂ lines, were sown in cell flats (128 cells per tray, 30 × 60 cm) filled with vermiculite. At sowing time, the cell flats were put on a 60 cm-wide row with 5 cm interval in the open-sided plastic house, in which N, P₂O₅ and K₂O were applied to the soil at 20 kg · 10 a⁻¹. To enable root development into the soil from basal hole of the cell flat, the seedlings were thoroughly irrigated from surface of cell flats for 2 weeks after sowing. Thereafter, water was applied by irrigation between the rows.

**First screening for low oxalate variants in M₂ generation**

On 15 August, 2003, one leaf disc with 6 mm diameter was taken from the apical part of the largest leaf blade in each of 1607 M₂ plants at 6-8 leaf stage. Samples consisting of leaf discs from 2 plants were put into 2 ml microtubes. 1.9 ml 1N H₂SO₄ solution was added, and the tubes left for 24 h at room temperature. Each extracted solution was considered to form one specimen for analysis of total oxalate content, and a total of 813 specimens were prepared. From the extracted solution, 0.1 ml supernatants were diluted to 1:10 with 0.9 ml of distilled water and these solutions were analyzed with a HPLC system by using a Shim-pack SCR-102H column (3 × 300 mm, Shimadzu, Kyoto) connected to a guard column (3 × 50 mm, Shimadzu, Kyoto). Detection was done by using a
SPD-6A UV/visible spectrophotometer (Shimadzu, Kyoto), set at 210 nm. The mobile phase was 1% phosphoric acid and the flow rate was 1.5 ml·min⁻¹. The temperature of the column was set at 80°C and 10 μl of the sample was loaded. The resulting chromatograms were analyzed with a data processing program (BROWN, JASCO, Tokyo).

**Second screening for low oxalate variants in M₁ plants selected in the first screening**

From the first analysis of total oxalate content, 9 specimens with high oxalate content and 13 specimens with low oxalate content were isolated from the 813 specimens. The 9 specimens consisted of 18 plant samples, while the 13 specimens among the low oxalate group consisted of 24 plant samples; 4 plants were discarded due to poor growth. On 22 December, 2003, (about 2 months after the first analysis), one leaf disc with 6mm diameter was taken from the apical part of the 4th-expanded leaf blade of each plant. The second analysis of total oxalate content was carried out as described above.

**Culture and oxalate analysis in M₁ progenies of plants selected in M₁ generation**

From the second analysis of total oxalate content, 3 plants with low oxalate content (596, 650 and 658 mg · 100 g FW⁻¹) and 3 plants with high oxalate content (2593, 2005 and 1966 mg · 100 g FW⁻¹) were selected. In April, 2004, the plants were covered with transparent bags to prevent cross-pollination, and M₂ seeds were harvested in early June. M₂ seeds were obtained in plants with 658 mg · 100 g FW⁻¹ and 1966 mg · 100 g FW⁻¹ oxalate content at the second analysis, because the other plants did not form hermaphroditic flowers. Sixteen seeds of each M₂ progeny were sown on cell flats (128 cells per tray, 30 × 60 cm) filled with vermiculite. These cell flats were put in growth chamber set to 12h photoperiod, 280 μmol · m⁻² · s⁻¹ and 23/17 °C. Plants were fertigateded everyday with a complete nutrient solution (Ohtsuka A-Solution, Ohtsuka Chemical, Osaka) from 7 days after sowing. At 1 month after sowing, one leaf disc with 6mm diameter was taken from the apical part of 4 th leaf blade at 6-8 leaf stage, and the analysis of total oxalate content in each plant was carried out as above.

**Results**

The frequency of survived M₁ seedlings reduced dramatically with increase of radiation doses above 100 Gy of gamma-ray, 20 Gy of ¹²C⁺⁺ and 200 Gy of ¹²He⁺⁺ (Fig. 1A). The LD₅₀ was estimated to be about 150, 30 and 250 Gy for gamma-ray, ¹²C⁺⁺ and ¹²He⁺⁺ irradiation, respectively. Based on this result, relative

![Fig. 1 Effect of irradiation source and dose on survival rate (A), gynomonoecy (B) and selfed-seed production (C) in spinach. Survival rate shows the number of survived plants per germinated plants. Frequency of gynomonoecious plants shows the number of gynomonoecious plants per cultivated plants. The rate of self-fertilized seed production shows the number of plants producing self-fertilized seeds per sown seeds.](image)
biological effectiveness (RBE) on lethality was 5 in $^{12}$C$^+$ and 0.6 in $^4$He$^+$ as compared with gamma-ray. The frequency of gynomonoecious plants was 60% at 100 Gy of gamma-ray and 48% at 5 Gy of $^{12}$C$^+$ and it decreased with increase in radiation dose (Fig. 1B). This frequency was 63–72% at 50–150 Gy and 38–56% at 200–300 Gy of $^4$He$^+$ irradiation. The rate of selfed-seed (self-fertilized seed) production was 31% at 100 Gy of gamma-ray and 41% at 5 Gy of $^{12}$C$^+$ and this value also decreased with increase of radiation dose (Fig. 1C). The rate was 40–44% at 50–150 Gy of $^4$He$^+$ irradiation and reduced with the increase of irradiation dose above 150 Gy.

