“NUTRITIONAL ENHANCEMENT OF LETTUCE USING MUTATIONAL BREEDING”

By

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Ilya Raskin

And approved by

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ABSTRACT OF THE THESIS

"Nutritional enhancement of lettuce using mutational breeding"

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Rutgers Scarlet Lettuce (RSL), a dark red lettuce variety with an exceptionally high content of health-promoting polyphenols, was selected as the starting material to develop new non-transgenic varieties of lettuce with improved nutritional content, through the use of ethyl methanesulfonate (EMS) seed mutagenesis followed by phenotype screening. The project focused on identification of a green phenotype which retained the high polyphenol content of its red RSL parent. An in-house approach for simple, efficient and large-scale production, identification and chemical analysis of mutagenized candidate plants was established and optimized. This approach generated a large seed collection from 2000 individual $M_2$ families with the potential to express both dominant and recessive mutations, which are being screened for traits of interest. A number of green plants were identified and chemically characterized and one of them was found to retain the high polyphenol trait. Successful self-pollination of this plant produced a collection of 10000 seeds of green high polyphenol lettuce. Analysis of their phytochemical profile suggests accumulation of colorless anthocyanin precursors brought on by blocking a later step in the anthocyanin biosynthetic pathway.

## ACKNOWLEDGEMENTS

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Last, but not least, I am immensely grateful to the Fulbright Program and the Rutgers Graduate School for providing me with this priceless opportunity for professional and personal growth. Indeed I am no longer the same person who arrived in the USA in 2012.

*To all left behind*.

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## BACKGROUND AND RESEARCH GOALS

Lettuce (*Lactuca sativa* L.) is a leafy herbaceous annual plant of the family Asteraceae, grown worldwide for its consumption, most commonly raw as a salad green. It is a self-fertilized diploid species (2n= 18)[1], first domesticated in the Eastern Mediterranean[2]. A hardy cool weather crop, it grows best at temperatures between 15 and 20 °C, under full sun, and in loose well-drained soils with a pH between 6 and 6.7[3]. The leaves form either a dense head or loose rosette with a very short stem during its vegetative phase, its height and diameter measuring between 15 and 30 cm; once the plant reaches the maturity phase it bolts, developing a flower stalk up to 1 m high, and is no longer suitable for consumption due to its bitter flavor. The most popular varieties include romaine, loose leaf and head lettuce, and its leaves are most commonly green but sometimes red or variegated[4]. It is commonly sold either as whole heads or as pre-washed baby greens.

From an industrial perspective, the Food and Agriculture Organization of the United Nations estimated worldwide production of lettuce (combined with chicory) to be almost 25 million metric tons in 2012[5], with the USA ranking as the second highest producer (17%) and exporter (19%) worldwide[4]. In the USA lettuce is the second leading vegetable crop in terms of production value, with production in 2012 valued at nearly $1.9 billion[4] and 28 pounds consumed per capita in the year 2010[6].

Considering its wide consumer base, lettuce seems like an ideal candidate for the delivery of nutrients and other compounds of interest to the population. However lettuce is not often associated with health-promoting properties, and despite its popularity it is not as rich in certain nutrients as other less popular leafy greens: 100 g of green leaf lettuce

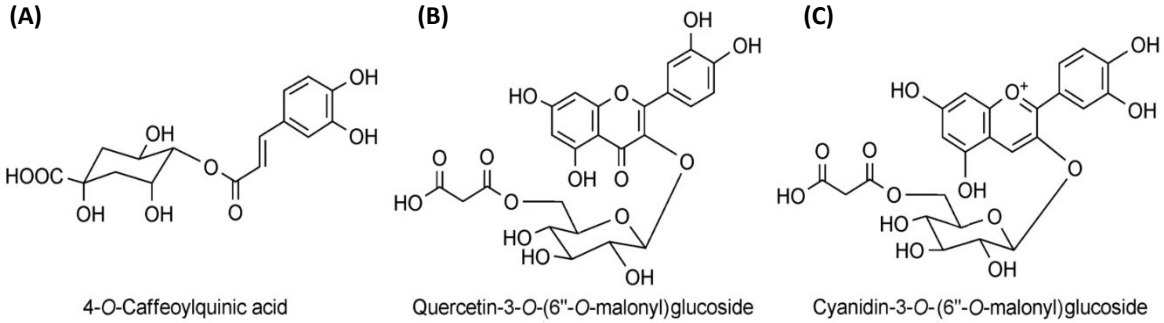
provide just 15% of the recommended daily intake of vitamin C versus 47% in the case of spinach, 72% in the case of watercress and 200% in the case of kale. For calcium these values are 4% versus 10%, 12% and 15%, respectively. The trend is similar for other nutrients and the differences are even higher when considering the more popular iceberg lettuce instead[7]. In combination with the current consumer awareness of the importance of healthier eating and the growing interest for functional foods, this can potentially translate into a niche market for nutritionally enhanced lettuce, such as varieties naturally biofortified in vitamins, minerals, or phytoactives.

Phytoactive compounds are plant secondary metabolites which accumulate in plant tissues in high amounts and therefore can constitute a significant part of diets rich in fruits and vegetables, although unlike traditional micronutrients they are not strictly essential[8,9]. Among phytoactives, polyphenols are a structural class of organic chemicals characterized by having at least one aromatic ring with one or more hydroxyl groups attached. This chemical structure lends polyphenols the ability to scavenge free-radicals, and a strong correlation between polyphenol content and *in vitro* antioxidant activity has been well established[8,10,11]. There is also a growing body of evidence that polyphenols, possibly through their antioxidant and antiinflammatory effects, play an important role in the beneficial effects of a diet rich in fruits and vegetables, having been shown to reduce the risk of chronic non-communicable diseases such as diabetes, cardiovascular disease, cancer or stroke[8,12–21]. The development and consumption of functional foods enriched in polyphenols could therefore play a part in the prevention of chronic metabolic disease worldwide, and there is a projected increase in demand for food rich in antioxidants[22].

Rutgers Scarlet Lettuce (RSL) comprises a number of high polyphenol dark red lettuce varieties recently developed through somaclonal variation followed by selection in tissue culture. The total polyphenol content of RSL is among the highest ever reported for common fruits and vegetables – 8.7 mg gallic acid equivalents (GAE) per g of fresh weight (FW), versus 5.3, 6.6 or 7.1 mg GAE/g FW in cultivated blueberries, blackberries and cranberries, respectively, and approximately 1.3 mg GAE/g FW in normal lettuce varieties[11,12]. RSL has been shown to inhibit glucose production when tested on rat H4IIE hepatoma cells and to improve glucose metabolism and attenuate liver lipid accumulation when tested on high-fat diet-induced obese mice[13]. Regular consumption of RSL as part of the diet may therefore contribute to the prevention and treatment of metabolic syndrome. However, despite its health benefits and its development through non-transgenic techniques, from the marketing perspective the striking deep burgundy color of its leaves may still pose consumer resistance.

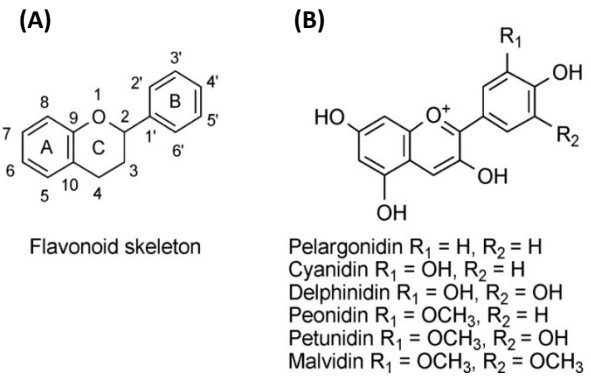
The three phenolic compounds which accumulate in the highest amounts in the leaves of RSL are chlorogenic acid, quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside[12] – representing the polyphenol classes of hydroxycinnamic acids, flavonols and anthocyanins, respectively **(Fig. 1).** Anthocyanins are water-soluble pigments found in the plant cell vacuole, which appear pink, red, purple, blue or colorless depending on the pH of the medium, and which give blueberries, red grapes and many other fruits and vegetables their characteristic bright color[23]. Functionally, anthocyanins serve as protective compounds against DNA damage caused by free radicals and UV radiation, and are therefore synthesized by plants in response to environmental stresses[24–29], as well as

to provide bright colors for attraction of pollinators and seed dispersers. Structurally, anthocyanins are glycosides of anthocyanidins, which have the typical $C_6$–$C_3$–$C_6$ or flavonoid skeleton structure containing a heterocyclic benzopyran ring, a fused aromatic ring and a phenyl constituent[8] (Fig. 2).




**(Figure adapted from Crozier et al., 2009[8])**

**Fig. 1. Chemical structure of the most abundant polyphenols in Rutgers Scarlet Lettuce**. **(A)** Chlorogenic acid, up to 27.6 mg/g dry weight in RSL[12], refers to a mixture of hydroxycinnamic acids most commonly present in the form of conjugates such as caffeoylquinic acid[8]. **(B)** Quercetin malonyl-glucoside and **(C)** cyanidin malonyl-glucoside are present in RSL in levels up to 35.7 mg/g dry weight and 20.5 mg/g dry weight respectively[12].



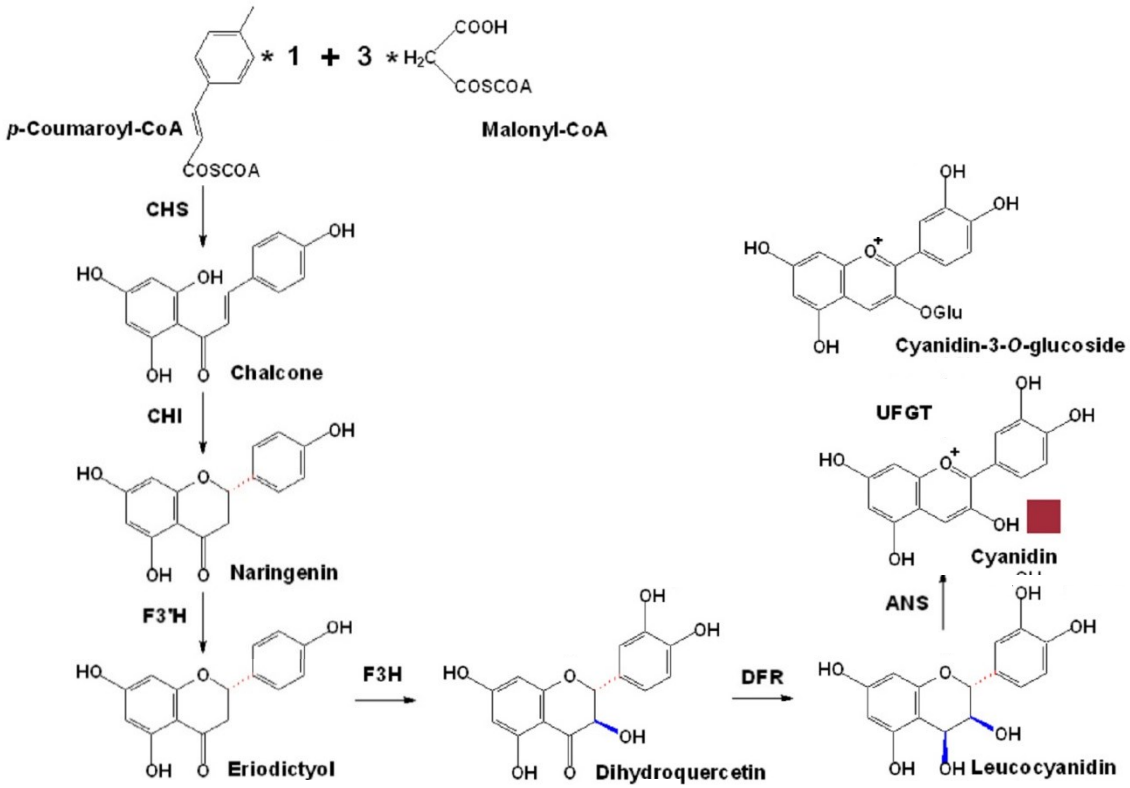

**(Figure adapted from Crozier et al., 2009[8])**

**Fig. 2. Chemical structure of the flavonoid skeleton and the most common anthocyanidins. (A)** The $C_6$–$C_3$–$C_6$ structure of flavonoids denotes two six-carbon aromatic rings connected by a three-carbon bridge. The heterocyclic benzopiran ring or C-ring and the aromatic ring or A-ring are fused and connected to the phenyl constituent or B-ring. **(B)** The most common anthocyanidins only differ in their substitutions at $C_3$ and $C_5$. In plant tissues anthocyanidins are invariably found as sugar conjugates known as anthocyanins, commonly glycosylated on $C_3$[8].

The biosynthesis of anthocyanidins and their stabilization as glycosylated anthocyanins takes place via the flavonoid pathway[8,23,30] – a long, complex process which

is mediated by ultraviolet radiation **(Fig. 3).** Transcription of fundamental structural genes in the pathway such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), flavanone 3β-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS), requires sufficient levels of stimulation by UV-A or UV-B[24,27,31,32]. When under adequate lighting conditions, anthocyanins accumulate in the plant cell vacuole, predominantly in the epidermis of leaves and petals and the skin of fruits[8] – and it has been observed that when growing pigmented crops such as eggplant, primula flowers or red lettuce under UV absorbing materials, poor pigmentation develops[24].

As demonstrated in grapes, mutations in genes which code a key step in the conversion from the colorless precursor to colored anthocyanin eliminate the pigment and produce a colorless revertant[33]. The step in the pathway which leads to transformation of the last colorless precursors into an anthocyanidin is mediated by anthocyanidin synthase (ANS), a highly specific enzyme encoded by one single active gene[23,30]. Other genes encode similarly specific enzymes which mediate anthocyanidin glycosylation (flavonoid glucosyltransferase, UFGT), methylation (O-methyltransferase, OMT) or acylation (anthocyanin acyltransferase, ACT), steps which are required for anthocyanin stability and accumulation. Additionally a few enzymes mediate controlled anthocyanin degradation, and a number of transcription factors, such as *VvmybA1*, regulate the whole process[23]. A mutation in any of these genes would block anthocyanin production or stability, potentially leading to a redirection of the pathway and the accumulation of upstream colorless precursors, thus producing a high polyphenol colorless mutant.




**(Figure adapted from Crozier et al., 2009[8])**

**Fig. 3. Basic flavonoid pathway for the biosynthesis of cyanidin 3-malonyl glucoside.** The main anthocyanin present in Rutgers Scarlet Lettuce, cyanidin malonyl-glucoside, is synthesized by glycosylation of the cyanidin precursor with a malonyl-glucoside moiety. Chlorogenic acid is synthesized from p-Coumaryl-CoA or its precursors, and quercetin malonyl-glucoside is synthesized from dihydroquercetin. CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid 3'-hydroxylase; F3H, flavanone 3β-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid glucosyltransferase.

The goal of this research was to develop an easily marketable and green-colored high polyphenol lettuce variety by blocking the later steps of the flavonoid pathway in RSL to produce a green revertant plant that retains its high phenolic content as described. Given that the dark red color of RSL is due to the abundance of only one type of anthocyanin – the very abundant cyanidin 3-malonyl glucoside and minor amounts of its direct precursor cyanidin 3-glucoside –, this could be theoretically achieved by knocking out *Ans* or *Ufgt*, or by altering the expression of the key regulator *VvmybA1*, among other possibilities. In

order to circumvent the regulatory hurdles faced by genetically engineered products it was decided to follow a breeding approach, inducing genetic variability through random mutagenesis and then visually selecting the trait of interest – green leaves or sectors – among mutagenized plants growing under adequate lighting conditions for good anthocyanin biosynthesis. Given that random mutagenesis may potentially also produce green candidate plants with a low phenolic content by blocking an early step in the biosynthesis pathway instead of a later step, the identification of any green plants must be followed by the measurement of their total phenolic content and the characterization of their chemical profile. Once the desired phenotype has been confirmed it would be necessary to obtain viable seeds from the plant, confirm the transmission and segregation of the trait, and if needed carry out rounds of selection across generations until the trait becomes sufficiently stable.

Overall, as this approach was likely to require screening of thousands of plants before producing results, from a practical standpoint the project focused on the development of a long-term in-house setup for simple and efficient production, identification and phytochemical characterization of candidate green plants obtained from RSL.

# PART I – OPTIMIZATION OF CONDITIONS FOR MUTATIONAL BREEDING OF LETTUCE

## SECTION A – EVALUATION OF MUTAGEN TYPE AND DOSE

### Introduction

The introduction of genetic variation is a crucial element for the development of new plant varieties through selective breeding, more so given that most crop species have had a large of portion of their genetic material fixed through centuries of selection. Random genetic variation is routinely introduced through treatments which induce DNA damage and mutation, such as ionizing radiation, chemical mutagens or somaclonal variation in tissue culture. This approach has been used for many decades to successfully develop thousands of new crop varieties with useful traits, from disease resistance to nutritional enhancement, for most commonly used species and all around the world[1,34,35].

For the purpose of the current research the first goal is to determine the most advantageous mutagen to use in order to introduce genetic variation into Rutgers Scarlet Lettuce (RSL) for subsequent screening and selection, and which optimal dose of said mutagen must be used to introduce the maximum amount of genetic variation which is compatible with healthy plant development and viable seed set. Out of the mutagens most commonly used and those more readily available for the study, it was decided to evaluate the effects of gamma radiation and ethyl methanesulfonate (EMS) on RSL.

Gamma radiation is a type of ionizing radiation produced by the decay of high energy atomic nuclei such as the radioisotopes $^{60}Co$ and $^{137}Cs$. The extremely high frequency of gamma rays allows them to penetrate tissue and ionize molecules, causing a spectrum of biological effects[36]. Most typically gamma radiation induces DNA damage through random

double-strand breaks (DSBs) and single-strand breaks (SSBs), although abasic sites, cross-linking and a variety of base modifications are also abundant; and it is common for such damage to appear in clusters making it harder for the cell to repair[36,37], leading to very strong genotoxicity.

EMS is an organic compound with formula $CH_3SO_3C_2H_5$ that acts as an alkylating agent of DNA, and which has been found to be strongly mutagenic on a wide variety of biological systems[38]. It induces random point mutations through the reaction of its ethyl group with the bases in the DNA, most commonly guanine. The modified bases are incorrectly recognized during cellular replication, leading to nucleotide substitution, point insertions or deletions, and in some cases SSBs as well[38] at a rate of $5x10^{-4}$ to $5x10^{-2}$ per gene, without substantial killing[39].

The mutagenic potential of ultraviolet (UV) radiation, which is not commonly used in crop mutational breeding, was also tested as part of this study due to its convenience versus dependence on external gamma irradiation facilities or its safety versus handling a potentially carcinogenic solution such as EMS. UV radiation comprises the region of the electromagnetic spectrum between 100 and 400 nm, and its relatively low energy and frequency makes it unable to ionize atoms. It can however still induce DNA damage, most commonly through the formation of pyrimidine dimers which lead to point mutations during replication, but also through cross-linking or producing SSBs[28,37]. UV-B, which corresponds to the 315–280 nm range of the spectrum, is the main responsible for inducing these effects in nature, and UV-C, comprising the 100-280 nm range of the spectrum[27,37], causes DNA damage so efficiently that it is commonly used as a germicide.

