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Research Article

Genetics Materials for Experimental Class of Mendel's 3rd Law Using Dihybrid Crosses of Lettuce Cultivars in High School

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Mendel's Law of Independent Assortment was included in biology education in high school in many countries. Gregor Mendel performed a dihybrid cross by using two true-breeding peas having homozygous dominant or recessive alleles for each trait in a plant: one with yellow round seeds (YYRR) and one with green wrinkled seeds (yyrr). Mendel obtained the F_2 phenotypic ratio 9: 3: 3: 1 of the yellow round: green round: yellow wrinkled: green wrinkled seed plants. In this study, we performed dihybrid crosses using lettuce cultivars having homozygous dominant and recessive alleles for each trait in a plant: one with green oak leaf (rrOO) and other plants with red lobed leaf (RRoo). We obtained the F_2 phenotypic ratio the same as Mendel's experiment result, *i.e.* 9: 3: 3: 1 of red oak: green oak: red lobed: green lobed leaf plants. The results of this experiment and Mendel's experiment result of a dihybrid cross can be used to explain that the alleles of two different genes are sorted into gametes independently of one another. Lettuce crossing techniques and experimental methods used in this study are suitable for biological experiments in high school.

Key words: Dihybrid cross, lettuce, Mendel's Law of Independent Assortment, high school biological experiments, leaf traits

INTRODUCTION

Lettuces were classified in the family Asteraceae, genus *Lactuca* L. Lettuces are vegetative vegetables and famous plant materials for genetic study such as Chin *et al.* (2001) used lettuce to study recombination and spontaneous resistance genes, Jeuken *et al.* (2002) investigated the genetics and specificity of *Bremia* resistance in *L. saligna*, Christopoulou *et al.* (2015) dissected two complex clusters of resistance genes in *L. sativa*, and other articles reported their study on disease resistance genes in lettuce (Landry *et al.*, 1987; Kuang *et al.*, 2006). Lettuces have many traits derived from natural and induced mutations that are useful in genetic studies (Mou, 2011).

The use of lettuce to trace Mendel's law of inheritance was also performed by some researchers. Lindqvist (1960) crossed lettuce to trace Mendel's law of segregation (2nd law). Truco *et al.* (2007) reported the result of intra-specific crosses of lettuce species to study on integrated genetic linkage map that the majority of markers segregated match to Mendelian expectations. Even though Mendel used garden peas to conduct experiments that led to the definition of the law of inheritance, genetics experiments for junior high school or senior high school in the world

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should use usable plant materials. Recently, we introduced the use of tomato cultivars to trace Mendel's 1st and 2nd laws (Mam *et al.* 2020). This study suggested that the dwarf tomato plant was a good plant material to be used by students to research to study genetic inheritance following Mendel's 1st and 2nd laws because the dwarf trait was expressed at an early stage of tomato growth and it was easy to be recognized by students. Mendel's laws of inheritance including the 3rd law, "Law of Independent Assortment", are being studied in high school in Japan and Cambodia (Yihoop *et al.,* 2009; Arima *et al.*, 2016; Tsukada *et al.*, 2016). However, only Mendel's experiment results are being studied in these biology textbooks, there were no other experiments used to explain Mendel's Law of Independent Assortment in those biology textbooks, and there was no experimental class introduced in the textbooks. The phenotypic ratio of Mendel's 3rd law is 9:3:3:1 in the combinations of alleles of two different genes. The lowest rate is 1/16 as double recessive type in F_2 segregation. Theoretically, when teachers want students to observe 5 double recessive plants, teachers have to keep more than 80 plants until plants expressed the target characters. In tomato plants, while several fruit character differences were introduced for genetic study (Yui *et al.*, 2011), they are very difficult to keep more than 80 tomato plants until getting fruits in the schools.

In this study, we performed dihybrid crosses using lettuce cultivars to produce study materials for the experimental class of Mendel's Law of Independent Assortment. Lettuces were used in this study because lettuce leaf characters could be identified easily, their growth cycles were short and also many seeds can be obtained in a lettuce plant.