Among 337 $M_s$ lines, there was one line segregating albino plants at 10 Gy $^{12}$C$^+$ treatment, one line segregating xantha plants at 250 Gy $^4$He$^+$ treatment and two lines segregating viridis plants in each treatment of 10 Gy $^{12}$C$^+$ and 50 Gy $^4$He$^+$ at cotyledonal stage (Table 1).

The first analysis of total oxalate content was conducted in one-month-old 1607 $M_s$ plants. Leaf discs from 2 plants were bulked and analyzed as one specimen, but there were some specimens which consisted of single plants because specimens were prepared in each irradiation source and dose. Thus, a total of 813 specimens were analyzed. The variation of total oxalate content ranged from 0.57–1.76 $\mu$mol/leaf disc continuously, and mean value was 1.11 $\mu$mol/leaf disc (Fig. 2). Black bars in Fig. 2 show 13 specimens which consist of low oxalate content below 0.7 $\mu$mol/leaf disc and account for 1.6% of all the specimens. We isolated 13 specimens consisting of 24 plants as low in oxalate content. Four of these plants, however, were discarded because of poor growth. White bars in Fig. 2 show 9 specimens with high oxalate content of over 1.7 $\mu$mol/leaf disc and account for 1.1% of all the specimens. We also selected 9 specimens consisting of 18 plants as high in oxalate content. The result of second analysis of total oxalate content in these selected plants is shown in Fig. 3. Plants selected as low in oxalate content in the first analysis (shown as black bars) had oxalate content ranging between 596–1969 mg $\cdot$ 100 g FW$^{-1}$. On the other hand, plants selected as high in oxalate content in the first analysis (shown as white bars) had oxalate content ranging between 821–2593 mg $\cdot$ 100 g FW$^{-1}$. In the second analysis, there was consistency in the distribution of low and high oxalate content corresponding to the

![Image](image.jpg)

Fig. 2  Frequency distribution of total oxalate content in the first screening for bulked leaf discs from two plants.

<table>
<thead>
<tr>
<th>(Gy)</th>
<th>No. of $M_s$ seeds used for $M_2$ progeny</th>
<th>No. of $M_1$ seeds with chlorophyll mutation in $M_2$ progeny</th>
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<tbody>
<tr>
<td></td>
<td>Albino</td>
<td>Xantha</td>
</tr>
<tr>
<td>$\gamma$-ray</td>
<td>100</td>
<td>31</td>
</tr>
<tr>
<td>$^{12}$C$^+$</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>0</td>
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<td>20</td>
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<tr>
<td>25</td>
<td>8</td>
<td>0</td>
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<tr>
<td>30</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>$^4$He$^+$</td>
<td>50</td>
<td>41</td>
</tr>
<tr>
<td>100</td>
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<td>0</td>
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<tr>
<td>300</td>
<td>9</td>
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first screening.

The distribution of total oxalate content mostly ranged from 1300–1900 mg/100 g FW⁻¹ in M₂ progeny of plants selected as low or high oxalate content (Fig. 4). There was one plant with oxalate content of 841 mg/100 g FW⁻¹ in the progeny of plant selected as high oxalate content.

![Graph](image-url)

**Fig. 3** Frequency distribution of total oxalate content in the second screening for each individual plant selected in the first screening.

![Graph](image-url)

**Fig. 4** Distribution of oxalate content in M₂ progeny of the plants selected as low and high oxalate content in M₂ generation. Black and white bars show progeny of the plant selected as low oxalate plants (658 mg/100 g FW⁻¹) and high oxalate plants (1966 mg/100 g FW⁻¹) in M₂ generation, respectively.