Unlike gamma radiation and EMS, which can induce damage in the plant embryo DNA by penetrating through the seed coat, allowing the seeds to be treated directly, the low penetration potential of UV radiation makes treatment of seeds insufficient to induce biological effects. Therefore UV treatment requires irradiation after germination, which can then be followed by cell de-differentiation and shoot regeneration in tissue culture to visualize phenotypical changes in plantlets regenerated from any mutated cells.

## Materials and methods

*Chemicals and reagents*

Murashige & Skoog[40] modified basal salts with Gamborg vitamins, micropropagation grade agar, 6-benzylaminopurine solution, α-naphthaleneacetic acid solution and indole-3-butyric acid solution were purchased from PhytoTechnology Laboratories (Overland Park, KS, USA). Ethyl methanesulfonate solution and sucrose were purchased from Sigma (St. Louis, MO, USA).

*Gamma irradiation treatment*

Seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) were treated in a gamma irradiator (Radiation Machinery Corporation, Parsippany, NJ, USA) located in the Rutgers Environmental Health and Safety department (Piscataway, NJ, USA). Irradiation was provided by $^{137}Cs$ at a nominal center-line dose rate of approximately 190 cGy/minute until the desired irradiation dose was reached. Four independent experiments evaluating the effects of gamma irradiation doses between 10 and 1000 Gy were carried out.

*Ethyl methanesulfonate treatment*

Batches of approximately 1000 g of seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) were placed inside 50 ml plastic centrifuge tubes containing 40 ml of EMS dilutions in distilled water. Seeds were soaked overnight at room temperature in a rotary shaker; after 12 h the EMS solution was decanted, seeds were washed five times with 50 ml of distilled water and were then blotted dry before sowing. Six independent experiments evaluating the effects of treatment at concentrations between 0.025% and 2% (v/v) EMS versus untreated plants were carried out. After determining an optimal dose range for EMS treatment of Rutgers Scarlet Lettuce, the effects of this dose range were tested on a number of green lettuce cultivars. Two additional experiments evaluating the effects of treatment at concentrations between 0.025% and 0.2% (v/v) EMS were carried out on seeds of the loose leaf green lettuce cultivars 'Lettony', 'Green Star', 'Two Star', 'Black Seeded Simpson' and 'Waldmann's Dark Green', and of the romaine lettuce cultivars 'Green Towers', 'Paris White' and 'Winter Density' (Johnny's Selected Seeds, Winslow, ME, USA; High Mowing Organic Seeds, Wolcott, VT, USA; Burpee Seeds, Warminster, PA, USA).

*UV irradiation treatment*

Seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) were surface sterilized by immersion in 70% ethanol for 1 min, followed by a 1.2% sodium hypochlorite solution for 12 min and rinsed three times with sterile distilled water. Sterile seeds were placed inside Petri dishes (VWR, Radnor, PA, USA)

| Media | MS | Sucrose | Cytokinins | Auxins |
|---|---|---|---|---|
| Germination | 1/2x | 1% | - | - |
| Shoot induction | 1x | 3% | 2 mg/l BA | 0.1 mg/l NAA |
| Root induction | 1x | 3% | - | 1 mg/l IBA |

**Table 1. Original composition of RSL tissue culture media.** MS, Murashige & Skoog modified basal salts with Gamborg vitamins; BA, 6-benzylaminopurine; NAA, α-naphthaleneacetic acid; IBA, indole-3-butyric acid. All media pH was adjusted to 5.7, solidified with 0.7% agar and autoclaved at 121 °C and 103 kPa for 20 min.

containing 40 ml of solid MS germination medium[40] **(Table 1)**. Three days after germination seedlings were placed inside a C-10 dark cabinet and irradiated with a handheld 6W UVGL-58 UV lamp (UVP, Upland, CA, USA) set to emit UV-B and UV-C between 254 and 320 nm. Four independent experiments evaluating the effects of UV exposure between 15 min and 3 h were carried out.

*Tissue culture conditions*

Immediately after UV irradiation the cotyledons were aseptically excised and placed inside Petri dishes containing 40 ml of solid MS shoot induction medium **(Table 1)**, and were kept inside a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) kept at 22 °C, under a PAR light intensity of 10.6 ± 1.7 mol/$m^2$d provided by full spectrum Philips F32T8/DX fluorescent lamps (Philips, Andover, MA, USA) and a UV light intensity of 0.4 ± 0.1 mol/$m^2$d provided by UV 26W Exo Terra Repti-Glo 5.0/T8 fluorescent lamps (Rolf C. Hagen, Mansfield, MA, USA), and under a 16/8 h (light/dark) photoperiod. Petri dishes were kept protected from light for 2-3 weeks, to induce callus formation and to minimize light-driven DNA repair by photolyases[37,41]. After 2-3 weeks, Petri dishes were uncovered and checked weekly for development of shoots, which typically started 4 weeks after explant excision. Explants with shoots were

subcultured into SteriCon-8 culture vessels (PhytoTechnology Laboratories, Overland Park, KS, USA) containing 80 ml of MS root induction medium (Table 1) until e roots developed. When plantlets reached a height of 5-7 cm they were transferred to growing mix.

*Plant growth conditions*

Gamma irradiated and EMS treated seeds were sown inside trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA) and kept in a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) in the Rutgers New Jersey Agricultural Experiment Station (NJAES) Research Greenhouse facility. Gamma or EMS treated plants which reached a height of 5-7 cm, as well as UV treated plantlets produced in tissue culture were transplanted into pots (diameter 10 cm, volume 414 $cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA) to observe their development. Growing conditions were 18/15 °C (day/night), 65% relative humidity, a PAR light intensity of 18.8 ± 1.4 mol/$m^2$d and a UV light intensity of 0.4 ± 0.1 mol/$m^2$d, both provided by Sylvania F96/T12/CW/VHO fluorescent lamps (Osram Sylvania, Danvers, MA, USA), and a 16/8 h (light/dark) photoperiod. Plants were hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks.

*Experimental design and analysis of results*

The effect of each mutagen was evaluated in at least four independent experiments which tested progressively narrower ranges of doses, aiming to determine the upper dose limit to allow healthy plant development and viable seed set. In order to determine the effect of each mutagen treated plants were visually compared to untreated control plants

or cotyledon explants of the same age and kept under the same conditions. Any changes in germination percentage, growth speed and plant height were noted, and the frequency of phenotypical changes, and more specifically chlorophyll mutations – a common marker used to assess mutagenic effectiveness – were estimated by counting all affected leaves.

## Results

*Gamma irradiation treatment*

Gamma irradiation induced no changes on the germination rate of lettuce (Table 2). Doses of gamma irradiation between 10 and 200 Gy induced small decreases in plant height and growth speed when compared to untreated controls, and doses above 400 Gy arrested the development of seedlings right after germination of the pre-formed embryo (Fig. 4). Doses between 200 and 400 Gy induced inconsistent effects in different experiments, making it difficult to determine an upper limit dose of irradiation that still allowed healthy plant development (Fig. 5). No phenotypical changes except for plant height and growth speed were observed during the course of any of the experiments.

| Gamma irradiation (Gy) | Germination % | EMS % (v/v) | Germination % |
|---|---|---|---|
| 0 | 89.4 | 0 | 93.4 |
| 10 | 86.2 | 0.1 | 92.6 |
| 50 | 91.9 | 0.2 | 93.5 |
| 100 | 99.2 | 0.3 | 89.3 |
| 150 | 86.7 | 0.4 | 87.7 |
| 200 | 84.3 | 0.6 | 84.7 |
| 400 | 87.4 | 0.7 | 83.2 |
| 600 | 85.7 | 0.8 | 58.7 |
| 800 | 88.7 | 1 | 25.3 |
| 1000 | 90.2 | 2 | 0.0 |

**Table 2. Effects of gamma radiation and EMS treatment on RSL seed germination.** Germination rate was not affected by gamma irradiation of seeds, however their treatment with EMS induced a clear dose-response effect. n = 200 seeds were sown per treatment group.

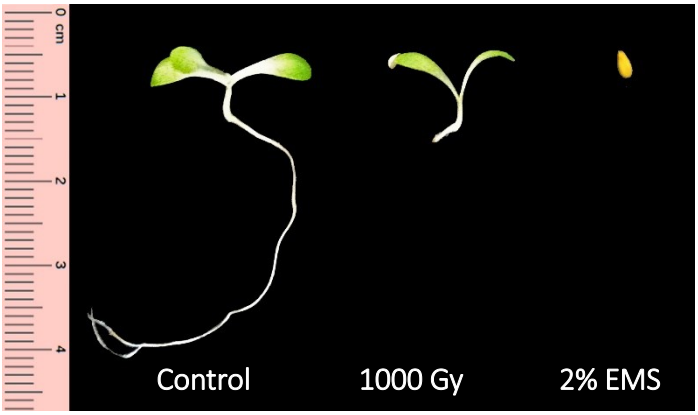
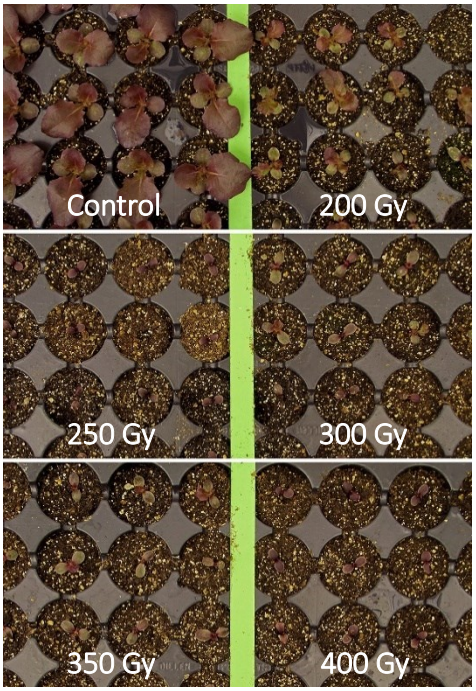



**Fig. 4. Effects of high doses of gamma radiation and EMS treatment on RSL seedling development.** Very high doses of gamma irradiation allow germination of the pre-formed plant embryo present inside the seed, but development does not continue, suggesting the inhibition of apical and root meristems. Very high doses of EMS prevent germination altogether. Seedlings shown at day 14.



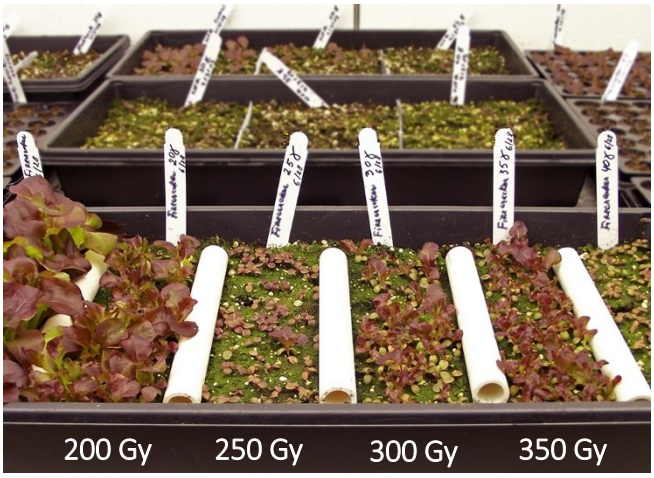




**Fig. 5. Effects of moderate doses of gamma radiation on RSL seedling development.** The upper limit dose of gamma irradiation to allow plant growth was initially estimated as 200 Gy (left), however subsequent experiments showed some plant development after gamma irradiation with up to 350 Gy (right).

*Ethyl methanesulfonate (EMS) treatment*

EMS induced a very strong decrease in the germination rate of seeds treated with doses above 0.7% **(Table 2)** with no seeds at a dose of 2% **(Fig. 4)**, as well a clear decrease in plant height and growth speed and abundant morphological changes in doses above 0.2%, and especially above 0.4%, when compared to untreated control plants **(Fig. 6)**. These plants looked overall unhealthy when compared to untreated plants and eventually their development was arrested: plants treated with doses 0.4% and above never reached

maturity, and even though some plants treated with doses between 0.2% and 0.4% were able to bolt, they produced sterile seeds only. A dose between 0.1% and 0.2% was determined as the upper limit to allow healthy plant development and viable seed production in RSL **(Fig. 7)** and similar results were obtained when testing the same range of doses on a variety of green lettuce cultivars **(Table 3)**. Doses between 0.025% and 0.1% induced no differences in plant growth speed and only minor morphological changes.

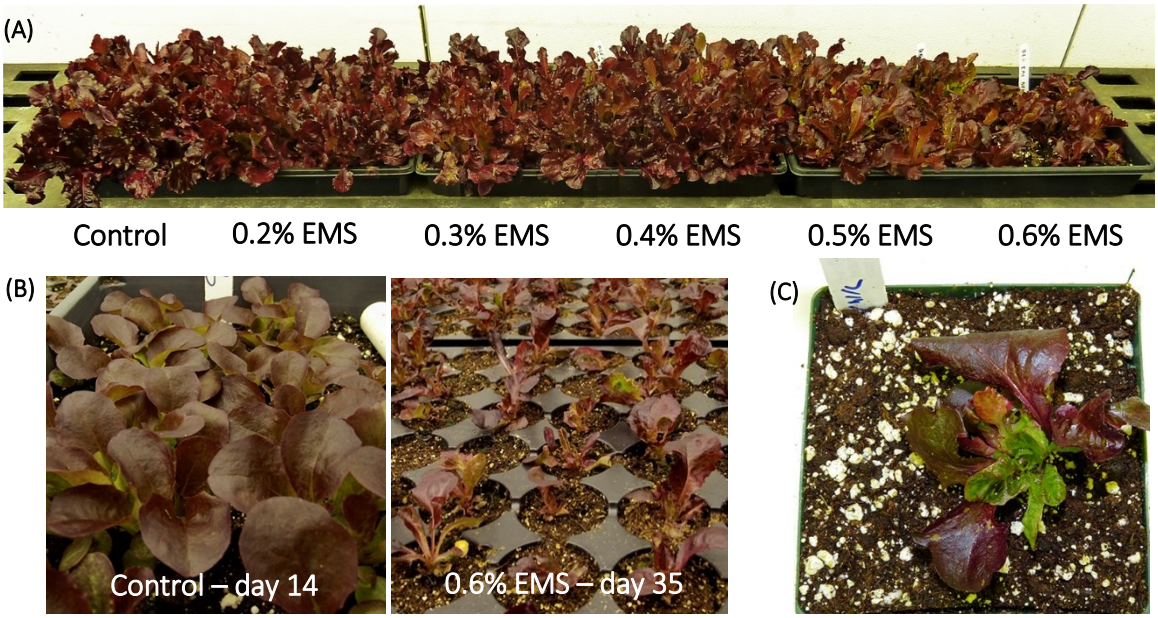



**Fig. 6. Effects of moderate EMS treatment on RSL development. (A)** Treatment with EMS doses between 0.2% and 0.8% induced a clear dose-response effect on plant height, growth speed and abundance of morphological changes. **(B)** Control plants reached a height of 3 cm in two weeks, whereas 0.6% EMS treated plants took over four weeks to reach that same size, and **(C)** often displayed aberrant morphological features.

(A)

| Cultivar name | 0.1% EMS | 0.15% EMS | 0.2% EMS |
|---|---|---|---|
| RSL NFR | 5.56 | 11.11 | 16.67 |
| Lettony | 3.4 | 3.1 | 5.6 |
| Green Towers | 3.1 | 6.3 | 9.7 |
| Paris White | 5.0 | 7.2 | 7.8 |
| Winter Density | 3.4 | 7.8 | 7.8 |

(B)

| Cultivar name | 0.1% EMS | 0.15% EMS | 0.2% EMS |
|---|---|---|---|
| RSL NFR | 0.56 | 1.67 | 1.11 |
| Lettony | 0.3 | 0.9 | 0.6 |
| Green Towers | 0.6 | 1.6 | 2.5 |
| Paris White | 1.3 | 1.6 | 2.2 |
| Winter Density | 0.9 | 4.4 | 3.4 |

**Table 3. Phenotypic variation % observed in 1 month old EMS treated lettuce plants.** All leaves showing phenotypical variation were collected from all plants and percentages were calculated using an average of 7 leaves per plant and n = 30 plants per treatment group. The total % of affected leaves (A) and total % of leaves with chlorophyll mutations (B) were estimated as measures of EMS mutagenic activity.

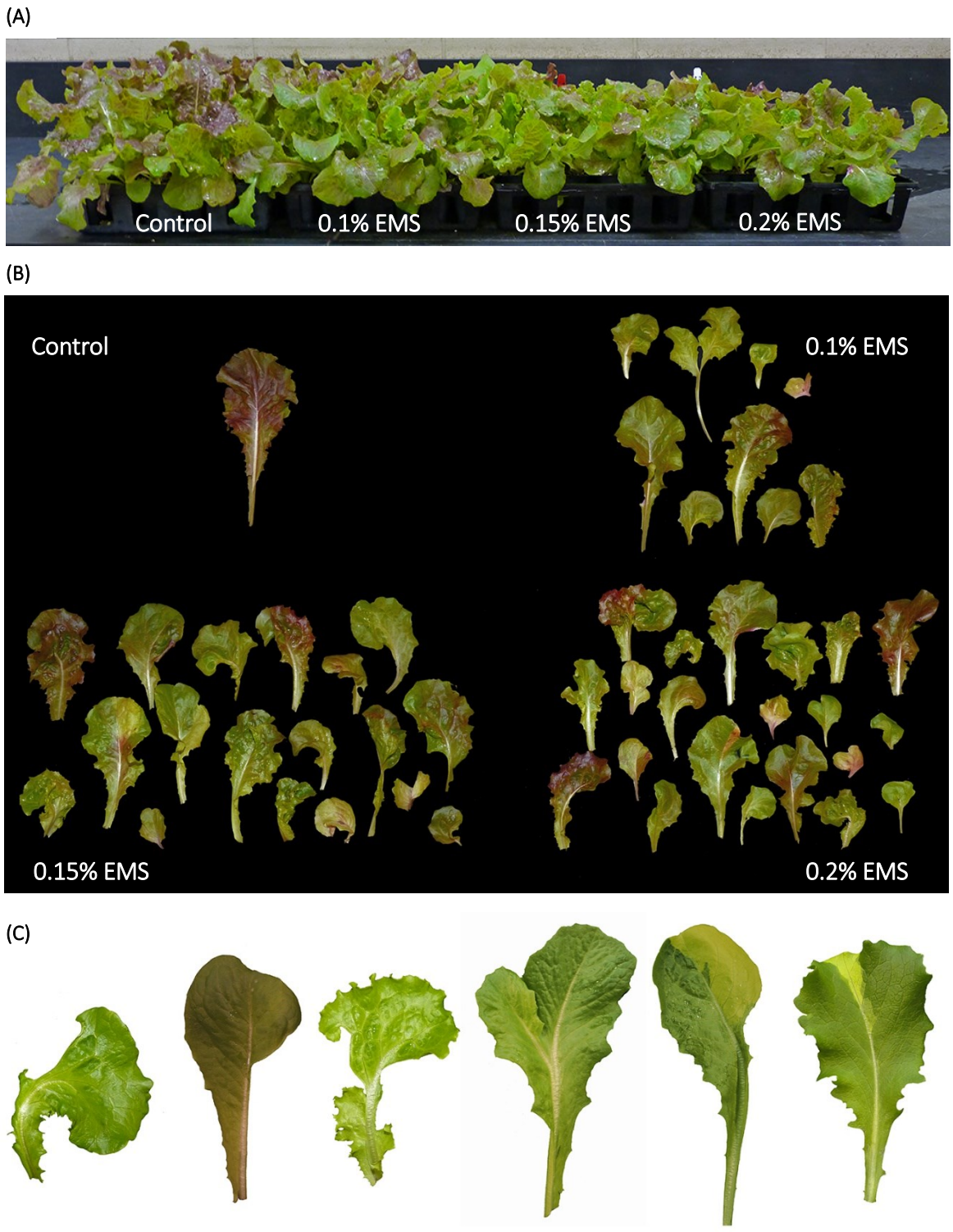



**Fig. 7. Effects of low EMS treatment on lettuce development.** (A) Treatment with EMS doses between 0.1% and 0.2% induced a slight height decrease as well as a small percentage of leaves with morphological changes and/or color sectors, but produced overall healthy plants. (B) All leaves showing phenotypical variation were collected from each treatment group at 1 month after germination to estimate the differences in EMS mutagenic activity. (C) Common changes observed were leaf growth in a curved shape, leaves with missing sections, midribs splitting into two, and chlorotic sectors.