MATERIALS AND METHODS

Plant Materials

Lettuce cultivars (cv.) purchased in Japan were used in this experiment. Cv. Furiru with green oak leaf and other 3 cultivars, cvs. Red sunstar, Sunny, and Vitamin, with red lobed leaf, were used in this dihybrid cross experiment.

Crossing Techniques

Reciprocal crosses between cv. Furiru and cv. Red sunstar, cv. Sunny or cv. Vitamin were performed. The crossing followed the YouTube Video (Work with Nature, 2014). From the female plant (ovule parent), the flower bud was selected in the early morning at around 6:00 AM (Figure 1a), the yellow part of the flower was cut (Figure 1b), the flower cut was kept for about 3 hours to make style elongate (Figure 1c) and then the flower was sprayed or soaked in water to remove their pollens. From male plant (pollen parent), at the crossing time, a full-bloomed flower was selected (Figure 1d and Figure 1e), the petals of the flower were cut to make naked style with pollen (Figure 1f), and then this flower was tapped on the female flower (Figure 1c) to pollinate. The fertilized fruit could be observed a few days after crossing (Figure 1g).

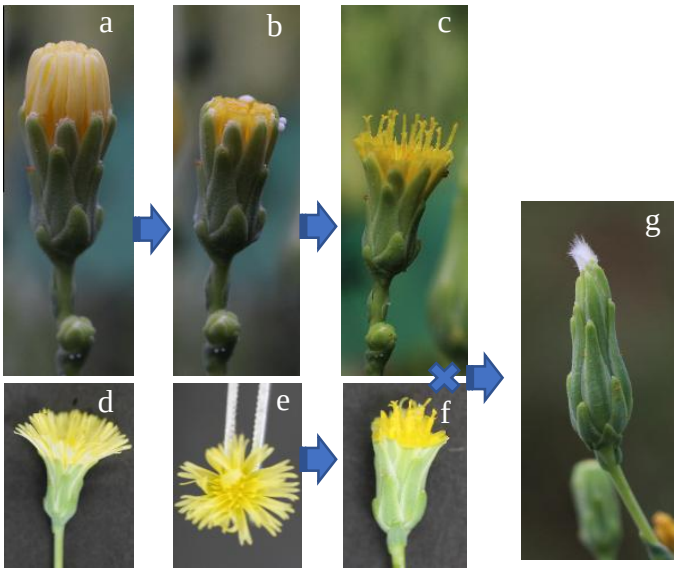

Figure 1: Crossing technique: a: flower bud, b: cut flower bud, c: pistil elongated from flower bud, d: full bloomed flower, e: top view of bloomed flower showing pistil with pollen, f: bloomed flower after cutting petal, g: young lettuce fruit.