**Discussion**

The frequency of survived M₃ seedlings was observed to reduce dramatically with the increase of radiation doses above 100 Gy of gamma-ray, 20 Gy of ¹²C⁺⁺ and 200 Gy of ²³He⁺⁺ (Fig. 1A). The RBE of lethality was estimated to be 5 in ¹²C⁺⁺ and 0.6 in ²³He⁺⁺ based on the LD₅₀. Komai et al.¹² also reported that ¹²C⁺⁺ was more effective than ²³He⁺⁺ in suppressing germination and flowering in spinach 'Jiromaru', but they did not investigate the effectiveness of gamma-ray. Our result, in which the RBE of ²³He⁺⁺ was lower than 1, was different from the results in other plant species such as carnation¹⁶, chrysanthemum¹⁹, rose¹⁷, salvia¹⁹ and tobacco¹⁹.

Komai et al.¹² reported that 6.3 - 50.0 % gynomonoecious plants were induced from gynoeccious seeds of spinach 'Jiromaru' by exposure to ¹²C⁺⁺ and ²³He⁺⁺, whereas unexposed control seeds consisted of 100 % gynoeccious plants. They suggested that in gynomonoecy, exposure to ion beams might release male flowers from being systematically suppressed on a gynoeccious plant. However, in our study, the frequency of gynomonoecious plants did not increase with seed irradiation of ¹²C⁺⁺ and ²³He⁺⁺ or with gamma-ray (Fig. 1B).

The rate of selfed seed production tended to decrease with increase of radiation dose in each radiation source (Fig. 1C). Based on the plant development from seedling survival and production of self-fertilized M₃ seeds, optimum dosage for seed irradiation was determined to be about 100 Gy, 15–20 Gy and 150–200 Gy in gamma-ray, ¹²C⁺⁺ and ²³He⁺⁺, respectively.

In mutation breeding schemes, 20 M₂ seeds per M₁ plant are generally used for screening of M₃ generation. In this study, 337 M₂ lines were tested by sowing 16 seeds per M₂ line. There was one line segregating albino plants, one line segregating xantha plants and two lines segregating viridis plants at the cotyledonary stage (Table 1). This result indicated that recessive mutations could be expressed in M₂ generation by self-fertilization of gynomonoecious plants in dioecious spinach.

We screened for plants with low oxalate content at 6–8 leaf stage. It is known that oxalate content in spinach varies among plant organs and tissues analyzed. Libert and Franceschi¹⁴ and Tone and Uchiyama²⁰ reported that leaf blade contained higher oxalate than leaf petiole in spinach. Furthermore, oxalate content within same leaf blade of spinach was higher at the apex than at the base²¹. When oxalate content in the parts known to have high oxalate
content is analyzed, it may be easy to isolate the plants with low oxalate content. Therefore, in this study, oxalate content in the apical part of leaf blade was analyzed in all plants.

In order to save labor in preparing the samples, oxalate was extracted by immersing the leaf disc in 1N-H$_2$SO$_4$ without homogenization. Spinach contains water-soluble oxalate and water-insoluble oxalate. The sum of water-soluble and water-insoluble oxalate can be extracted and evaluated as total oxalate at once by extraction with acid solution. Oxalate was extracted by immersing 1 disc in 5ml 3N-HCl for 24 h$^{13}$ and 10 discs in 1ml 2N-HCl for 16h$^{13}$. In this study, 2 discs were immersed in 1.9ml 1N-H$_2$SO$_4$ for 24h, and the resulting value of oxalate content was consistent with the reports.

Bulk-method can be more effective for the reduction of labor and time for analysis in many plants. Gachotte et al.$^{13}$ reported that a mutant deficient in sterol biosynthesis was isolated from 22000 plants of commercial M$_2$ population of *Arabidopsis thaliana* by first analyzing 1100 pools of 20 plants which was followed by second analysis of 20 individual plants in one pool presenting an abnormal profile. This mutant was characterized by the accumulation of three sterols which were not detected in wild-type leaves concomitantly with the decrease of three corresponding sterols which are the end products of the sterol pathway in wild-type leaves. Since this mutant was due to a single recessive nuclear mutation, it is assumed that the existence of mutants could be recognized qualitatively in the first analysis even if bulked leaves from 20 individuals were analyzed as one specimen.

It is not clear whether the character of low oxalate content in spinach expresses as a qualitative phenotype. Therefore, in this study, the first screening was conducted by the analysis of bulked leaves from 2 plants as one specimen, and selected individual plants in the first screening were subsequently analyzed. Data analyzed in the first screening indicates mean value of two individual plants; a specimen, in which one of the two may contain extremely low oxalate or both individuals may contain low oxalate. It is, however, possible that a plant with low oxalate content may be obscured in the first screening if the other in the pair has a very high oxalate content. Nevertheless, we preferred the two-step selection to non-bulked method because of the 50% reduction of labor and time for analysis.