*UV irradiation treatment*

Shoots and plantlets regenerated from UV irradiated cotyledon explants showed no abnormal phenotypical characteristics regardless of the UV exposure dose used. The regenerated $M_1$ plantlets were grown to maturity and seeds from those exposed to UV for 3 h, the highest dose tested, were collected to screen for mutations in the following generation. However the $M_2$ seedlings showed no phenotypical changes when compared to untreated plants.

## Discussion

Since the biological effects of ionizing radiation usually correlate closely with dose and rate[42], a gradual dose-response in plants treated with increasing levels of gamma radiation, such as that reported by other similar studies[43], was expected. However the effects observed on RSL seem to follow more of an all-or-nothing trend, with no phenotypical changes observed in doses below 200 Gy, no seedling development observed in doses above 350 Gy, and doses in between seeming to either affect growth speed only moderately or to prevent plant growth altogether. This would be consistent with gamma irradiation inducing clustered damage on the DNA consisting mostly of strand breaks, whose cellular repair mechanisms can get saturated relatively quickly. Increases in exposure time past a certain threshold would therefore lead to rapid accumulation of DNA damage and a drastic decrease in plant viability such as the one observed. Further narrowing of the dose might have yielded the expected dose-response behavior, but given the inconsistency repeatedly observed across experiments and the problems derived from depending on a separate facility to schedule irradiation, this approach was abandoned.

As for UV irradiation, it induced no apparent effect on RSL at the doses tested. This might be due to the existence of an additional light-independent repair mechanism for UV-induced DNA damage, which efficiently corrects point mutations and mismatches[37], due to the range of exposure doses tested being too low to induce effects, or due to a combination of both. The duration of the exposure was limited by the delicate tissue of the lettuce cotyledons, which after 3 h under UV already showed signs of dryness, decreasing its viability for shoot regeneration. Shorter exposure treatments using a lamp which provides a higher intensity of UV radiation might prevent tissue dryness and therefore allow exploring a wider range of irradiation doses. However due to the inability to selectively block the light-independent DNA repair mechanism, as well as the laboriousness of the process, this approach was abandoned as well.

Unlike gamma and UV radiation, soaking seeds in increasingly concentrated EMS dilutions induces a very clear and consistent dose-response effect. The decrease in plant height and the increase in phenotypical variation at higher doses are in agreement with an accumulation of point mutations which are not corrected efficiently enough, leading to progressively impaired plant growth and viability. This response, in combination with the consistency across independent experiments and the convenience and low price of the mutagenic treatment, make EMS mutagenesis at the optimized dose of 0.1% – 0.2% (v/v) the optimal treatment choice for inducing variability on RSL for phenotype selection.

## SECTION B – IMPROVEMENT OF SHOOT REGENERATION EFFICIENCY

### Introduction

The success of plant genetic manipulation methods, from *Agrobacterium*-mediated gene transfer to transformation of the chloroplast genome by particle bombardment, ultimately rests on the ability to regenerate whole plants from the transformed cells in a reliable and efficient manner using direct shoot regeneration in tissue culture. Tissue culture is the process of aseptically isolating pieces of plant tissue, or explants, and culturing them on an adequate mixture of nutrients and growth regulators in order to induce cell de-differentiation and subsequent re-differentiation into a plant organ or a plant embryo – known as organogenesis and somatic embryogenesis, respectively –. Both organogenesis and somatic embryogenesis can occur either directly and fast from explant tissue, or indirectly and more slowly through an intermediate callus stage[44].

The success of a tissue culture approach for direct, fast shoot regeneration depends first and foremost on the selection of a cultivar with adequate regeneration efficiency, followed by optimization of explant source and age, and lastly adaptation of the regeneration medium for the given species and genotype. Optimization of the medium composition involves selecting a salt and vitamin mixture, selecting a carbon source, and most importantly selecting cytokinins and auxins and adjusting their amounts and relative proportion. It has been generally observed that a high cytokinin to auxin ratio leads to shoot regeneration, a low cytokinin to auxin ratio leads to root regeneration, and an intermediate cytokinin to auxin ratio mostly leads to cell de-differentiation, producing unorganized masses of cells or callus[44] – however this is not the case for all species or

genotypes – especially given that under certain circumstances explants may produce additional growth regulators of their own – so medium adjusting is always needed.

In lettuce, shoots have been obtained directly and indirectly by culturing 3 to 7-day old cotyledon explants on a variety of solid media[45–50]. Most combinations use the cytokinin benzylaminopurine (BA) and the auxin naphthaleneacetic acid (NAA), and a medium containing 0.44 µM (0.09 mg/l) BA and 0.54 µM (0.1 mg/l) NAA, reported to produce up to 72% direct shoot regeneration[47,48], is the most commonly used. Minor variations of this medium have allowed development of important agronomic traits in lettuce such as herbicide resistance[51], virus resistance[52], water stress resistance[53], yield enhancement[54], increased leaf calcium content[55] or accumulation of pharmaceutical proteins[56,57]. Nevertheless most studies on lettuce tissue culture have reported the regeneration response to be extremely variable across experiments and statistically dependent on genotype[46,49,50]. Therefore medium optimization is critical for success.

Regeneration of RSL using the original MS shoot induction medium containing 2 mg/l BA and 0.1 mg/l NAA yielded an average shoot induction rate of approximately 10%, and this rate became even lower when using EMS mutagenized seeds as starting material (unpublished data). Regeneration on this medium occurred slowly and indirectly through an intermediate callus stage, so the first shoots appeared 4-6 weeks after explant excision (Fig. 8). Given the need to screen thousands of mutagenized shoots for the phenotype of interest, breeding and selection in tissue culture under these low efficiency regeneration conditions introduces a major bottleneck. An adequate screening throughput therefore rests on optimization of the medium to induce high efficiency direct shoot regeneration.

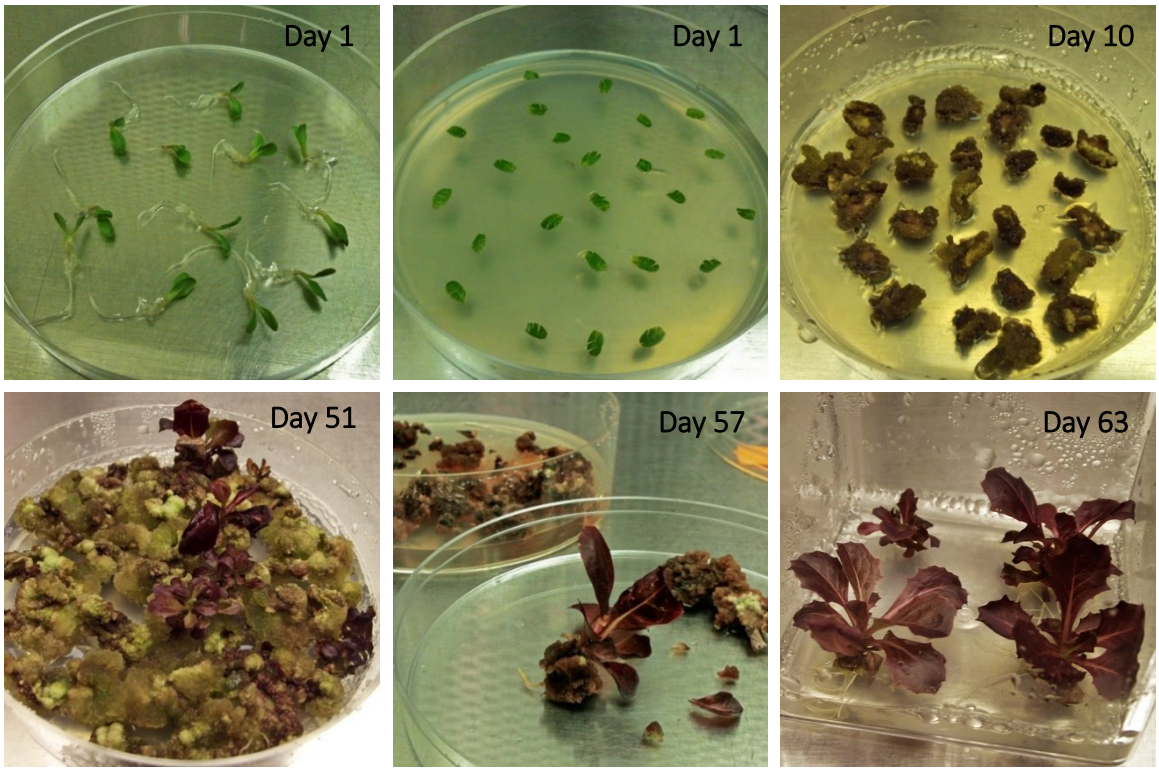



**Fig. 8. Original steps and timeline of tissue culture shoot induction in RSL.** Cotyledons were aseptically excised from 3-day old seedlings and placed on the surface of solid MS shoot induction medium. Within the first week the presence of 2 mg/l BA and 0.1 mg/l NAA started to induce swelling and extending of all explants, and during weeks 2 to 4 abundant friable callus developed. The first shoots developed from this callus 4 to 6 weeks since explant excision with an efficiency of approximately 10%, each explant commonly producing a maximum of one shoot only. Shoots developed slowly but showed normal morphology, allowing development of healthy plantlets. When a shoot showing a phenotype of interest reached a height of 1-2 cm, approximately 2 months since explant excision, it was transferred to solid MS root induction medium. Plantlets with mature roots and a height of 5-7 cm were transferred to growing mix.

Regarding nutritional composition of the medium, the effect of the Schenk and Hildebrandt (SH) basal salt mixture, commonly used in lettuce shoot induction[46,58], as an alternative to the Murashige & Skoog (MS) basal salt mixture, was evaluated. A 1992 study by Teng et al. describing rapid regeneration of lettuce in suspension culture and using 1.5% glucose as a carbon source concluded that glucose induced more shoot induction and less callus growth than sucrose[58], and this possible effect was tested as well. Regarding hormonal composition, the most common medium for lettuce direct shoot induction

described in the literature, containing 0.09 mg/l BA and 0.1 mg/l NAA[47,49,58], was evaluated. Additionally, any differential effects of the common cytokinins kinetin, thidiazuron and zeatin were tested side by side with BA, and a possible effect of the total amount of hormones added was studied by evaluating concentrations of BA ranging from 1 mg/l to 9 mg/l and their corresponding amounts of NAA to maintain the original ratio (20:1). Lastly, activated charcoal (AC) was added to the medium in a range of concentrations between 20 mg/l and 2000 mg/l. AC is one of the many natural substances that can be added to regeneration media to induce beneficial effects[59,60], and it has been reported to aid in shoot induction in concentrations ranging from 1 mg/l to 10000 mg/l depending on the species[59]. The total amounts of BA and NAA were also increased when testing these media combinations to offset their adsorption by AC. All media variations were tested on the cultivar of interest 'RSL NFR' as well as on 'Winter Density', a green romaine cultivar with extremely low shoot induction efficiency. The MS shoot induction medium originally used for development of RSL was tested side by side with all new combinations as a control.

## Materials and methods

*Chemicals and reagents*

Murashige & Skoog[40] modified basal salt mixture with Gamborg vitamins, Schenk & Hildebrandt[61] modified basal salt mixture with vitamins, micropropagation grade agar, benzylaminopurine solution, kinetin solution, thidiazuron solution, zeatin solution, naphthaleneacetic acid solution and indolebutyric acid solution were purchased from PhytoTechnology Laboratories (Overland Park, KS, USA). Activated charcoal, glucose and sucrose were purchased from Sigma (St. Louis, MO, USA).

*Tissue culture conditions*

Seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) and of romaine 'Winter Density' lettuce (Johnny's Selected Seeds, Winslow, ME, USA) were surface sterilized by immersion in 70% ethanol for 1 min, followed by a 1.2% sodium hypochlorite solution for 12 min and rinsed three times with sterilized distilled water. Sterile seeds were placed inside deep Petri dishes (VWR, Radnor, PA, USA) containing 40 ml of solid MS germination medium[40] (Table 1). Three days after germination the cotyledons were aseptically excised and placed inside deep Petri dishes containing 40 ml of different types of solid shoot induction media (Table 4). Petri dishes were kept inside a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) at 22 °C, under a PAR light intensity of 10.6 ± 1.7 mol/$m^2$d provided by full spectrum 32W Philips F32T8/DX fluorescent lamps (Philips, Andover, MA, USA) and a UV light intensity of 0.4 ± 0.1 mol/$m^2$d provided by UV 26W Exo Terra Repti-Glo 5.0/T8 fluorescent lamps (Rolf C. Hagen, Mansfield, MA, USA), and under a 16/8 h (light/dark) photoperiod. Petri dishes were checked weekly for shoot development and any other changes.

*Experimental design and analysis of results*

Each treatment group comprised 15 cotyledon explants in a single Petri dish and all treatments were replicated a minimum of three times, totaling at least n = 45 explants per treatment group. The length of time until the earliest signs of shoot induction was noted for each Petri dish, and the effects of each medium on shoot induction and quality were evaluated at week 6 and week 8 since explant excision. The percentage of explants with at least one shoot was recorded for each Petri dish. Size, growth speed, morphology and

| Media | Basal salts | Sugar | Cytokinins | Auxins | Additions |
|---|---|---|---|---|---|
| Control | 1x MS | 3% sucrose | 2 mg/l BA | 0.1 mg/l NAA | - |
| S | 1x SH | 3% sucrose | 2 mg/l BA | 0.1 mg/l NAA | - |
| G | 1x MS | 1.5% glucose | 2 mg/l BA | 0.1 mg/l NAA | - |
| R | 1x MS | 3% sucrose | 0.09 mg/l BA | 0.1 mg/l NAA | - |
| C1 | 1x MS | 3% sucrose | 2 mg/l K | 0.1 mg/l NAA | - |
| C2 | 1x MS | 3% sucrose | 2 mg/l TDZ | 0.1 mg/l NAA | - |
| C3 | 1x MS | 3% sucrose | 2 mg/l Z | 0.1 mg/l NAA | - |
| H1 | 1x MS | 3% sucrose | 1 mg/l BA | 0.05 mg/l NAA | - |
| H2 | 1x MS | 3% sucrose | 3 mg/l BA | 0.15 mg/l NAA | - |
| H3 | 1x MS | 3% sucrose | 4 mg/l BA | 0.2 mg/l NAA | - |
| H4 | 1x MS | 3% sucrose | 5 mg/l BA | 0.25 mg/l NAA | - |
| H5 | 1x MS | 3% sucrose | 6 mg/l BA | 0.3 mg/l NAA | - |
| H6 | 1x MS | 3% sucrose | 7 mg/l BA | 0.35 mg/l NAA | - |
| H7 | 1x MS | 3% sucrose | 8 mg/l BA | 0.4 mg/l NAA | - |
| H8 | 1x MS | 3% sucrose | 9 mg/l BA | 0.45 mg/l NAA | - |
| AC1 | 1x MS | 3% sucrose | 2.5 mg/l BA | 0.125 mg/l NAA | 20 mg/l AC |
| AC2 | 1x MS | 3% sucrose | 5 mg/l BA | 0.25 mg/l NAA | 20 mg/l AC |
| AC3 | 1x MS | 3% sucrose | 5 mg/l BA | 0.25 mg/l NAA | 200 mg/l AC |
| AC4 | 1x MS | 3% sucrose | 10 mg/l BA | 0.5 mg/l NAA | 200 mg/l AC |
| AC5 | 1x MS | 3% sucrose | 5 mg/l BA | 0.25 mg/l NAA | 2000 mg/l AC |
| AC6 | 1x MS | 3% sucrose | 10 mg/l BA | 0.5 mg/l NAA | 2000 mg/l AC |

**Table 4. Types of tissue culture media tested for increasing shoot induction efficiency.** MS, Murashige & Skoog modified basal salts with Gamborg vitamins; SH, Schenk & Hildebrandt modified basal salts with vitamins; BA, benzylaminopurine; K, kinetin; TDZ, thidiazuron; Z, zeatin; NAA, naphthaleneacetic acid; AC, activated charcoal. All media pH was adjusted to 5.7, solidified with 0.7% agar and autoclaved at 121 °C and 103 kPa for 20 min. Solid MS shoot induction media originally developed for RSL NFR was used as control, and differences between each medium and the control medium are marked in red.

health of these shoots were also noted for each treatment group. The means and standard deviations (SD) were calculated from pooled data and all results are expressed as mean ± SD. Statistical significance of the differential effects of treatments versus control was determined using a general linear model analysis of variance (ANOVA) followed by a Dunnett test. A global significance level was set at $\alpha = 0.05$. All statistical procedures were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

*Modification of shoot induction medium basic composition*

Modification of the nutritional composition of the original shoot induction medium yielded no significant results **(Fig. 9)**. The use of glucose as a carbon source for regeneration **(Table 4, medium G)** induced changes in the texture and quality of the callus but no differences in shoot induction **(Fig. 10)**, and the SH basal salt mix **(Table 4, medium S)** produced identical results to those obtained with the MS basal salt mix.