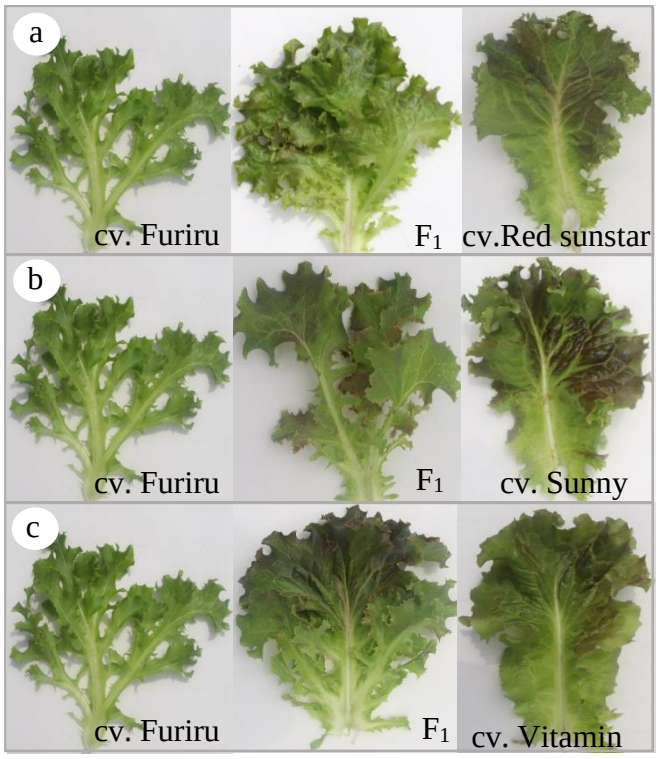

Figure 2: Leaf traits of F_1 plants and their parents at 6 months after transplanting to pots. A reciprocal F_1 lettuce leaf (middle) and the parents' leaf (left and right).

F_1 and F_2 Plant Cultivation

F_1 seedlings obtained from the cross combinations between cv. Furiru and the 3 lettuce cultivars were transplanted to the 20cm diameter pots with soil. Totally, twenty five F_1 seedlings from Furiru's pollen and 25 F_1 seedlings from Furiru's ovule in each cross combination of each cultivar were transplanted and the F_1 plants were kept producing F_2 seeds by self-pollination.

Approximately 300 F_2 seeds of an F_1 plant were sown on kitchen paper towels in 10 Petri dishes and then these seeds were irradiated with red light in order to maximize the seed germination. The seedlings germinated under red light irradiation in a room with a controlled temperature around 24^{o}C, 3 days after seed sowing, were adapted to sunlight and outside temperature for one day before transplanting to seedling trays. Around 230 to 250 seedlings were transplanted to seedling trays for one week before transplanting to the land in the total area of about 3 m^2 in a field.

Statistical analysis

The statistical test, probabilities of Chi-square was calculated by free online software for Calculation for the Chi-square test by Kristopher J. Preacher ©2010-2020.

RESULTS AND DISCUSSION

Lettuce leaf traits, leaf color and leaf form, could be identified from 3 weeks after transplanting to the field when the growth of lettuce had 4 to 5 true leaves. The leaf size was also different between parents but we did not adopt leaf size for genetic study because the leaf size is generally affected by the environment and the plant growth itself.

The F_1 lettuce plants were transplanted in a plastic greenhouse in early November and the leaf characters were checked 6 months after transplanting. Leaf of cv. Furiru expressed a green oak leaf with the deeply curled margins (Figure 2, left). The leaves of cv. Red sunstar (Figure 2a, right), cv. Sunny (Figure 2b, right) and cv. Vitamin (Figure 2c, right) were red lobed leaves. All F_1 plants survived, around 25 F_1 plants of each cross combination, expressed traits of red oak leaves with the deeply curled margins (Figure 2a, Figure 2b, and Figure 2c, middle). These results indicated that red leaf color might be the dominant trait inherited from cv. Red sunstar, cv. Sunny and cv. Vitamin, while the oak leaf might be the dominant trait inherited from cv. Furiru. The leaf form trait inheritance in this study was the same as those reported by Lindqvist (1958), *i.e.* the oak leaf trait was dominant on the lobed leaf character. The F_1 leaf color character in this study was the same as those reported by Thompson (1938). Both leaf traits inherited in this experiment followed Mendel's Law of Dominance that all F_1 offspring inherited one character from a female or male plant. The red and oak leaf characters that appeared in F_1 offspring are called dominant characters and the green and lobed characters remain hidden are called recessive characters.