The variation of oxalate content in the first screening ranged widely and continuously from 0.57-1.76 µmol/leaf disc (Fig. 2). This result is consistent with the report that there was wide and continuous variation of oxalate content in spinach$^{13}$ and rhubarb$^{13}$. In the first screening, we selected 13 specimens as low and 9 specimens as high in oxalate content, then the second screening was carried out for each individual plant. Values of the second screening tended to distribute in the range of lower or higher oxalate content corresponding to the first screening (Fig. 3). This result indicated that selection of low oxalate variants could be achieved by this two-step selection with half the labor and time for analysis as compared to non-bulked method.

There were no clear differences in distribution of oxalate content between M$_1$ progenies of plants selected as having low or high oxalate content (Fig. 4). Furthermore, plants containing relatively low oxalate ranging between 700-900 mg · 100g FW$^{-1}$ were observed in the progeny of the plant selected as having high oxalate content, whereas such plants were not observed in the progeny of the plants selected as having low oxalate content. From these results, low and high oxalate contents in plants selected in M$_1$ generation did not seem to be of a genetic origin. Kataoka et al.$^{13}$ also reported that cross-pollinated progeny of selected spinach plants with low oxalate contents of 599-704 mg · 100g FW$^{-1}$ contained 1088mg · 100g FW$^{-1}$ oxalate, which was almost the same value as cross-pollinated progeny of selected plants with high oxalate contents of 1309-1459mg · 100g FW$^{-1}$. These results suggest that environmental influence of oxalate content is large in spinach. It has also been reported that heritability in the characters of oxalate content was low in spinach$^{13}$ and rhubarb$^{13}$.

A number of pathways for oxalate production have been proposed. Precursors of oxalate in spinach were reported to be glyoxylate derived from photorespiration$^{23}$, oxaloacetate$^{28}$ and L-ascorbic acid$^{23}$. Although these pathways have been identified, it is still unknown how many genes are involved in oxalate production. It was recently reported that many mutants defective in calcium oxalate crystal formation were isolated in *Medicago truncatula*; each mutant was due to a single gene mutation$^{13}$, and there were some mutants with very low total oxalate content$^{13,16}$. Therefore, we emphasize that there is a very high potential for obtaining a qualitative trait of low oxalate content through induced mutation in spinach.

In conclusion, it is indicated that mutagenesis is achieved by seed-irradiation of gamma-ray and ion beam, and recessive mutation could express in M$_1$ generation by using the capacity of self-fertilization of gynonoecious plants in dioecious spinach. Further-
more, selection of low oxalate variants could be achieved through specimen bulking with half the labor and time for analysis as compared with the non-bulked method. The bulking method requires a two-step selection; the first analysis of bulked leaves from 2 plants and the second analysis of selected individual plants. Since environmental influence on oxalate content seems to be large in spinach, it is necessary to explore a variant with obvious deviation from the continuous variation of oxalate content in the M2 generation.

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References

ホウレンソウ雌性間性株における
突然変異誘発ならびに低シュウ酸個体の選抜

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本实验では、ホウレンソウ雌性間性株における突然変異誘発ならびに低シュウ酸個体の選抜を試みた。種子照射の変異原としては、γ線と220MeV 12C + などに50MeV 4He + イオンビームを用いた。照射の最適線量は、γ線はおよそ100Gy、12C + は15～20Gy、4He + は150～200Gyであった。M1株の自殖により得られたM2世代において、葉緑素突然変異体のアルビノ、キサンタを分離する系統が、それぞれ1系統、ピリディスを分離する2系統が確認された。このことから、雌雄異株植物であるホウレンソウにおいて、自殖性を有する雌性間性株を利用することにより、M2世代において効率の突然変異を発現させられることが示された。M2世代における低シュウ酸個体の選抜は、2個体分を1検体として二次分析し、低シュウ酸検体の各個体を二次分析するという2段階評価法により行い、労力の軽減を図った。813検体の二次分析により、低シュウ酸である13検体を選抜し、比較対照として高シュウ酸である9検体も選抜した。二次分析においても、低シュウ酸選抜個体は低シュウ酸側に、高シュウ酸選抜個体は高シュウ酸側に分布する傾向にあった。このことから、この2段階選抜によって、1個体ずつ分析する場合の半分の労力で、低シュウ酸個体を検定できることが示された。しかしながら、低シュウ酸として選抜したM3個体の後代では差が少なく、M2世代で選抜した個体ではシュウ酸含量が、遺伝的性質に起因しないことが示唆された。したがって、低シュウ酸突然変異個体を選抜するには、M2世代において、連続的なシュウ酸含量の集団とは異なる、非連続的な個体を、探し求める必要があると考えられた。

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