Changes to the hormonal composition of the original shoot induction medium produced several interesting results **(Fig. 9)**. The medium containing 0.09 mg/l BA and 0.1 mg/l NAA **(Table 4, medium R)** induced a strongly significant ($p \leq 0.0001$) increase in shoot

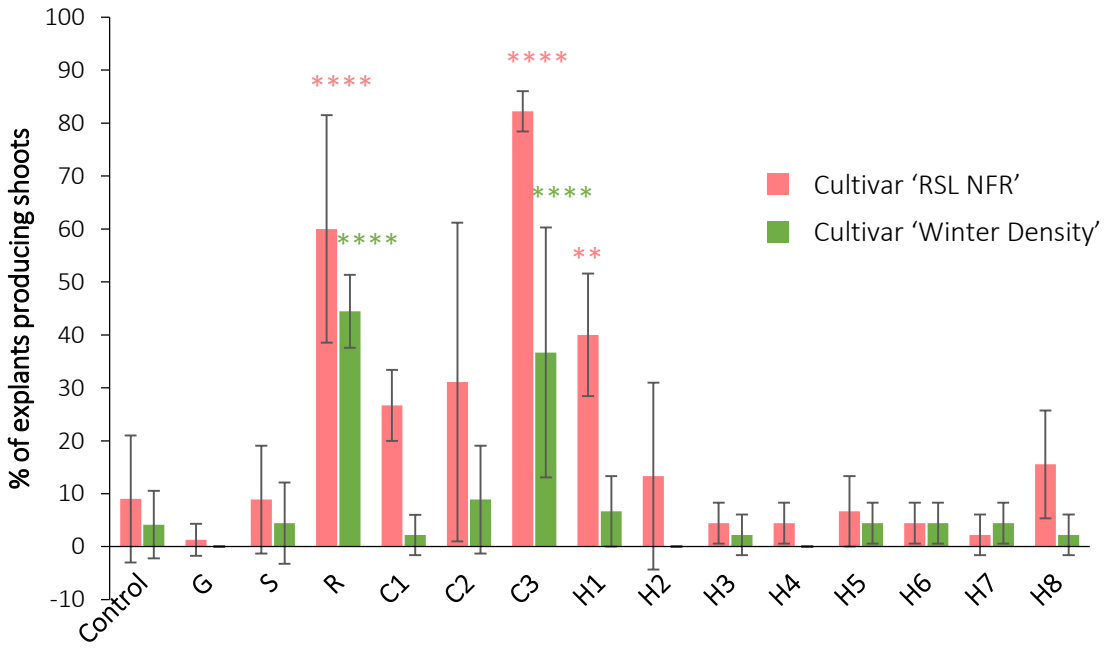



**Fig. 9. Shoot induction on different types of tissue culture media.** The number of explants with at least one shoot after 8 weeks since explant excision were quantified for all treatment groups and expressed as a percentage for each Petri dish. Explants per treatment group were n = 45. Data were pooled and are presented as the mean ± SD. Significant differences between the control group and each treatment group were analyzed by one-way ANOVA followed by a Dunnett test (** $p \leq 0.01$, **** $p \leq 0.0001$). Statistical data reflect the differences between the control and each treatment group for each cultivar, but not for each treatment between both cultivars. Data does not reflect the size, growth speed or overall quality of the regenerated shoots.

count, size and growth speed. These shoots developed directly from the explants, initiating development just 2 weeks after explant excision. Subsequently a low to moderate amount of callus developed as well. The morphology of the shoots, however, was markedly abnormal with extremely elongated petioles and very thin and narrow leaf blades **(Fig. 11)**.

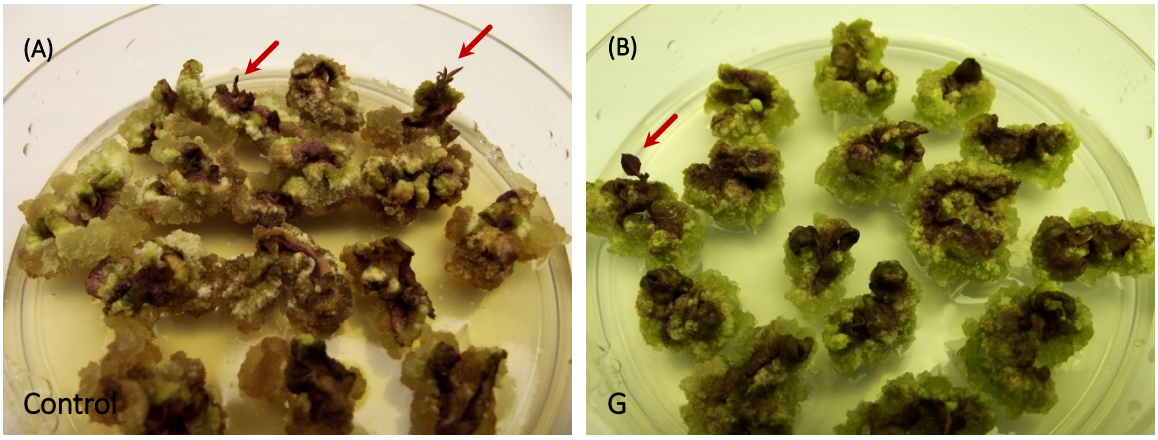



**Fig. 10. Shoot induction in RSL using sucrose versus glucose as a carbon source.** No significant differences in shoot induction were observed between the control medium with sucrose **(A)** and medium G with glucose **(B)**. Callus developed on the medium with glucose was consistently more compact and showed symptoms of hyperhydricity, which has been correlated with decreased indirect shoot induction. Explants shown at week 7 and small shoots marked with arrows.

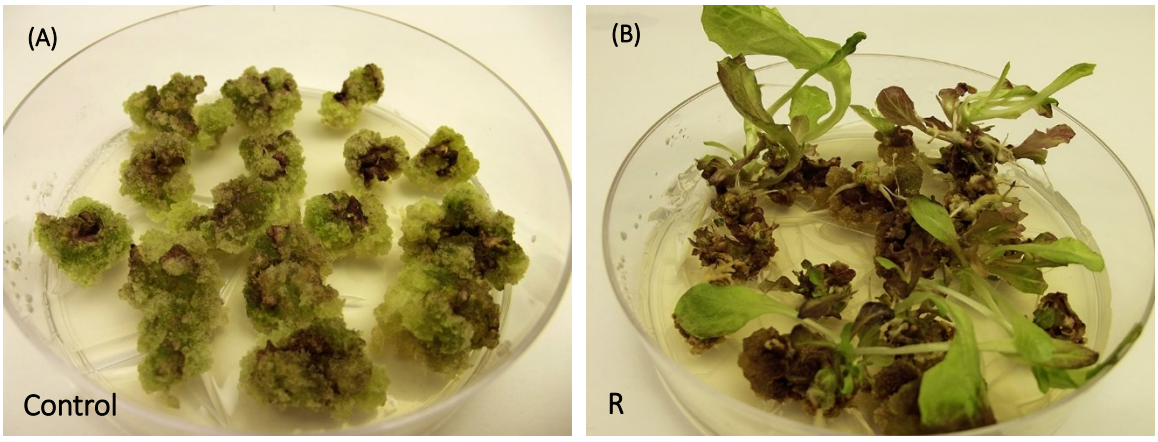



**Fig. 11. Shoot induction in RSL using a 20:1 versus a 1:1 cytokinin to auxin ratio.** A strongly significant ($p \leq 0.0001$) increase in shoot count, size and growth speed was observed between the control medium with 2 mg/l BA and 0.1 mg/l NAA **(A)** and medium R with an approximate 0.09 mg/l BA and 0.1 mg/l NAA **(B)**. Shoots were weak with extremely long petioles and thin and narrow leaf blades. Explants shown at week 7.

Comparison of the differential effects of common cytokinins **(Table 4, media C1-C3)** showed a strongly significant ($p \leq 0.0001$) increase in shoot count, size and growth speed on medium containing 2 mg/l zeatin instead of BA, kinetin or TDZ. Again these shoots initiated development just 2 weeks after explant excision without need for a previous callus stage, followed by later development of a moderate amount of roots and callus. The morphology of these shoots was also aberrant with elongated petioles and very irregularly shaped leaf blades **(Fig. 12)**.

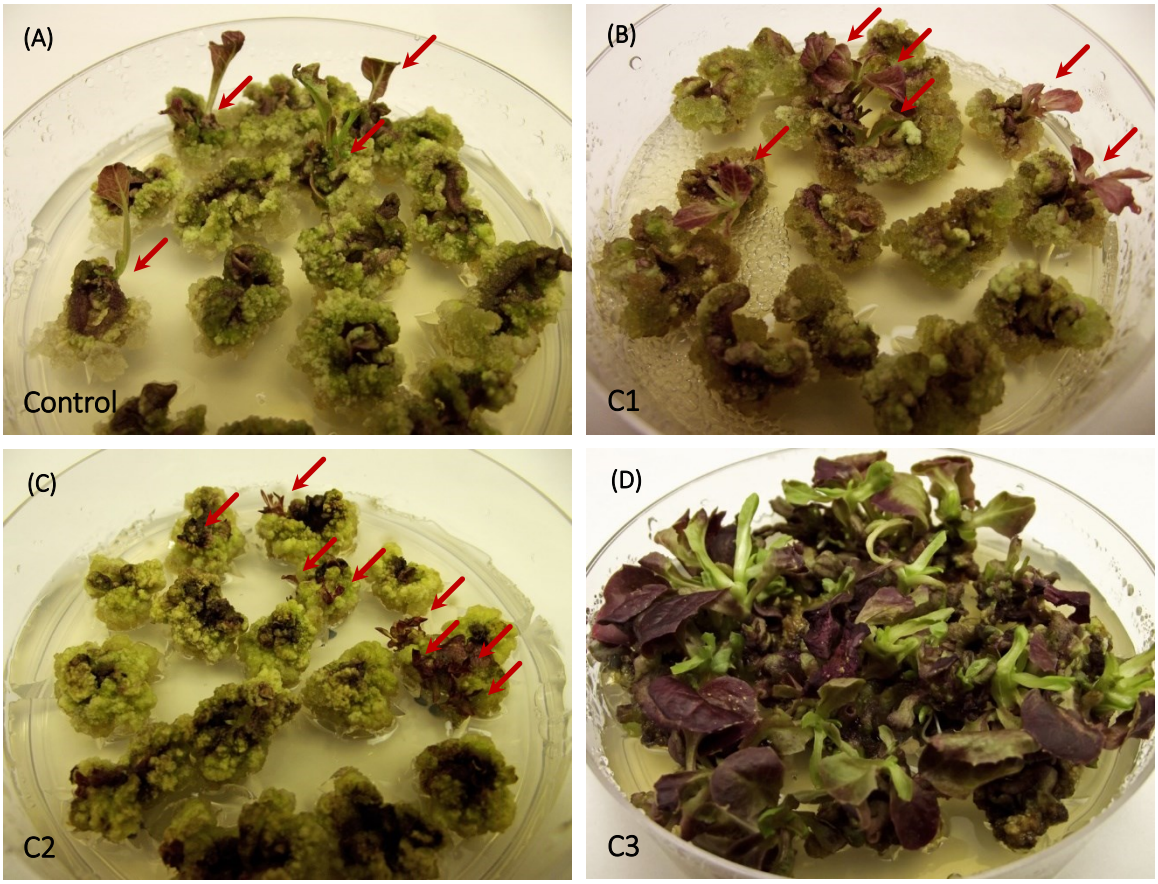



**Fig. 12. Shoot induction in RSL using different types of cytokinins.** No significant differences in shoot induction were observed between the control medium with BA **(A)**, medium C1 with kinetin **(B)** and medium C2 with TDZ **(C)**. Shoots induced by BA and kinetin were comparable in size, growth speed and morphology, whereas shoots induced by TDZ appeared later and developed much more slowly. In all three treatments, regeneration was mediated by a moderate to high amount of callus. A strongly significant ($p \leq 0.0001$) increase in shoot count, size and growth speed was observed on medium C3 with zeatin **(D)**, which resulted in the development of several shoots per explant, with long petioles and irregularly shaped leaves. Explants shown at week 7 and small shoots marked with arrows.

Lastly, modification of the absolute amounts of BA and NAA added to the medium while maintaining the 20:1 ratio **(Table 4, media H1-H8)** produced a moderately significant ($p \leq 0.01$) increase in shoot induction in 'RSL NFR' grown on 1 mg/l BA and 0.05 mg/l NAA. These shoots initiated development 2 weeks after explant excision directly from explant tissue, often grew in bundles, and overall looked weak, again showing long petioles and thin and narrow leaf blades. Media containing 3 mg/l BA and 0.15 mg/l NAA, and up to 9 mg/ml BA and 0.45 mg/l NAA however induced no significant changes and the few shoots that appeared developed indirectly from an intermediate callus stage, growing slowly and sometimes arresting their growth altogether **(Fig. 13)**.

*Supplementation of shoot induction media with activated charcoal*

Different amounts of AC induced very different effects on shoot development on 'RSL NFR' **(Fig. 14 and Fig. 15)**. Addition of 20 mg/l AC to medium containing 2.5 mg/l BA and 0.125 mg/l NAA **(Table 4, medium AC1)** produced a moderately significant ($p \leq 0.01$)

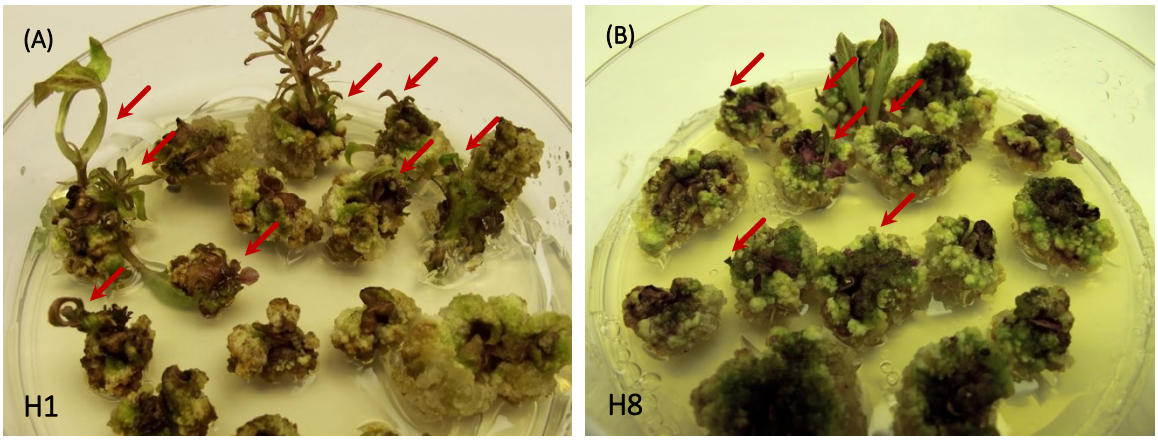



**Fig. 13. Shoot induction in RSL using different total amounts of BA and NAA.** A moderately significant ($p \leq 0.01$) increase in shoot induction was observed on medium H1 containing 1 mg/l BA and 0.05 mg/l NAA **(A)** when compared to the control medium containing 2 mg/l BA and 0.1 mg/l NAA. Shoot morphology was abnormal with extremely long petioles and very narrow leaf blades. No significant differences in shoot induction were observed on media containing 3 mg/l BA and 0.15 mg/l NAA up to media containing 9 mg/ml BA and 0.45 mg/l NAA **(B)**. Most shoots developed from intermediate callus after 6 weeks and developed very slowly. Explants shown at week 7 and small shoots marked with arrows.

increase in shoot count, size and growth speed, but the same amount of charcoal added to medium containing 5 mg/l BA and 0.25 mg/l NAA **(Table 4, medium AC2)** induced no significant changes. Addition of 200 mg/l AC, however, produced a striking and strongly significant ($p \leq 0.0001$) increase in shoot count, size and growth speed on media containing a wide range of BA and NAA **(Table 4, media AC3 and AC4)**. Most of these shoots initiated development just 10 days since explant excision and prior to any callus development, with most and sometimes all explants producing several bundles of healthy looking shoots, especially in the case of medium AC3. Addition of AC above 200 mg/l, however, again decreased shoot induction, and a dose of 2000 mg/l induced no reaction in the tissue at the tested doses of BA and NAA **(Table 4, media AC5 and AC6)**.

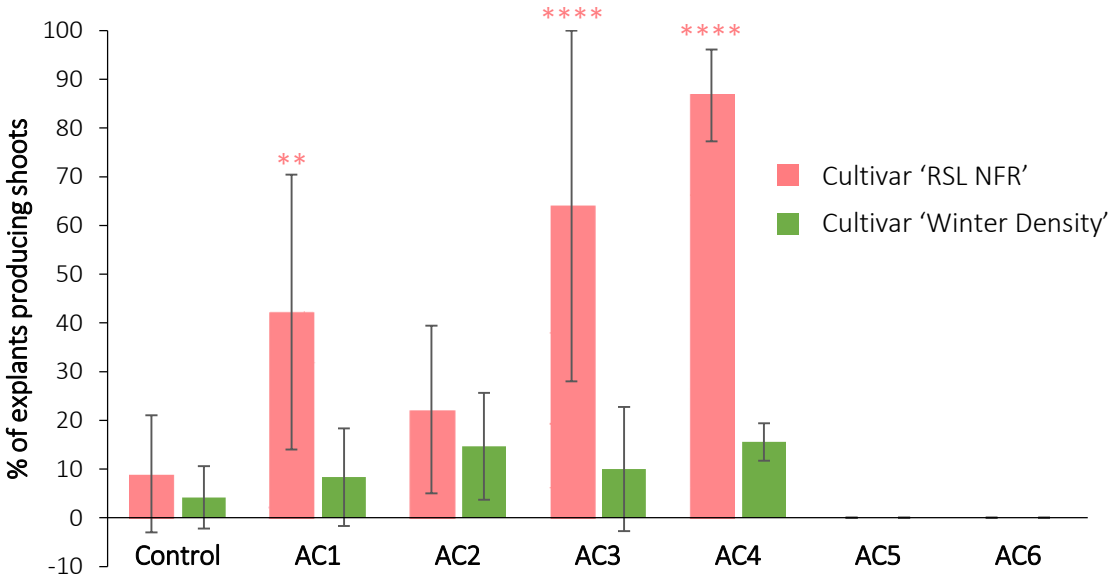



**Fig. 14. Shoot induction under different concentrations of activated charcoal.** The number of explants with at least one shoot after 8 weeks since explant excision were quantified for all treatment groups and expressed as a percentage for each Petri dish. Explants per treatment group were n = 45. No shoot regeneration was observed for media AC5 and AC6. Data were pooled and are presented as the mean ± SD. Significant differences between the control group and each treatment group were analyzed by one-way ANOVA followed by a Dunnett test (** $p \leq 0.01$, **** $p \leq 0.0001$). Statistical data reflect the differences between the control and each treatment group for each cultivar, but not for each treatment between both cultivars. Data does not reflect the size, growth speed or overall quality of the regenerated shoots.