In the F_2 populations, four different phenotypes: red oak leaf (RO), red lobed leaf (RL), green oak leaf (GO) and green lobed leaf (GL) appeared from each F_1 derived from each reciprocal cross combination (Figure 3). F_2 segregation ratios of all cross combinations matched as RO: GO: RL: GL= 9: 3: 3:1 (Table 1) which are the same as the results of Mendel's dihybrid crosses and statistical analysis of all combinations between cv. Furiru and 3 cultivars were confirmed without deviation from 9:3:3:1 by Chi square test (Table 1). Mendel crossed between round yellow seed and wrinkled green seed peas. He obtained only round yellow seed plants in the F_1 generation; and round yellow, wrinkled yellow, round green and wrinkled green seed plants in the phenotypic ratio 9: 3: 3: 1 in F_2 generation (Figure 4).

The dihybrid cross between the green oak leaf lettuce and red lobed lettuces obtained only red oak leaf plants in F_1 generation so the parents using this cross were homozygotes in leaf color alleles and leaf form alleles. In this lettuce leaf color, because Red leaf color expressed dominant and Green leaf color expressed recessive, the former allele was represented as "R" and the latter allele was represented as "r", tentatively. In the lettuce leaf form, because Oak leaf expressed dominant and Lobed leaf expressed recessive, the former allele was represented as "O" and the latter allele was represented as "o", tentatively. Therefore, the genotype of cv. Furiru with the green oak leaf becomes "rrOO" and the genotype of the three cultivars with the red lobed leaf lettuces becomes "RRoo". The reciprocal F_1 offspring which had the red oak leaf had the genotype "RrOo". According to the Law of Independent Assortment, combinations of alleles of two different genes gave phenotype ratio of 9: 3: 3: 1. Because our lettuce leaves in F_2 segregations were also approximately same as 9: 3: 3: 1 ratio, statistically, inheritance of either leaf color gene or leaf form gene was independent. The segregation of "R" and "r" is independent of the segregation of "O" and "o" in gametes formation. Therefore the F_1 hybrid formed 4 different genotypes of gametes, RO, Ro, rO, and ro. In the fertilization time, these gametes paired up to form the progenies of 9 different genotypes in F_2 generation and 4 different phenotypes including red oak leaf, green oak leaf, red lobed leaf, and green lobed leaf plants in the ratio 9: 3: 3: 1 (Figure 4). The results of lettuce dihybrid crosses in this experiment were the same as those of Mendel's dihybrid cross of pea plants (Figure 4).