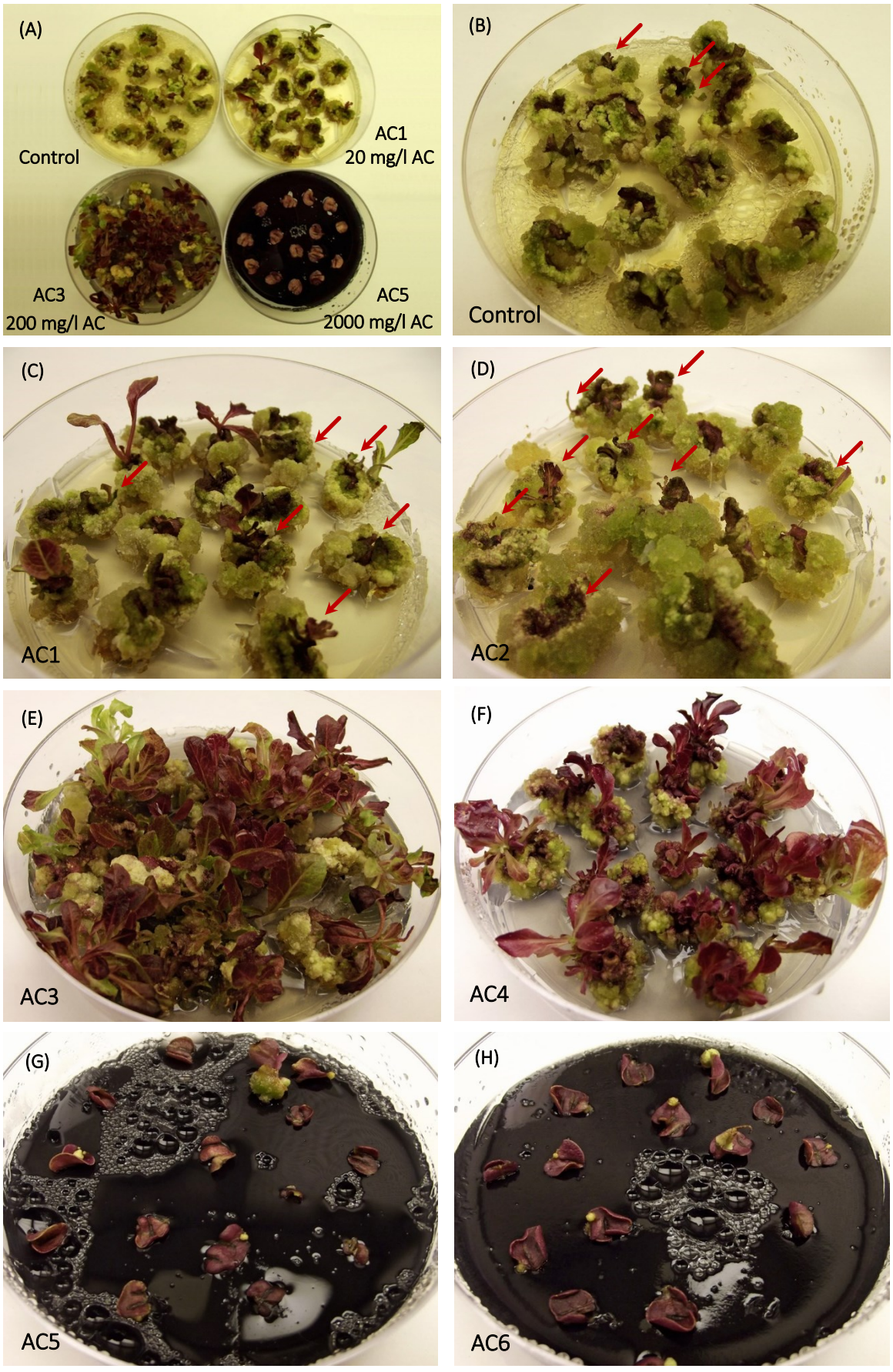

(A)
Control
AC1
20 mg/l AC
AC3
200 mg/l AC
AC5
2000 mg/l AC
(B)
Control
(C)
AC1
(D)
AC2
(E)
AC3
(F)
AC4
(G)
AC5
(H)
AC6

**Fig. 15. Shoot induction in RSL using activated charcoal (previous page).** Strong differences were observed between the control medium and the three concentrations of activated charcoal tested **(A)**. A strongly significant ($p \leq 0.0001$) increase in shoot count, size and growth speed was observed on media supplemented with 200 mg/l of AC **(E, F)**. A moderately significant ($p \leq 0.01$) increase in shoot induction was observed on medium containing 20 mg/l AC, 2.5 mg/l BA and 0.125 mg/l NAA **(C)** when compared to the control medium **(B)**. No significant differences were observed on medium containing 20 mg/l AC, 5 mg/l BA and 0.25 mg/l NAA **(D)**, and tissue did not react at all on media supplemented with 2000 mg/l AC **(G, H)**. Explants shown at week 7 and small shoots marked with arrows.

Medium AC3 was determined as the optimal for efficient regeneration of healthy shoots in all varieties of 'RSL', across all the media variations tested. Subsequent evaluation of the effects of medium AC3 on a range of different lettuce cultivars showed that even though results were not always consistent across genotypes, it often induced significant improvements **(Fig. 16)**.


**Fig. 16. Enhanced shoot induction on medium AC3 containing 200 mg/l activated charcoal.** The effects of medium AC3 were consistent in 'RSL NFR' **(A)** and significant improvements were also observed when tested on a number of other lettuce cultivars (data not shown) such as 'Paris White' **(B)**, 'Lettony', 'Simpson Elite' or 'Bambi'. The high rate of direct shoot induction allowed a much faster throughput when screening for the desired phenotype in regenerated shoots **(C)**. Explants shown at week 7.

## Discussion

Direct and efficient shoot regeneration was observed for both 'RSL NFR' and 'Winter Density' when using 0.09 mg/l BA and 0.1 mg/l NAA (medium R) and when using 2 mg/l zeatin and 0.1 mg/l NAA (medium C3), and in the case of 'RSL NFR' also moderately when using 1 mg/l BA and 0.05 mg/l NAA (medium H1). The high regeneration rate of 60% ± 21.5% obtained for 'RSL NFR' on medium R is consistent with rates of up to 72% previously reported for other cultivars in the literature[49], and the moderate regeneration rate of 40% ± 11.5% obtained for 'RSL NFR' on medium H1 is consistent with reports that low amounts of BA induce a decrease in callus production and an increase in direct shoot induction[49]. Nevertheless the abnormal and weak shoot morphology observed on these media might well lead to problems surviving transplantation into growing mix and producing viable seed, as it has been very consistently observed that plantlets developing under significant stress have very high seed abortion rates. As for medium C3, the very high regeneration rate of 82.2% ± 3.8% obtained for 'RSL NFR' is consistent with zeatin being considered the most potent cytokinin. However aberrant shoot morphology casts doubts on plant viability for seed production, which given the extremely high price of zeatin ($1,000/g zeatin versus $5/g BA) makes it a suboptimal choice for large scale applications.

Supplementation of the medium with moderate amounts of AC induced the best and most interesting results in 'RSL NFR'. The regeneration rate on media supplemented with 200 mg/l AC varied between 64% ± 36% when under 5 mg/l BA and 0.25 mg/l NAA (medium AC3) and 86.7% ± 9.4% when under 10 mg/l BA and 0.5 mg/l NAA (medium AC4). Despite the higher rate of explants producing shoots on medium AC4, medium AC3 consistently

produced a higher amount of more even looking shoots per single explant, making it the optimal choice for high throughput screening. These shoots showed a normal and healthy morphology, were able to successfully develop roots on MS root induction media and survived transplantation to growing mix with no strong signs of stress. It was observed that when AC was not distributed evenly while pouring, leading to locally higher concentrations in some Petri dishes, shoot induction decreased. This effect is consistent with an excessive adsorption of medium nutrients and growth regulators induced by too abundant AC, which also explains the lack of explant reaction on media with 2000 mg/l (media AC5 and AC6).

The lack of significant differences in media containing increased amounts of total BA and NAA (media H3 to H8) rules out the possibility that the results obtained in media AC3 and AC4 are due to the increased amount of hormones added to offset their adsorption by AC. It has been hypothesized that the beneficial effects of AC on shoot growth and development can be attributed to the adsorption of inhibitory substances in the medium, a decrease in the accumulation of toxic exudates from the explants, a gradual release of the nutrients and plant growth regulators initially adsorbed to its surface, or even a gradual release of other useful compounds which may be naturally present in AC particles[59].

Medium AC3 allows screening hundreds of mutagenized 'RSL NFR' candidate shoots for the desired phenotype in half the time required to obtain a few dozens of shoots using the original medium. This is also the first report of AC used for shoot induction in lettuce. The intensity of the beneficial effects is highly dependent on the cultivar used, which is consistent with previous studies[46,49,50]. Nevertheless, a significant increase in direct shoot induction was observed for a variety of other lettuce cultivars tested as well.

## SECTION C – OPTIMIZATION OF CONDITIONS FOR MAXIMUM POLYPHENOL CONTENT

### Introduction

Growing and management conditions are well known to have an enormous influence on the phytochemical profile of plants and RSL is no exception. During the course of the mutagen dose optimization experiments, it was observed that the tonality of RSL leaves was strongly affected by apparently minor changes in lighting conditions, which is consistent with the fact that the expression of most fundamental genes in the anthocyanin biosynthetic pathway is induced by UV radiation[24,27,32]. Given the need to establish optimal and standardized growing conditions to be able to maximize polyphenol levels and compare them across plant batches or experiments, it was decided to evaluate the effect of different types of light sources available on phytochemical production in RSL.

In addition to high light intensity and UV radiation, it has been well established in different plant species that exposure to other types of environmental stresses also increases production of anthocyanins and other polyphenols, possibly due to the protective effect these antioxidant compounds exert against free radicals and reactive oxygen species. Accumulation of anthocyanins has been associated with increased plant resistance to leaf wounding, chilling, freezing, heat shock, osmotic stress, drought stress, heavy metal contamination, certain nutritional stresses and infections[24,26,29,62]. In the case of lettuce, previous experiments have shown significant increases in leaf polyphenol content and antioxidant capacity as measured by DPPH, FRAP or TEAC after mechanical wounding[10], heat shock and chilling[63] or prolonged UV exposure[27]. It was therefore decided to study the potential of abiotic stresses to further increase polyphenols in RSL.

## Materials and methods

*Chemicals and reagents*

Folin-Ciocalteu phenol reagent, sodium carbonate salt, gallic acid, potassium chloride, sodium acetate, Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%], fluorescein sodium salt, 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (AAPH), phosphate buffered saline (PBS) 10x concentrate, quercetin 3-glucoside and organic solvents were purchased from Sigma (St. Louis, MO, USA). Chlorogenic acid and dicaffeoylquinic acid were purchased from ChromaDex (Irvine, CA, USA). Cyanidin 3-glucoside was purchased from Polyphenols (Sandnes, Norway).

*Light study plant growth conditions*

Seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) were sown inside plastic trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA) and kept in the Rutgers New Jersey Agricultural Experiment Station (NJAES) research greenhouse, constructed using Cyro/Degussa Deglas Impact Modified 16/32 UV stabilized acrylic panels (Evonik Corporation, Parsippany, NJ, USA) as glazing material, with light transmittance values ranging from 82% to 86% depending on the light angle. When plants reached a height of 5-7 cm they were transplanted into pots (diameter 10 cm, volume 414 $cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA). During this time, all plants were kept under Philips C400S51/Alto/ED18 400 W high pressure sodium (HPS) lamps (P.L. Light Systems Inc., Beamsville, ON, Canada). When plants reached 8 weeks old, they were separated into groups to undergo differential light treatments (Table 5). Two batches of

| | PAR intensity | | UV intensity | |
|---|---|---|---|---|
| | µmol/m²s | mol/m²d (DLI) | µmol/m²s | mol/m²d (DLI) |
| Sunlight | – | 18.8 ± 4.5 | – | 0.1 ± 0.0 |
| High pressure sodium lamps | 39.8 ± 3.4 | 1.6 ± 0.1 | 0.3 ± 0.0 | 0.0 ± 0.0 |
| Metal halide lamps | 92.6 ± 19.2 | 3.7 ± 0.8 | 6.7 ± 1.8 | 0.3 ± 0.1 |
| LED LumiBars | 120.5 ± 16.3 | 6.9 ± 0.9 | 0.1 ± 0.0 | 0.0 ± 0.0 |
| Fluorescent lamps | 455.8 ± 45.1 | 26.3 ± 2.6 | 12.3 ± 2.1 | 0.7 ± 0.1 |

**Table 5. Light sources and intensities evaluated on RSL**. Four types of light sources with different spectral distributions and wattage were tested. Daily light integral (mol/m$^2$d) measurements were estimated from averaged instantaneous µmol/m$^2$s readings collected using a MQ-200 quantum sensor and a MU-200 UV sensor, respectively (Apogee Instruments, Logan, UT, USA). Total DLI values for plants exposed to high pressure sodium lamps and metal halide lamps are the sum of DLI values from sunlight and DLI values of each corresponding lamp. DLI values for LED LumiBars and fluorescent lamps are independent from sunlight, since these lamps were located inside environmentally controlled growth chambers. Instantaneous measurements for sunlight not shown as the values were different at different times of the day. Sunlight DLI estimated for each day in the month of May 2015 and averaged. PAR, Photosynthetically Active Radiation; DLI, Daily Light Integral.

plants remained inside the greenhouse, under 400 W HPS lamps and under General Electric R1000 Multi-Vapor MVR1000/U 1000 W metal halide (MH) lamps (General Electric, Fairfield, CT, USA) respectively. A batch of plants was placed inside an environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) containing two 5 W LumiBars (LumiGrow, Novato, CA, USA) with red and blue light-emitting diodes (LED) adjusted to emit red and blue light in a ratio of 68:27, and a last batch of plants was placed inside a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) containing Sylvania F96/T12/CW/VHO fluorescent bulbs (Osram Sylvania, Danvers, MA, USA). The remaining conditions inside the greenhouse were 24.4 ± 1.7/19.7 ± 1.8 °C (day/night), 51 ± 12.3% relative humidity, and a 16/8 h (light/dark) photoperiod. All plants were hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks. Plants were kept under differential light conditions for 2 weeks before final collection for analysis.

*Stress study plant growth conditions*

Seeds of romaine 'Rutgers Scarlet Lettuce NAR' provided by Shamrock Seed Company (Salinas, CA, USA) were sown inside plastic trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA) and kept in a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA). When plants reached a height of 5-7 cm they were transplanted into pots (diameter 10 cm, volume 414 $cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA). Growing conditions were 22/18 °C (day/night), 65% relative humidity, a light intensity of 26.3 ± 2.6 $mol/m^{-2}d$ for PAR and 0.7 ± 0.1 $mol/m^{-2}d$ UV, provided by Sylvania F96/T12/CW/VHO fluorescent lamps (Osram Sylvania, Danvers, MA, USA), and a 16/8 h (light/dark) photoperiod. Plants were hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks.

*Environmental stress treatments*

When plants reached 8 weeks old they were treated with various short-term environmental stresses. All plants were watered to field capacity before the start of all treatments, and normal growing conditions were restored for 48 h after the end of each treatment to allow recovery and phytochemical changes. (1) Chilling stress was imposed by placing the plants inside a cold room kept at 4 °C for 24 h. (2) Drought stress was imposed by withholding water supply for several days until plants showed clear symptoms of wilt, soil volumetric water content (VWC) reached approximately 7% and relative water content of leaves (RWC) reached approximately 60%. Evaluation of plant wilt was performed visually every 8 h after initiation of drought stress. VWC was monitored using a

MAS-1 soil moisture probe (Decagon Devices, Pullman, WA, USA) connected to a HOBO U12-013 data logger (Onset, Bourne, MA, USA) from the beginning of each drought treatment and until plant collection. RWV was measured using the Barrs & Weatherley[64] method, based on recording the fresh weight (FW), turgid weight (TW), and dry weight (DW) of leaves and applying the formula (%) = ([FW - DW]/[TW - DW]) × 100. Briefly, pieces of all leaves measuring approximately 1 $cm^2$ were collected at the time of maximum wilt and their fresh weight was recorded immediately after harvesting. Samples were then wrapped in tissue paper and submerged in deionized water for 24 h at 4 °C. Leaf tissue was removed from water, blotted dry, and turgid weight was recorded. After oven drying samples at 80 °C for 72 h, dry weight was recorded as well. (3) Wounding stress was achieved by piercing the entire surface of all leaves using a toothed dissecting forceps. (4) UV exposure stress was imposed by placing each plant under a 26 W Exo Terra Repti-Glo 10.0 lamp (Rolf C. Hagensfield, MA, USA) uninterruptedly for 24 h. UV intensity was monitored using a MU-200 UV sensor with handheld meter (Apogee Instruments, Logan, UT, USA) to adjust plant position so all received equivalent amounts of radiation. Lamps were placed centered 5 cm above each canopy top and the average UV intensity at the center of the canopy during the duration of the treatment was 9.4 ± 0.3 $mol/m^2d$.

*Sample processing and extraction*

All leaves from the selected plants were collected, their fresh weights were recorded, and leaves were flash frozen using liquid nitrogen prior to lyophilization. Dried leaves were ground to a fine powder with a mortar and pestle. Leaf extracts were prepared using a methanolic solvent to efficiently extract polyphenols as described by Wu et al.[11] with minor

modifications. Briefly, 0.5 g of dried material was transferred to a 50 ml tube protected from light and 15 ml of extraction solvent (methanol/water/acetic acid; 85:14.5:0.5) was added. Samples were vortexed for 30 s, sonicated at room temperature for 5 min, vortexed for 30 s, then incubated at room temperature for 10 min. Samples were again vortexed for 5 s then centrifuged at 1699 rcf for 5 min. The supernatant was decanted into a clean 50 ml tube and the extraction process was repeated two more times. The supernatants were pooled and filtered through 0.45 mm PTFE filters (VWR, Radnor, PA, USA) prior to all analyses. Extracts were covered to protect from light and stored at –20 °C.

*Quantification of total polyphenols*

Total phenolic content was measured by a modified Folin-Ciocalteu method[12,65]. Briefly, filtered lettuce extracts were diluted 20x and fresh gallic acid standard dilutions (0–100 µg/ml) were prepared using double distilled water. 2N Folin-Ciocalteu phenol reagent was combined with 50% methanol (1:1). 200 µl of this Folin-Ciocalteu solution was added to 200 µl of gallic acid standard dilutions or diluted lettuce extracts, and incubated at room temperature for 10 min. Next, 300 ml of 20% sodium carbonate was added and samples were incubated in a 40 °C water bath for 20 min. Samples were then cooled on ice and centrifuged for 30 s at 7000 rcf. 100 µl of supernatant was transferred in triplicate to a 96-well plate and absorbance was measured at 760 nm using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). A regression line was generated by plotting the concentration of gallic acid standard dilutions by their respective absorbances. Results were expressed as gallic acid equivalents (GAE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of total monomeric anthocyanins*

Total monomeric anthocyanin content was determined according to the AOAC pH differential method[66]. Filtered lettuce extracts were diluted 10x in double distilled water and 800 µl of pH 1 (25 mM potassium chloride) or pH 4.5 (0.4 M sodium acetate) buffers were added to 200 µl of each diluted lettuce extract. 300 µl of each pH 1 and pH 4.5 solutions were transferred in triplicate to a 96-well plate and their absorbances were measured at 510 nm and 700 nm on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). Results were expressed as cyanidin 3-glucoside equivalents (CGE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of Oxygen Radical Absorption Capacity (ORAC)*

ORAC was measured as described by Prior et al.[12,67] with minor modifications. Briefly, filtered lettuce extracts were diluted 1000x and fresh Trolox standard dilutions (0–100 µM) were prepared using 75 mM PBS, pH 7.4. Fluorescein sodium salt was diluted in 75 mM PBS, pH 7.4, to a final concentration of 6 nM. Water was pipetted into the outer wells of a 96-well plate and then 150 µl of 6 nM fluorescein solution was added to each well. Next, 25 µl of Trolox standard dilutions or diluted lettuce extracts were added to each well in triplicate. The plate was preincubated at 37 °C for 30 min. A fresh 125 mM solution of AAPH was prepared and 25 µl added to each well after pre-incubation to a final concentration of 16 mM. Fluorescence readings were recorded every minute for 120 min on a Synergy HT Multi-Detection Microplate Reader (485 nm, 20 nm bandpass, excitation filter and 528 nm, 20 nm bandpass, emission filter). A regression line was generated by plotting the concentration of Trolox standard dilutions by their respective net area under the curve

(AUC). Results were expressed as µmol Trolox equivalents (TE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of phytochemicals by UPLC/MS/UV*

Filtered lettuce extracts were dried using a Savant SpeedVac AES2010 centrifugal evaporator (Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in methanolic extraction solvent to a final concentration of 10 mg/ml. Standards of chlorogenic acid, dicaffeoylquinic acid, quercetin 3-glucoside and cyanidin 3-glucoside were also prepared to generate regression lines for quantification. 1 µl from each sample or standard dilution was injected into a Dionex UltiMate 3000 RSLC system (Dionex Corporation, Sunnyvale, CA, USA) ultra-high pressure liquid chromatography for separation and analysis. The system consisted of a workstation running Dionex Chromeleon v.6.8, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector, the eluent flow was guided to a Varian 1200L (Varian Medical Systems, Palo Alto, CA, USA) triple quadrupole mass detector with electrospray ionization interface (ESI), operated in either negative or positive ionization modes. The voltage was adjusted to –4.5 kV, heated capillary temperature was 280 °C, and sheath gas was compressed air, zero grade for the negative ionization mode, and nitrogen gas, 5 kV capillary voltage for the positive ionization mode. The mass detector was used in scanning mode from 65 to 1500 atomic mass units (amu). Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v.6.9 SP2. Substances were separated on a Phenomenex C8 (Phenomenex, Torrance, CA, USA)

reverse phase column of size 150 x 2 mm, particle size 3 µm and pore size 10 nm. The mobile phase consisted of 2 components: solvent A (0.5% ACS grade acetic acid in double distilled de-ionized water, pH 3-3.5), and solvent B (100% acetonitrile). The mobile phase flow was 0.20 ml/min, and a gradient mode was used for all analyses.

For separation of the dicaffeoylquinic acid, quercetin 3-glucoside, quercetin 3-malonyl-glucoside and cyanidin 3-glucoside peaks the following gradient conditions were used: initial conditions of the gradient were 95% A and 5% B; for 30 minutes the proportion reached 5% A and 95% B, which was kept for 8 minutes, and during the following 4 minutes the ratio was brought back to initial conditions. An 8 minutes equilibration interval was included between subsequent injections. For separation of the chlorogenic acid and cyanidin 3-malonyl-glucoside peaks the following gradient conditions were used: initial conditions of the gradient were 95% A and 5% B; for 20 minutes the proportion reached 80% A and 20% B; for the next 3 minutes the proportion reached 5% A and 95% B, which was kept for the next 4 minutes. For the following 3 minutes the ratio was brought back to initial conditions. A 5 minutes equilibration interval was included between subsequent injections. The average pump pressure using these parameters was typically around 26.9 MPa for the initial conditions in both cases.

*Experimental design and statistical analysis*

All treatment groups comprised four plants chosen at random from the same cohort and all treatments were replicated three times, totaling n = 12 plants per treatment group. Unstressed plants were kept under the same growing conditions and collected at the same time as the stress treated plants to serve as controls. The means and standard deviations

(SD) were calculated from pooled data and all results are expressed as mean ± SD. Statistical significance of the differential effects of the treatments was determined using a general linear model analysis of variance (ANOVA) followed by a Tukey test for the light study treatment groups, and by a Dunnett test for the stress study treatment groups. A global significance level was set at $\alpha = 0.05$. All statistical procedures were performed using GraphPad Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

## Results

*Effects of light source and intensity*

'RSL NFR' plants initially growing in the greenhouse under HPS bulbs developed only moderate amounts of red color in their leaves. By the time samples were collected, after two weeks under differential light conditions, a clear gradient in pigmentation **(Fig. 17)**

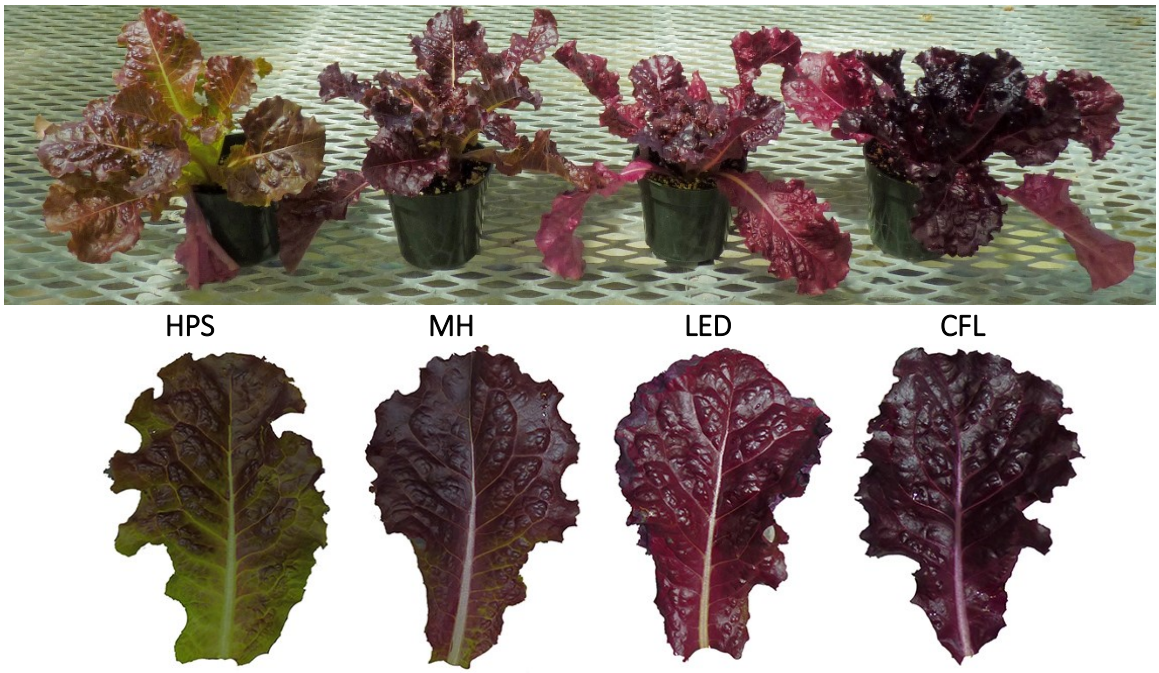



**Fig. 17. Effect of light intensity and spectrum on anthocyanin development in RSL**. An increase in leaf anthocyanin accumulation was visually apparent when plants were exposed to high light intensity (MH) or a light spectrum richer in short wavelengths (LED) than the HPS baseline, and was the strongest when under a combination of both high intensity and abundant short wavelengths (CFL). Plants shown at the moment of collection at 10 weeks old, after 2 weeks growing under different light conditions. HPS, high pressure sodium lamps; LED, light-emitting diodes; MH, metal halide lamps; CFL, cool white fluorescent lamps.

could be observed between groups. Plants which had remained under HPS lighting showed the lightest color and plants which had been kept under fluorescent lamps exhibited the darkest. Plants exposed to LED lamps and plants which had been kept in the greenhouse under MH lamps both developed a similar amount of red pigment, but their leaf color was different due to a lack of other pigments in plants grown under LED lamps. A small size difference could also be observed, where plants which had remained in the greenhouse under HPS and MH lighting were slightly bigger than plants kept inside either of the growth chambers, possibly due to the different temperature conditions.

The phytochemical characterization of the four groups revealed a similar trend, with total polyphenol content and ORAC antioxidant activity progressively increasing with anthocyanin accumulation **(Fig 18, Table 6)**. A very significant ($p \leq 0.0001$) increase in total polyphenols was observed between plants grown under fluorescent lamps and all other treatments, which also correlated with an increase in ORAC antioxidant activity ($p \leq 0.05$).

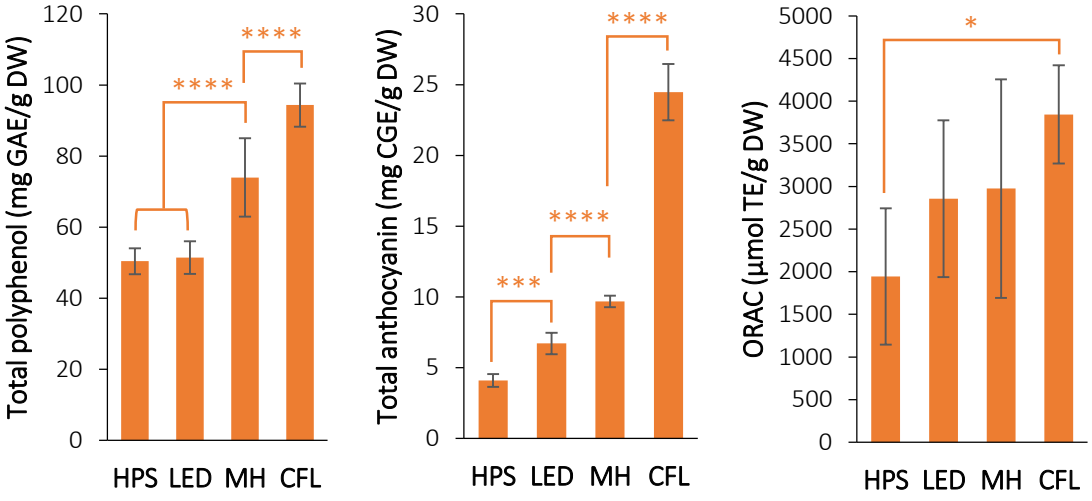



**Fig. 18. Phytochemical content of RSL exposed to different light conditions for two weeks**. n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside equivalents (CGE), and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Significant differences between groups were analyzed by one-way ANOVA followed by a Tukey test (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$). DW, dry weight basis; HPS, high pressure sodium lamps; LED, light-emitting diodes; MH, metal halide lamps; CFL, cool white fluorescent lamps.

| | Total polyphenols (mg/g) | | Total anthocyanins (mg/g) | | ORAC (µmol/g) | |
|---|---|---|---|---|---|---|
| | DW | FW | DW | FW | DW | FW |
| HPS | 50.4 ± 3.6 | 4.5 ± 0.3 | 4.1 ± 0.5 | 0.4 ± 0 | 1944 ± 800 | 173 ± 71 |
| LED | 51.4 ± 4.6 | 4.6 ± 0.4 | 6.7 ± 0.8 | 0.6 ± 0.1 | 2857 ± 920 | 254 ± 82 |
| MH | 74 ± 11 | 6.6 ± 1 | 9.7 ± 0.4 | 0.9 ± 0 | 2975 ± 1280 | 265 ± 114 |
| CFL | 94.3 ± 6.1 | 8.4 ± 0.5 | 24.5 ± 2 | 2.2 ± 0.2 | 3845 ± 578 | 342 ± 51 |

**Table 6. Phytochemical content of RSL exposed to different light conditions for two weeks**. n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside (C3G) equivalents, and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Fresh weights estimated from dry weights assuming a water content in RSL of 91.1%[12]. DW, dry weight basis; FW, fresh weight basis; HPS, high pressure sodium lamps; LED, light-emitting diodes; MH, metal halide lamps; CFL, cool white fluorescent lamps.

Plants grown under MH lamps also showed a very significant increase in total polyphenols when compared to both HPS and LED treatments. No significant differences were found between the total polyphenol levels in plants exposed to HPS and LED light.

UPLC/UV quantification of the major chemical components normally present in RSL **(Fig. 1)** show increasing levels of the major RSL phytochemical component quercetin 3-malonyl glucoside with anthocyanin accumulation **(Fig. 19, Table 7)**. A significant difference in quercetin 3-malonyl glucoside content was observed between plants exposed to fluorescent and HPS light ($p \leq 0.001$), as well as plants exposed to LED and MH light ($p \leq 0.01$). This increase correlated with a significant increase in ORAC antioxidant activity as well. However no significant difference was found between the chlorogenic acid levels of any of the treatment groups.

*Effects of short-term abiotic stress*

Unlike the changes readily observed on 'RSL NFR' exposed to different light sources, the effect of the abiotic stress treatments was not visually apparent on 'RSL NAR' plants prior to collection. Plants chilled at 4 °C for 24 h did not show any visual signs of being

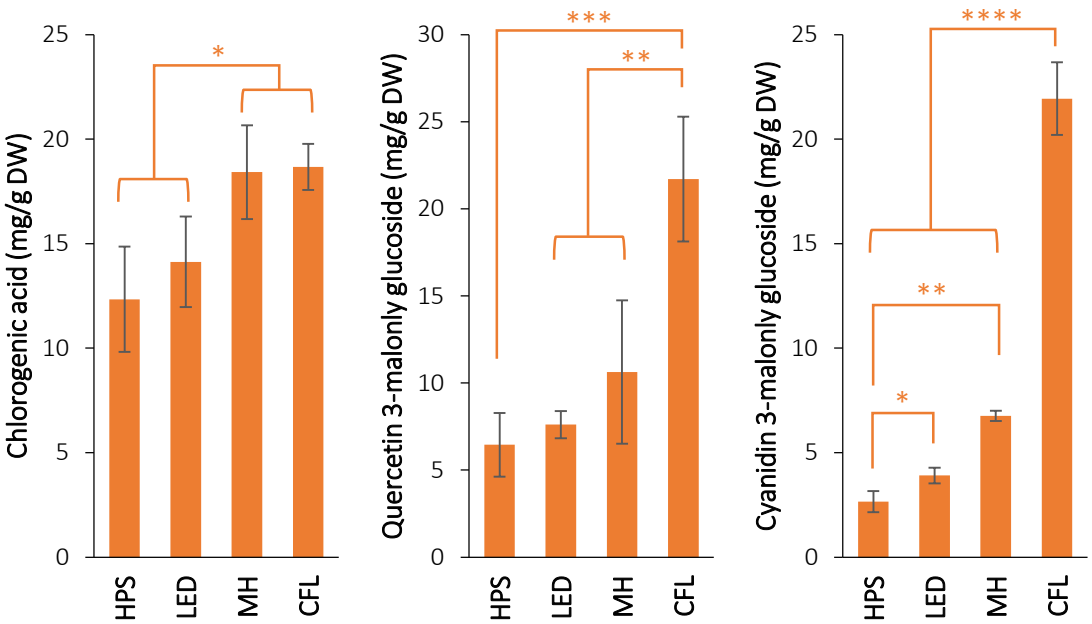



**Fig. 19. Major phytochemical components of RSL exposed to different light conditions for two weeks**. n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. Quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside values estimated using quercetin 3-glucoside and cyanidin 3-glucoside as standard compounds, respectively. All values estimated using UV spectroscopy. Significant differences between groups were analyzed by one-way ANOVA followed by a Tukey test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. DW, dry weight basis; HPS, high pressure sodium lamps; LED, light-emitting diodes; MH, metal halide lampss; CFL, cool white fluorescent lamps.

| Compound (mg/g DW) | HPS | LED | MH | CFL |
|---|---|---|---|---|
| Chlorogenic acid | 12.3 ± 42.5 | 14.1 ± 2.2 | 18.4 ± 2.2 | 18.7 ± 1.1 |
| Dicaffeoylquinic acid | 1.2 ± 0.5 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.8 ± 0.2 |
| Quercetin glucoside | 0.7 ± 0.3 | 1.1 ± 0.1 | 1.3 ± 0.6 | 3.7 ± 0.7 |
| Quercetin malonyl glucoside | 6.4 ± 1.8 | 7.6 ± 0.8 | 10.6 ± 4.1 | 21.7 ± 3.6 |
| Cyanidin glucoside | 0.4 ± 0.2 | 0.9 ± 0.1 | 0.7 ± 0.2 | 2.3 ± 0.4 |
| Cyanidin malonyl glucoside | 2.7 ± 0.5 | 3.9 ± 0.4 | 6.8 ± 0.2 | 21.9 ± 1.7 |

**Table 7. Major phytochemical components of RSL exposed to different light conditions for two weeks.** n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. All results are expressed as mg per gram of dry weight. Quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside values estimated using quercetin 3-glucoside and cyanidin 3-glucoside as standard compounds respectively. All values estimated using UV spectroscopy. HPS, high pressure sodium lamps; LED, light-emitting diodes; MH, metal halide lampss; CFL, cool white fluorescent lamps.

affected by the treatment. Plants subjected to drought stress for several days were wilted by the time the treatment was stopped, but had regained their turgor completely and looked healthy by the time they were sampled two days later. Plants subjected to leaf

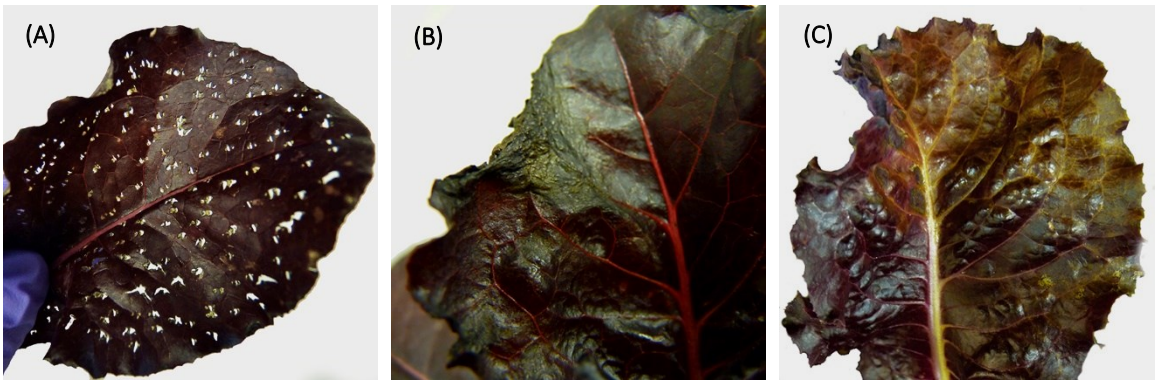



**Fig. 20. RSL leaves after wounding and UV exposure stress treatments. (A)** Plants wounded by piercing the surface of the leaves using a toothed forceps maintained a relatively high turgor at the time of collection. **(B)** Outer leaves exposed to a mixture of UV-A and UV-B radiation for 24 h often developed burnt edges and **(C)** discoloration.

wounding did initially lose leaf turgor, but looked normal at the time of sampling. Plants exposed to UV radiation for 24 h showed burnt edges and discolorations in some outer leaves, but seemed healthy otherwise **(Fig. 20)**. No changes in color when visually compared to the control plants were observed for any of the treatment groups.

Phytochemical characterization of all samples revealed no significant differences between the control plants and any of the treatment groups for either total polyphenol content, total anthocyanin content or ORAC antioxidant capacity **(Fig 21, Table 8)**. UPLC/MS/UV quantification of major RSL compounds was carried out on a subset of samples to look for any possible shifts in the phytochemical profile, but no significant differences were observed either **(Fig 22, Table 9)**.