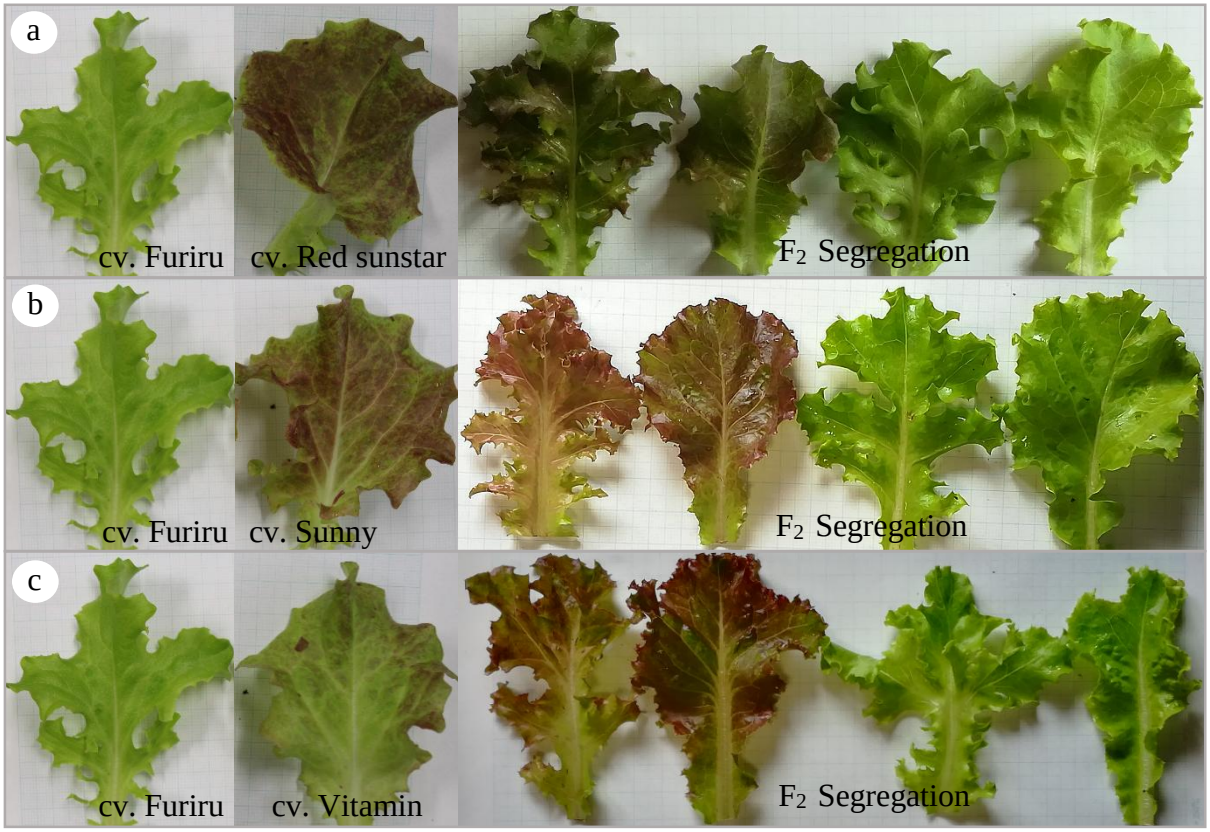

Figure 3: Segregation of F_2 plants' leaf traits comparing to their parents' leaf traits at 3 weeks after transplanting to pots for parents' plant (in winter), and to field for F_2 plants (in summer). Two leaves at the right side of each figure are the parent plants' leaves. Four leaves at the left side are F_2 plants' segregated leaves.

Table 1: The segregation of F_2 lettuce plants (Dihybrid characters), the ratio 9: 3: 3: 1

Reciprocal F_1 hybrid with cv. Furiru	Number of F_2 Plants	Segregation of F_2 plants								Chi-square test		
		Red oak leaf		Red lobed leaf		Green oak leaf		Green lobed leaf		Chi-square	Degree of freedom	p-value
		Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected			
cv. Red sunstar	245	140	137.8	35	45.9	54	45.9	16	15.3	4.085	3	0.252
cv. Sunny	103	52	57.9	21	19.3	22	19.3	8	6.4	1.529	3	0.676
cv. Vitamin	172	85	96.8	37	32.3	37	32.3	13	10.8	3.254	3	0.354

Mendel crossed between pure line pea plants of dominant traits in a plant "yellow round, YYRR" and recessive traits in another plant "green wrinkled, yyrr". But in our study, we crossed lettuce plants of both dominant and recessive traits in a plant, "red lobed leaf, RRoo" and "green oak leaf, rrOO" (Figure 4). The dihybrid crosses in this study and Mendel's dihybrid cross could obtain the same results of F_2. These results can confirm Mendel's Law of Independent Assortment that during a dihybrid cross, an assortment of each pair of traits is independent of the other. The gametes of lettuce plant having both dominant and recessive alleles "Ro and rO" while the gametes of pea plants having the dominant or recessive alleles "YR and yr", the F_1 of these crosses were heterozygous for both traits, *i.e.* RrOo and YyRr for a red oak leaf lettuce and a yellow round pea respectively (Figure 4).

POSSIBILITY SCHOOL EDUCATION APPLICATION METHODS

The use of experiments as a teaching methodology in science lessons is preferable, but not all experiments are suitable to be applied to the classroom. The experiments introduced in this study apply to biology education as a research activity in fieldwork, and the experimental data and figures are the biological source for studying Mendel's Law of Independent Assortment.

Application to field-work

The use of lettuce to trace Mendel's law of inheritance is not difficult for students and teachers, but they should pay attention to 4 factors leading to the success of their experiments including lettuce cultivar selection, temperature, light, and number of F_2 plants.