## Discussion

The phytochemical results obtained for the different light sources and intensities correlate with the fact that transcription of fundamental genes in the phenylpropanoid pathway leading to anthocyanin production requires both (1) sufficient levels of light

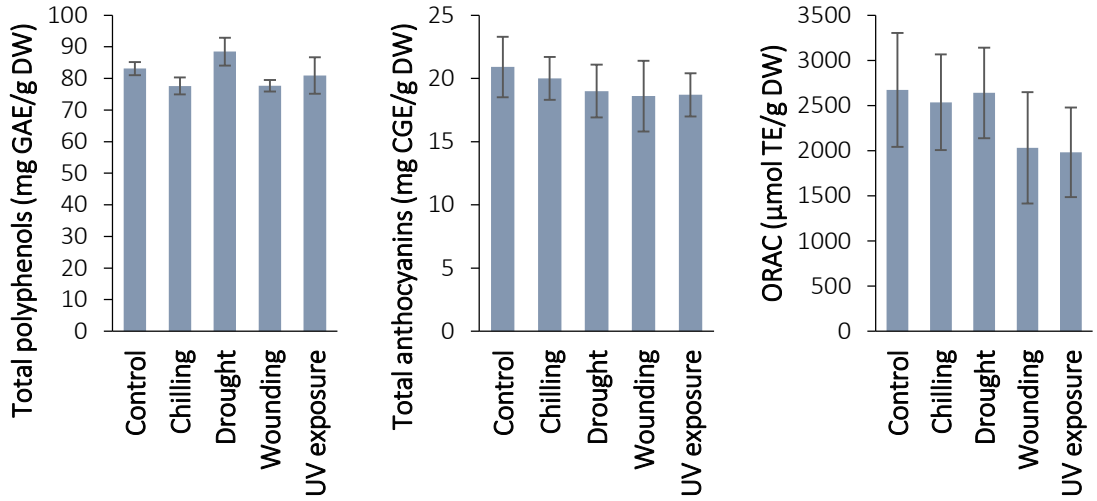



**Fig. 21. Phytochemical content of RSL grown subjected to different abiotic stresses.** n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside equivalents (CGE), and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Significant differences between treatment groups and controls were analyzed by one-way ANOVA followed by a Dunnett test. DW, dry weight basis.

| | Total polyphenols (mg/g) | | Total anthocyanins (mg/g) | | ORAC (µmol/g) | |
|---|---|---|---|---|---|---|
| | DW | FW | DW | FW | DW | FW |
| Control | 83.1 ± 2.1 | 7.4 ± 0.2 | 20.9 ± 2.4 | 1.9 ± 0.2 | 2672 ± 630 | 238 ± 56 |
| Chilling | 77.6 ± 2.7 | 6.9 ± 0.3 | 20 ± 1.7 | 1.8 ± 0.2 | 2536 ± 531 | 226 ± 47 |
| Drought | 88.5 ± 4.4 | 7.9 ± 0.4 | 19 ± 2.1 | 1.7 ± 0.2 | 2639 ± 500 | 235 ± 45 |
| Wounding | 77.7 ± 1.8 | 6.9 ± 0.2 | 18.6 ± 2.8 | 1.7 ± 0.2 | 2032 ± 617 | 181 ± 55 |
| UV exposure | 80.9 ± 5.8 | 7.2 ± 0.5 | 18.7 ± 1.7 | 1.7 ± 0.2 | 1980 ± 496 | 176 ± 44 |

**Table 8. Phytochemical content of RSL subjected to different abiotic stresses**. n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside equivalents (CGE), and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Fresh weights estimated from dry weights assuming a water content in lettuce of 91.1%[12]. DW, dry weight basis; FW, fresh weight basis.

intensity and (2) stimulation by UV-A or UV-B[24,27,31,32]. The moderately intense 400 W HPS lamps commonly used as supplemental lighting in greenhouses are rich in the red section of the spectrum mostly, in order to allow efficient photosynthetic rates and growth despite low sunlight intensity, but are not meant to induce polyphenol production in crops and therefore barely emit any UV radiation **(Table 5)**. LED lamps emit discrete wavelengths

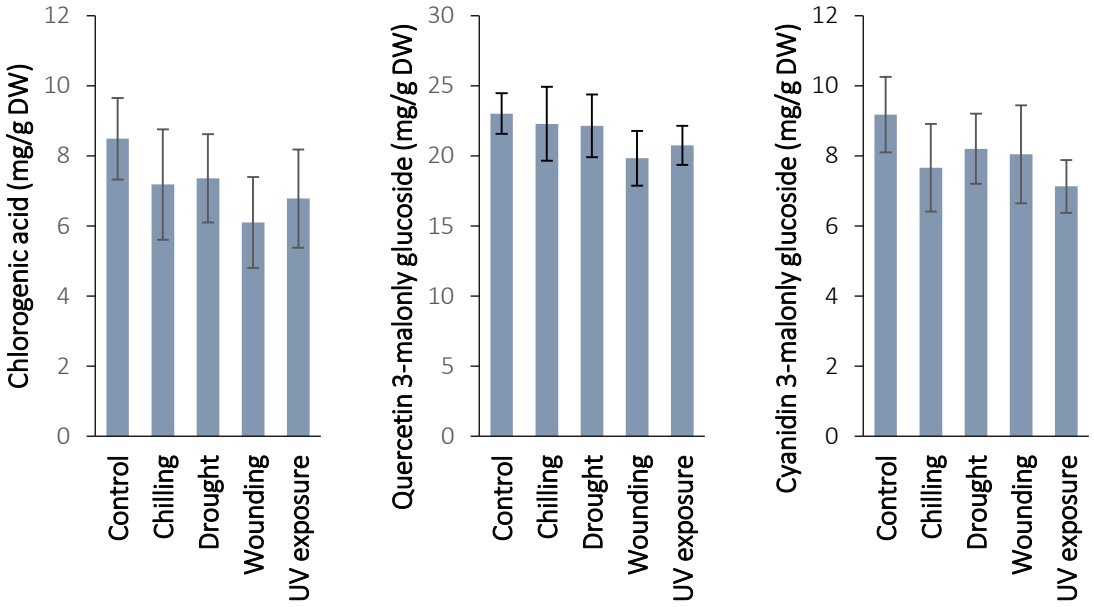



**Fig. 22. Major phytochemical components of RSL subjected to different abiotic stresses**. n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. All results are expressed as mg per gram of dry weight. Quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside values estimated using quercetin 3-glucoside and cyanidin 3-glucoside as standard compounds respectively. All values estimated using UV spectroscopy. Significant differences between treatment groups and controls were analyzed by one-way ANOVA followed by a Dunnett test. DW, dry weight basis.

| Compound (mg/g DW) | Control | Chilling | Drought | Wounding | UV exposure |
|---|---|---|---|---|---|
| Chlorogenic acid | 8.5 ± 1.2 | 7.2 ± 1.6 | 7.4 ± 1.3 | 6.1 ± 1.3 | 6.8 ± 1.4 |
| Dicaffeoyl quinic acid | 2.4 ± 1.5 | 2.0 ± 0.7 | 3.4 ± 1.1 | 3.1 ± 1.3 | 3.1 ± 0.5 |
| Quercetin glucoside | 4.7 ± 0.2 | 3.3 ± 0.4 | 4.5 ± 0.4 | 3.3 ± 0.2 | 4.0 ± 0.4 |
| Quercetin malonyl glucoside | 23.0 ± 1.4 | 22.3 ± 2.6 | 22.1 ± 2.2 | 19.8 ± 1.9 | 20.7 ± 1.4 |
| Cyanidin glucoside | 2.8 ± 0.4 | 1.9 ± 0.5 | 2.5 ± 0.4 | 1.9 ± 0.5 | 2.1 ± 0.4 |
| Cyanidin malonyl glucoside | 9.2 ± 1.1 | 7.7 ± 1.2 | 8.2 ± 1.0 | 8.0 ± 1.4 | 7.1 ± 0.8 |

**Table 9. Major phytochemical components of RSL grown subjected to different abiotic stresses.** n= 12 plants per treatment group. Data were pooled and are presented as the mean ± SD. All results are expressed as mg per gram of dry weight. Quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside values estimated using quercetin 3-glucoside and cyanidin 3-glucoside as standard compounds respectively. All values estimated using UV spectroscopy. DW, dry weight basis.

whose ratios can be manipulated in order to induce either efficient biomass production or synthesis of phytoactives[68]. Therefore, an LED setup can be enriched with short

wavelength blue light and induce relatively high polyphenol production, despite the lack of UV. A previous study reported that a blue to red LED ratio of 65 to 35 induced increases in polyphenol content and TEAC antioxidant capacity in leaves of loose leaf green lettuce cultivar 'Grand Rapid TBR' and loose leaf red lettuce cultivar 'Sunmang'[68]. In the present study,which is a very similar blue to red LED ratio of 68:27, a significant increase was found in the anthocyanin content under LED lamps when compared to the baseline levels produced under HPS lamps, but there were no significant increases in total polyphenols or ORAC content. This discrepancy with the aforementioned study could very well be due to the high baseline polyphenol content of RSL, given that the maximum polyphenol values reported in said study are 0.7 mg GAE/g FW – whereas even under moderate polyphenol inducing HPS lamps, the total polyphenol values of RSL can already reach 4.5 mg GAE/g FW (Table 6). The lack of significant increases in polyphenol content under LED lamps could also be explained by the overall low light intensity provided by only two 5 W LumiGrow LumiBars. Evaluation of the effects of a blue to red LED ratio of 47:53 on RSL, which rendered the highest increase in polyphenol production in the aforementioned study by Son et al[68], would be an interesting follow-up experiment.The 1000 W MH lamps provided both relatively high levels of light intensity and a spectrum much richer in blue and UV emissions than the HPS lamps, which might explain the increase of total polyphenol content up to 6.6 mg GAE/g FW. However, it is the extremely high levels of light intensity and UV radiation provided by very high output fluorescent lamps that induce the most drastic accumulation of polyphenols, reaching levels of up to 8.4 mg GAE/g FW, and which will be used for any further RSL screening or production. However, given the observed

results, LED lamps could also provide an alternative for color screening in RSL, at a small fraction of the electrical costs, and MH lamps could be used for high polyphenol RSL production, even if not up to the levels produced under fluorescent lamps.

Having confirmed the optimal light conditions for efficient polyphenol production in RSL, the question arose whether this amount can be further increased, potentially improving its beneficial properties through stress induction prior to harvest. For this purpose, the variety of RSL with the highest reported levels of polyphenols, 'RSL NAR', was selected as starting material. A significant increase in total polyphenols and TEAC antioxidant capacity had been previously reported for the romaine green lettuce cultivar 'Baronet' within just 1 h of chilling treatment at 4 °C [63]. Leaf wounding in romaine and iceberg lettuce had been reported to induce increases in phenolic content and DPPH antioxidant capacity of up to 290% and 255%, respectively, when measured two days after treatment[10]. As for prolonged UV exposure, 24 h under UV-B induced significant increases in total polyphenols and TEAC antioxidant capacity on the loose leaf red cultivar 'Hongyeom'[27]. No studies evaluating the effect of drought on polyphenol production in lettuce were found, but prolonged lack of water and the subsequent osmotic stress are well documented to induce phenolic compounds in other species[24,29,62]. Given that the same stress treatments at the same doses or higher induced no changes on 'RSL NAR', it can be concluded that, under the optimized light conditions provided by high intensity fluorescent lamps, polyphenol production in RSL seems to have reached a saturation level.

In addition, it would be interesting to study whether the application of abiotic stresses on RSL growing under light conditions which induce non-saturating levels of polyphenol

production, such as HPS, LED or MH, can induce an increase in total polyphenols similar to those reported when using fluorescent lamps. The power consumption required for the high light intensity conditions provided by the fluorescent lamps in the growth chamber was 14400 W (400 W ballast consumption for 2 lamps, with a total of 72 lamps covering an area of 9 $m^2$), whereas the power consumption required by HPS, LED and MH set ups covering areas of a similar size was approximately 1860 W, 1850 W and 3240 W, respectively. From the agronomic perspective, it would therefore be of great interest to determine the viability of producing very high polyphenol RSL using low electric energy consumption lighting supplemented by a short stress treatment prior to harvest.

## SECTION D – LARGE SCALE MUTATIONAL BREEDING IN GREENHOUSE

### Introduction

Several hundreds of mutagenized RSL shoots were produced and screened in tissue culture over a 3 to 4 month period using regeneration medium AC3 **(Table 4)**, but no changes in color were observed. Given that shoots regenerated from mutagenized material can only express dominant mutations and that the screening throughput in tissue culture still remained too low to obtain fast results despite enhancement of the medium, all greenhouse resources available were optimized in order to pursue a classical large-scale breeding approach instead. Batch screening of $M_1$ seedlings would be followed with batch production of $M_2$ seeds and screening of the individual $M_2$ families produced, in order to allow expression of recessive mutations as well.

Transmission of a genetic change from one generation to the next requires the mutation to happen in the cells of the germline that contribute to the formation of gametes, and therefore offspring. At the time of the mutation, these cells may be one, two or more depending on the species and the timing of the treatment. The number of these cells at a particular developmental stage is known as the genetically effective cell number (GECN)[69], and it will influence the segregation ratio in the progeny. Assuming a fixed GECN of 2 for mature lettuce seeds undergoing treatment and 100% self-pollination for all $M_1$ plants, the segregation of dominant versus recessive individuals in the $M_2$ generation will be 7 to 1, and the number of seedlings from each $M_2$ family that must be screened to recover one recessive mutant can be obtained through the equation $P = 1 - f^n$, where "P" is any given probability, "f" is the fraction of the progeny without the mutation, and "n" is

the number of individuals that must be screened[69]. For our particular scenario this number is 23 and 35 seedlings from each $M_2$ family for a probability of 95% and 99%, respectively, even though it would be recommended to sow seeds in excess of the desired number to account for any decreases in germination rate.

## Materials and methods

*$M_0$ seed EMS mutagenesis*

Seeds of loose leaf 'Rutgers Scarlet Lettuce NFR' provided by Shamrock Seed Company (Salinas, CA, USA) were soaked in distilled water containing 0.1% or 0.15% (v/v) EMS and treated overnight at room temperature in a rotary shaker. After 12 h the EMS solution was decanted, seeds were washed five times with 50 ml of distilled water and blotted dry.

*$M_1$ plant growth conditions*

EMS treated seeds were sown inside trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA) and kept in the Rutgers New Jersey Agricultural Experiment Station (NJAES) research greenhouse. When plants reached a height of 5-7 cm they were transplanted into pots (diameter 10 cm, volume 414 $cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA), which were kept inside pot carrying trays to maximize bench space. Plants were hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks. Growing conditions were 24.4 ± 1.7/19.7 ± 1.8 °C (day/night), 51 ± 12.3% relative humidity, a PAR light intensity of 22.5 ± 5.3 mol/$m^2$d and a UV light intensity of 0.4 ± 0.1 mol/$m^2$d, provided by sunlight supplemented with 1000 W metal halide lamps, and a 16/8 h (light/dark) photoperiod.

*$M_2$ seed collection and storage*

Once 50% of flowers in each inflorescence had produced mature seeds, approximately five and a half months since the sowing date, inflorescences were manually harvested, placed upside down inside open paper bags and left to dry at room temperature for 1 to 2 months. Seeds from each dry inflorescence were manually threshed, screened and winnowed, and clean $M_2$ seed sets from each individual plant, or $M_2$ families, were placed in separate paper envelopes and kept at 4 °C for long-term storage. The germination rate was evaluated on wet paper before proceeding to screening in the greenhouse.

*$M_2$ seedling screening*

Approximately 50 seeds from each individual $M_2$ family were sown inside trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA) and kept in a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) in the Rutgers New Jersey Agricultural Experiment Station (NJAES) research greenhouse. At least 23 $M_2$ seedlings from each family were screened for green color or green leaf sectors between weeks 1 and 2 and were discarded if they showed a normal phenotype. Seedlings were hand watered as needed, and growing conditions were 18/15 °C (day/night), 65% relative humidity, a PAR light intensity of 26.3 ± 2.6 mol/$m^2$d and a UV light intensity of 0.7 ± 0.1 mol/$m^2$d provided by Sylvania F96/T12/CW/VHO fluorescent lamps (Osram Sylvania, Danvers, MA, USA), and a 16/8 h (light/dark) photoperiod.

## Results

Approximately 1000 $M_1$ plants treated with 0.1% EMS and 1000 $M_1$ plants treated with

0.15% EMS were screened for green leaf sectors during the course of 9 months, but no variations in color were observed other than the occasional chlorotic sector. Plant morphology was normal except for a small number of phenotypical changes already described during EMS dose optimization **(Fig. 7)**, which did not affect plant viability. Most plants bolted as expected, producing stalks up to 1 m high and dense inflorescences with healthy flowers **(Fig. 23)**. Keeping plants inside 10 cm diameter pots for their whole lifespan to induce mild stress produced faster bolting and allowed bench space maximization.


**Fig. 23. RSL $M_1$ screening and $M_2$ seed production in the NJAES greenhouse.** When $M_1$ seedlings reached a height of 5-7 cm they were transferred to pots and screened for green color or green leaf sectors under 1000 W metal halide lamps. After bolting, plants reached a height of up to 1 m and produced full, healthy looking flower heads. The pictured setup used 15 cm diameter pots, allowing growth of approximately 200 plants per bench. Later setups used 10 cm diameter pots, allowing growth of approximately 400 plants per bench.

After self-pollination, $M_2$ seeds were collected from approximately 1800 $M_1$ plants. $M_1$ plants treated with 0.1% EMS produced approximately 50% viable seeds and 50% abortive seeds per flower, and each individual $M_2$ family from these plants contained between 1000 and 2000 viable seeds. $M_1$ plants treated with 0.15% EMS produced approximately 30% viable seeds and 70% abortive seeds per flower, and each $M_2$ family from these plants contained between 300 and 1000 viable seeds. The germination rate of all seeds was normal (96% ± 2%) when evaluated on wet paper, although much lower (55% ± 15%) in growing mix **(Fig. 24)**.

**Fig. 24. Flower morphology and seed quality in RSL mutagenized with 0.1% (v/v) EMS**. Flower morphology was normal and most individual flowers produced approximately 50% viable seeds (blue arrows) and 50% abortive seeds (red arrows). The germination rate on wet paper was comparable to that of untreated seeds (data not shown).

At the time of writing $M_2$ screening for green color is still underway, 50 families having been evaluated so far, with one green seedling recently identified **(Fig. 25)**.

## Discussion

Given that the phenotype of interest is, in principle, just as likely to be produced by a dominant mutation – such as the overexpression or modulation of a regulatory gene –, or by a recessive mutation – such as a knockout of both alleles required for the biosynthesis of the key enzymes ANS or UFGT **(Fig. 3)** –, a screening system that gives visibility to both is of great advantage. Efforts to establish a simple and cost-effective in-house RSL mutational breeding setup have yielded, within a short time, a large and valuable collection of $M_2$ seeds which can be stored long-term and be screened for a variety of phenotypes of interest. Identification of a first green seedling having screened less than 5% of the available $M_2$ families points to the potential success of the approach, and additional valuable candidate plants are likely to be identified as screening continues.