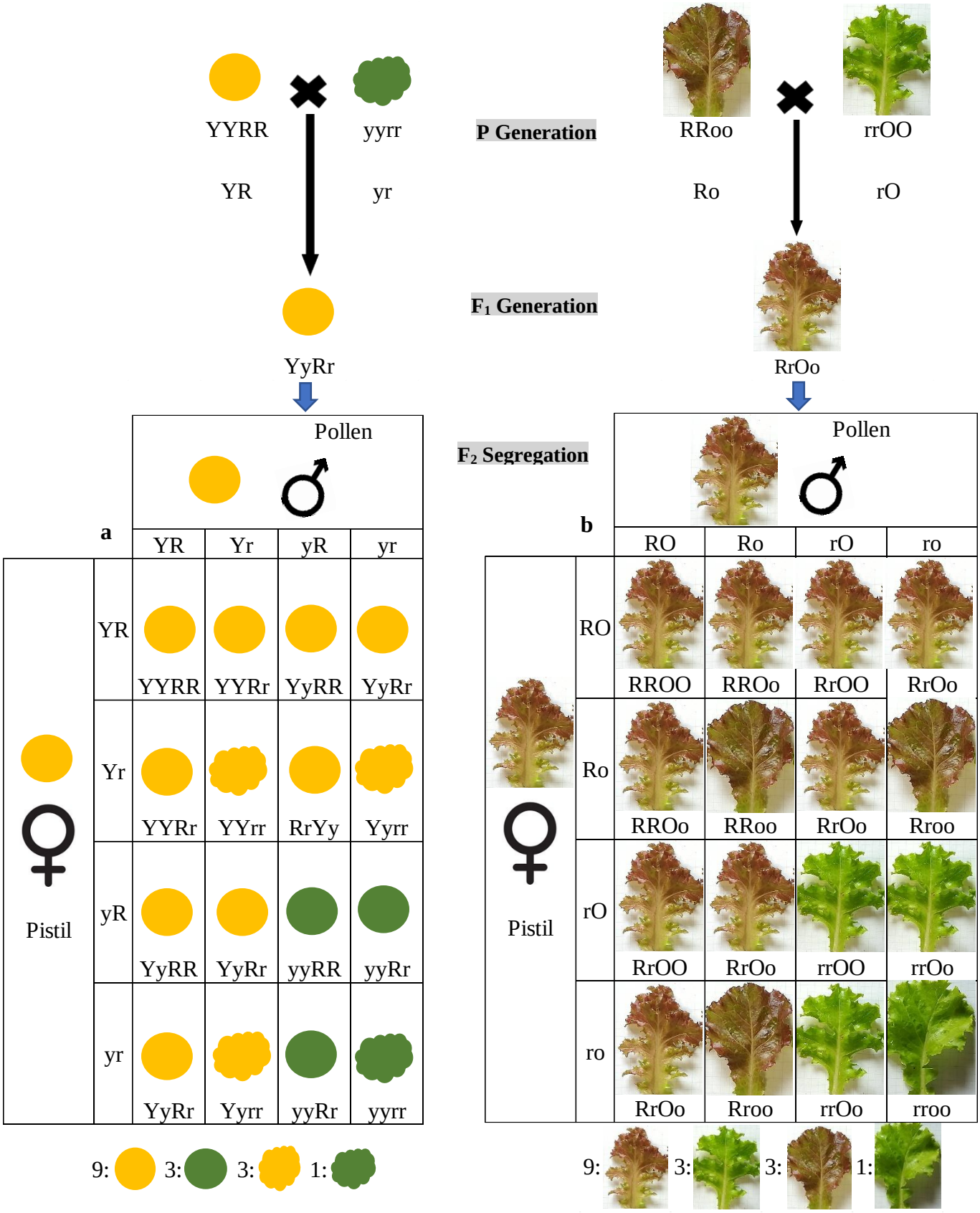

Figure 4: Dihybrid crosses and their F_2 segregation, a: Mendel's cross between yellow round seed pea and green wrinkled seed pea (Data source from Yihoop *et al.*, 2009 and Gautum, 2018), b: A cross between the red lobed leaf lettuce and green oak leaf lettuce.

1. The selection of lettuce cultivars is an essential factor for the success of the experiment and to achieve the objective. For example, if teachers want students to study the dihybrid traits, parent plants that have two different leaf traits such as leaf color and leaf form are required. In this study, we selected cv. Furiru with green oak leaves and other 3 cultivars with red lobed leaves to conduct experiments. Lettuce cultivars with homozygous alleles for target traits might not be difficult to select at the market because most of commercial cultivars might be produced by self- fertilization in order to keep the same traits.

2. The appropriate seasons are required to start lettuce growing. In our study lettuce grew well, vegetative growth, in a plastic greenhouse in winter which has cool temperature and short day length in Japan. This result was the same as Lee *et al.* (2015), and the optimum temperature for lettuce growing is 18.5^{0}C (Wallace *et al.*, 2012). In our study of lettuce inheritance, the vegetative growth is not a preference factor. We need lettuce plants to induce flowers as soon as possible. Lettuce plants elongated stems and induced flowers within 3 months in summer and it did not induce flowers in the winter. Wallace *et al.* (2012) also reported that lettuce induced flowers in the temperature between 21 to 27^{0}C, and Waycott (1995) reported that lettuce elongated stem and initiated flowers in the long day length and high temperature. Therefore, lettuce plants cultivation should be started at the end of winter because the plants will grow well at that time and they will induce stem and followers in the following 2 or 3 months and the cultivation of F_1 and F_2 plants will be continued consequently in order to finish the field activities within one year. In a tropical country such as Cambodia, the temperature and day length might not be the problem because the temperature did not change a lot within the year varying from 22 to 35^{0}C and the day length varied from 11.30 to 13.00 hours. Therefore, students and teachers might be able to start their lettuce cultivation any time in the year.

3. Light affects on red leaf color. In this study the red leaf color was difficult to identify when the plants were grown in a plastic greenhouse, but the red leaf color could be observed clearly when the plants were moved to the outside. Ohashi-Kaneko *et al.* (2007) reported that the pigment content in lettuce leaves was affected by different kinds of light. Therefore, lettuce plants should be grown in the field that plants can be exposed to sunlight.

4. The larger number of F_2 plants are better for this experiment because the result should meet the 9:3:3:1 ratio. The number of F_2 plants should remain at least around 100 at the time of leaf identification. Therefore, about 120 to 150 F_2 plants should be transplanted to the field. Lettuce plants can grow in a small space. We can grow up to 250 lettuce plants in the land area about 3 m^2 in a field in this study (Figure 5). Because lettuce can be grown indoors with LED (Light Emitting Diode) illumination in Japan, it is a good plant to be able to cultivate in a small space.

Figure 5: F_2 plants cultivation at the field 3 weeks after transplanting

Application to classroom

The data and figures in this study can be used as a reference for Mendel's law lesson in addition to the content in the biology textbooks. The results of this experiment are additional information to students understanding Mendel's Law of Independent Assortment. A teacher should ask students to compare Mendel's dihybrid cross and the lettuce dihybrid cross in Figure 4. The teacher should propose two questions for students to inquire: "What are the different genotypes at parent generation of the lettuce cross in this experiment and Mendel's pea cross?", and "Why the F_2 result of the lettuce cross and Mendel's cross have the same phenotypic ratio?"

CONCLUSION

The experimental methods and the crossing technique used in this study are suitable to be introduced to biological experiments in high school levels. Lettuce is an easy plant to grow and they are also easy to fertilize. The results of these dihybrid crosses are good evidence to explain Mendel's Law of Independent Assortment.

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