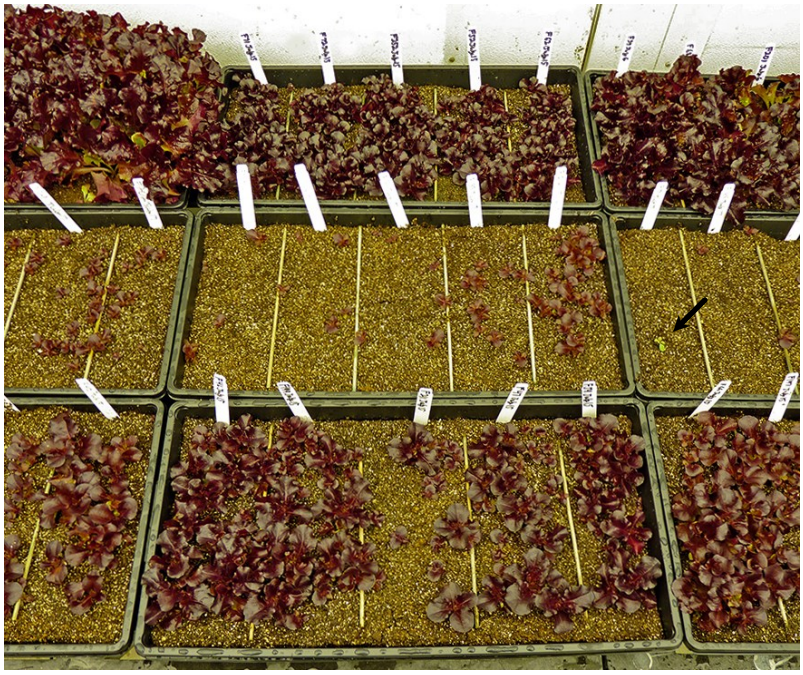


**Fig. 25. RSL $M_2$ screening in environmentally controlled growth chamber**. 50 seeds from each $M_2$ family were sown to evaluate the color phenotype of at least 23 seedlings. Each tray pictured contains seedlings from 5 individual $M_2$ families in different stages of plant development. Seedlings were kept under high light intensity to induce rapid anthocyanin development and faster screening. The recently identified green seedling is marked with an arrow.

## PART II – CHARACTERIZATION OF HIGH POLYPHENOL GREEN LETTUCE REVERTANTS

### Introduction

Screening of both untreated and mutagenized lettuce during the course of this research produced a total of three green colored revertant plants. The first of these, found among $M_1$ 'RSL NFR' seedlings treated with a gamma irradiation dose of 200 Gy during the experiments for optimization of mutagen type and dose, eventually arrested its growth and died before producing any seeds. Another was very recently found during early screening of the $M_2$ 'RSL NFR' seedlings. The third, a spontaneous green revertant with red spotted leaves, was found among untreated 'RSL NAR' plants **(Fig. 26)**.

Phytochemical analysis of mature leaves from this 'RSL NAR' green revertant revealed very similar total polyphenol levels to those of red 'RSL NAR' plants, as well as increased levels of the pathway precursors chlorogenic acid and dicaffeoylquinic acid. Even though this is the desired phenotype, successful and stable transmission of the trait to the following generation is still necessary, and depending on its segregation pattern, selection across several generations might also be required to produce sufficient numbers of seeds.


**Fig. 26. Green revertant RSL plants identified during the course of the project. (A)** 'RSL NFR' $M_1$ seedling treated with 200 Gy of gamma irradiation; **(B)**, 'RSL NFR' $M_2$ seedling obtained from large scale breeding of EMS mutagenized plants; **(C)** 'RSL NAR' natural green revertant with red spotted leaves.

## Materials and methods

*Chemicals and reagents*

Folin-Ciocalteu phenol reagent, sodium carbonate salt, gallic acid, potassium chloride, sodium acetate, Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%], fluorescein sodium salt, 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (AAPH), phosphate buffered saline (PBS) 10x concentrate, quercetin 3-glucoside and organic solvents were purchased from Sigma (St. Louis, MO, USA). Chlorogenic acid and dicaffeoylquinic acid were purchased from ChromaDex (Irvine, CA, USA). Cyanidin 3-glucoside was purchased from Polyphenols (Sandnes, Norway).

*'RSL NAR' green revertant plant growth conditions*

The green seedling was identified among a batch of red untreated 'RSL NAR' plants sown in a GC-96 CW walk-in environmentally controlled growth chamber (EGC, Chagrin Falls, OH, USA) and grown under Sylvania F96/T12/CW/VHO fluorescent lamps (Osram Sylvania, Danvers, MA, USA). When the plant reached a height of 5-7 cm it was transplanted into a pot (diameter 10 cm, volume 414 $cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA), and when it reached a height of 10-12 cm it was transplanted into a bigger pot (diameter 15 cm, volume 1770 $cm^3$) to minimize stress. Growing conditions in the environmentally controlled chamber were 18/15 °C (day/night), 65% relative humidity, a light intensity of 26.3 ± 2.6 mol/$m^2$d and a UV light intensity of 0.7 ± 0.1 mol/$m^2$d for UV respectively, and a 16/8 h (light/dark) photoperiod. When the plant was 12 weeks old, four mature leaves were collected for phytochemical analysis and the plant was then transferred to the NJAES research greenhouse to induce faster bolting.

Growing conditions in the greenhouse were 24.4 ± 1.7/19.7 ± 1.8 °C (day/night), 51 ± 12.3% relative humidity, a PAR light intensity of 20.4 ± 5.0 mol/$m^2$d provided by sunlight supplemented with 400 W high pressure sodium lamps, and a 16/8 h (light/dark) photoperiod. The plant was hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks.

*$M_2$ seed collection and storage*

Flowers were plucked from the green revertant one by one as they matured in order to minimize seed loss. Seeds were manually threshed, screened and winnowed, divided in batches of 0.5 g, placed in separate paper envelopes and kept at 4 °C for long-term storage. The germination rate was evaluated on wet paper for several of the collected batches before proceeding to sowing in growing mix.

*$M_2$ plant growth conditions*

Approximately 100 $M_2$ seeds collected from the 'RSL NAR' green revertant parent plant were sown in batches inside plastic trays containing Sunshine Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, Agawam, MA, USA). Some of these seeds were kept in the growth chamber and grown Sylvania F96/T12/CW/VHO fluorescent lamps (Osram Sylvania, Danvers, MA, USA) and some were kept in the Rutgers New Jersey Agricultural Experiment Station (NJAES) research greenhouse and grown under 400 W high pressure sodium (HPS) lamps, to observe whether the differential effect of the light source and intensity on polyphenol production observed for 'RSL NFR' (Figure 18) as well as observed in the parent cultivar 'RSL NAR', was present in these revertants as well. When plants reached a height of 5-7 cm they were transplanted into pots (diameter 10 cm, volume 414

$cm^3$) containing Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA). Growing conditions in the walk-in environmentally controlled chamber were 18/15 °C (day/night), 65% relative humidity, a light intensity of 26.3 ± 2.6 mol/$m^2$d and 0.7 ± 0.1 mol/$m^2$d for PAR and UV respectively, and a 16/8 h (light/dark) photoperiod. Growing conditions in the NJAES research greenhouse were 24.4 ± 1.7/19.7 ± 1.8 °C (day/night), 51 ± 12.3% relative humidity, a PAR light intensity of 20.4 ± 5.0 mol/$m^2$d provided by sunlight supplemented with 400 W high pressure sodium lamps, and a 16/8 h (light/dark) photoperiod. Plants were hand watered as needed and supplemented with 300 ppm N of 20-20-20 general purpose fertilizer (Everris NA, Dublin, OH, USA) every two weeks.

*Sample processing and extraction*

Entire 8 week-old $M_2$ plants were collected, their fresh weights were recorded, and leaves were flash frozen using liquid nitrogen prior to lyophilization. Dried leaves were ground to a fine powder with a mortar and pestle. Leaf extracts were prepared using a methanolic solvent to efficiently extract polyphenols as described by Wu et al.[11] with minor modifications. Briefly, 0.5 g of dried material was transferred to a 50 ml tube protected from light and 15 ml of extraction solvent (methanol/water/acetic acid; 85:14.5:0.5) was added. Samples were vortexed for 30 s, sonicated at room temperature for 5 min, vortexed for 30 s, then incubated at room temperature for 10 min. Samples were again vortexed for 5 s then centrifuged at 1699 rcf for 5 min. The supernatant was decanted into a clean 50 ml tube and the extraction process was repeated two more times. The supernatants were pooled and filtered through 0.45 mm PTFE filters (VWR, Radnor, PA, USA) prior to all analyses. Extracts were protected from light and stored at –20 °C.

*Quantification of total polyphenols*

Total phenolic content was measured by a modified Folin-Ciocalteu method[12,65]. Briefly, filtered lettuce extracts were diluted 20x and fresh gallic acid standard dilutions (0–100 µg/ml) were prepared using double distilled water. 2N Folin-Ciocalteu phenol reagent was combined with 50% methanol (1:1). 200 µl of this Folin-Ciocalteu solution was added to 200 µl of gallic acid dilutions or diluted extracts, and incubated at room temperature for 10 min. 300 ml of 20% sodium carbonate was added and samples were incubated in a 40 °C water bath for 20 min. Samples were cooled on ice and centrifuged for 30 s at 7000 rcf. 100 µl of supernatant was transferred in triplicate to a 96-well plate and absorbance was measured at 760 nm using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). A regression line was generated by plotting concentrations of gallic acid standard dilutions by their respective absorbances. Results were expressed as gallic acid equivalents (GAE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of total monomeric anthocyanins*

Total monomeric anthocyanin content was determined according to the AOAC pH differential method[66]. Filtered lettuce extracts were diluted 10x in double distilled water and 800 µl of pH 1 (25 mM potassium chloride) or pH 4.5 (0.4 M sodium acetate) buffers were added to 200 µl of each diluted lettuce extract. 300 µl of each pH 1 and pH 4.5 solutions were transferred in triplicate to a 96-well plate and their absorbances were measured at 510 nm and 700 nm on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). Results were expressed as cyanidin 3-glucoside equivalents (CGE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of Oxygen Radical Absorption Capacity (ORAC)*

ORAC was measured as described by Prior et al.[12,67] with slight modifications. Filtered lettuce extracts were diluted 1000x and fresh Trolox standard dilutions (0–100 µM) were prepared using 75 mM PBS, pH 7.4. Fluorescein sodium salt was diluted in 75 mM PBS, pH 7.4, to a final concentration of 6 nM. Water was pipetted into the outer wells of a 96-well plate and then 150 µl of 6 nM fluorescein solution was added to each well. Next, 25 µl of Trolox standard dilutions or diluted lettuce extracts were added to each well in triplicate. The plate was preincubated at 37 °C for 30 min. A fresh 125 mM solution of AAPH was prepared and 25 µl added to each well after pre-incubation to a final concentration of 16 mM. Fluorescence readings were recorded every minute for 120 min on a Synergy HT Multi-Detection Microplate Reader (485 nm, 20 nm bandpass, excitation filter and 528 nm, 20 nm bandpass, emission filter). A regression line was generated by plotting the concentration of Trolox standard dilutions by their respective net area under the curve (AUC). Results were expressed as µmol Trolox equivalents (TE) on a dry weight (DW) or fresh weight (FW) basis.

*Quantification of phytochemicals by UPLC/MS/UV*

Filtered lettuce extracts were dried using a Savant SpeedVac AES2010 centrifugal evaporator (Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in methanolic extraction solvent to a final concentration of 10 mg/ml. Standards of chlorogenic acid, dicaffeoylquinic acid, quercetin 3-glucoside and cyanidin 3-glucoside were also prepared to generate regression lines for quantification. 1 µl from each sample or standard dilution was injected into a Dionex UltiMate 3000 RSLC system (Dionex Corporation, Sunnyvale, CA,

USA) ultra-high pressure liquid chromatography for separation and analysis. The system consisted of a workstation running Dionex Chromeleon v.6.8, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector, the eluent flow was guided to a Varian 1200L (Varian Medical Systems, Palo Alto, CA, USA) triple quadrupole mass detector with electrospray ionization interface (ESI), operated in either negative or positive ionization modes. The voltage was adjusted to –4.5 kV, heated capillary temperature was 280 °C, and sheath gas was compressed air, zero grade for the negative ionization mode, and nitrogen gas, 5 kV capillary voltage for the positive ionization mode. The mass detector was used in scanning mode from 65 to 1500 atomic mass units (amu). Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v.6.9 SP2. Substances were separated on a Phenomenex C8 (Phenomenex, Torrance, CA, USA) reverse phase column of size 150 x 2 mm, particle size 3 µm and pore size 10 nm. The mobile phase consisted of 2 components: solvent A (0.5% ACS grade acetic acid in double distilled de-ionized water, pH 3-3.5), and solvent B (100% acetonitrile). The mobile phase flow was 0.20 ml/min, and a gradient mode was used for all analyses.

For separation of the dicaffeoylquinic acid, quercetin 3-glucoside, quercetin 3-malonyl-glucoside and cyanidin 3-glucoside peaks the following gradient conditions were used: initial conditions of the gradient were 95% A and 5% B; for 30 minutes the proportion reached 5% A and 95% B, which was kept for 8 minutes, and during the following 4 minutes the ratio was brought back to initial conditions. An 8 minutes equilibration interval was

included between subsequent injections. For separation of the chlorogenic acid and cyanidin 3-malonyl-glucoside peaks the following gradient conditions were used: initial conditions of the gradient were 95% A and 5% B; for 20 minutes the proportion reached 80% A and 20% B; for the next 3 minutes the proportion reached 5% A and 95% B, which was kept for the next 4 minutes. For the following 3 minutes the ratio was brought back to initial conditions. A 5 minutes equilibration interval was included between subsequent injections. The average pump pressure using these parameters was typically around 26.9 MPa for the initial conditions in both cases.

*Experimental design and statistical analysis*

25 $M_2$ revertants were chosen at random from the cohort growing in the growth chamber under high light intensity, and plants were pooled in batches of 5 for analysis, totaling n = 5 batches of 5 plants each. Two red 'RSL NAR' plants sown at the same time as the revertants and kept under the same growing conditions were pooled and extracted in the same manner to serve as control. 25 $M_2$ revertants were chosen at random from the cohort growing in the NJAES research greenhouse under moderate light intensity and pooled in the same manner. The means and standard deviations (SD) were calculated from pooled data and all results are expressed as mean ± SD. Statistical significance of the differential effects of the treatments versus the controls was determined using a general linear model analysis of variance (ANOVA) followed by a Dunnett test. A global significance level was set at $\alpha = 0.05$. All statistical procedures were performed using GraphPad Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

## Results

*$M_2$ seed production and storage*

The 'RSL NAR' green revertant plant underwent self-pollination successfully and started bolting 4 months after the sowing date, producing a thick stem approximately 50 cm tall with a dense and healthy inflorescence. Individual plucking of the flowers stimulated more flower production and prolonged the life of the plant **(Fig. 27)**. However,


**Fig. 27. Growth and seed production in 'RSL NAR' green revertant plant. (A)** The plant grew normally and remained green even under intense anthocyanin inducing conditions, save for the red sectors in the leaves **(B)**, which nevertheless were small (1-5 mm) and hardly noticeable. **(C)** After approximately four months the plant bolted, producing a dense inflorescence with abundant, healthy looking flowers **(D)**.

the proportion of abortive seeds per flower progressively increased as the plant became older, and after seven months the whole inflorescence was harvested. A total of approximately 10000 $M_2$ seeds were collected and stored in batches. The germination rate of all seeds was normal (98% ± 1%) when evaluated on wet paper **(Fig. 28)**.

*Analysis of high polyphenol trait in $M_2$ generation*

Out of the approximately 100 $M_2$ seeds sown to study transmission and segregation of the trait, one plant showed reversion to the normal red phenotype but all others showed the same variegated phenotype as the parent. The distribution and size of the red sectors varied greatly across plants, sometimes barely visible and sometimes covering almost an entire leaf **(Fig. 29)**. Plants kept inside the growth chamber under high intensity light showed dark red sectors, grew slower than the red 'RSL NAR' controls, and developed leaves with a dark green color and a thick wax cuticle. Plants kept inside the NJAES research greenhouse growing under moderate light intensity showed less intense red sectors, grew at a normal speed and developed leaves with a normal green color and cuticle **(Fig. 30)**.

The phytochemical characterization of both groups of $M_2$ revertant plants revealed that the high polyphenol trait had been successfully transmitted to the progeny **(Fig 31, Table 10)**. No significant differences were found between total polyphenol levels in the revertant plants grown in the growth chamber under high light intensity (6 ± 0.6 mg GAE/g FW) and those of the red 'RSL NAR' controls also grown under high light intensity (6.5 ± 0.5 mg GAE/g FW); total polyphenol levels in the revertant plants grown in the greenhouse under moderate intensity light were slightly lower (4.8 ± 0.5 mg GAE/g FW) but still well above the range of regular green lettuce (1.3 ± 0.4 mg GAE/g FW[11]). Total anthocyanin

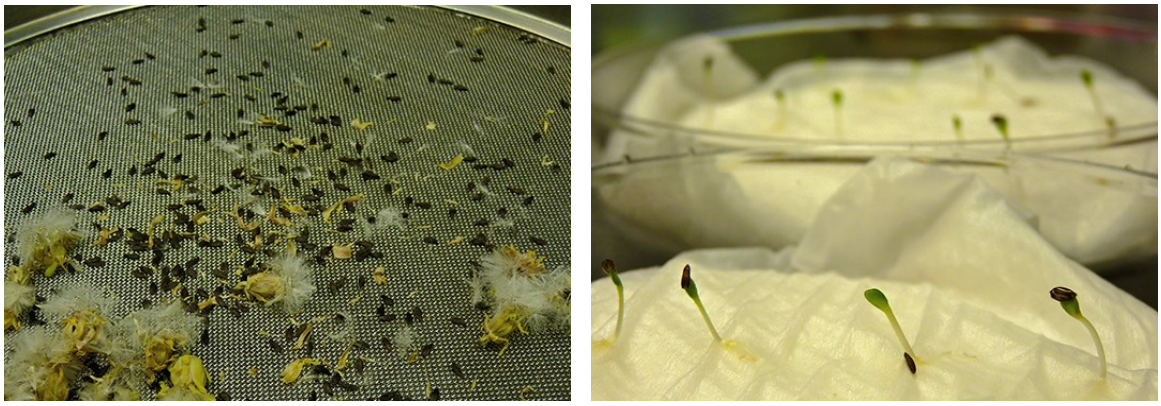


**Fig. 28. Quality of $M_2$ seed produced by 'RSL NAR' green revertant plant.** Seeds were manually separated from chaff and stored in batches at 4 °C. The germination rate on wet paper was comparable to that of normal seeds although slightly lower in growing mix. Seeds collected early during flowering also showed a slightly higher germination rate than those collected late in the life of the parent plant (data not shown).

**Fig. 29. Different variegation patterns in $M_2$ 'RSL NAR' green revertant plants.** All green revertants screened presented irregular red sectors in most leaves, and most of these were between 1 and 5 mm in diameter, small enough not to be noticeable from afar. A few plants had sectors larger in size, but these were not as common. The variations in pigment distribution were three-dimensional, as it was observed that some of the sectors were only visible on the adaxial or abaxial surface of the leaves, but not both at the same time.

**Fig. 30. $M_2$ 'RSL NAR' green revertant plants grown under different light intensities.** Plants grown under high light intensity (right) developed slower and showed darker green leaves with a thicker wax cuticle. Plants grown under moderate light intensity (left) developed at normal speed and had lighter green leaves of normal thickness. The intensity of the red sectors was light inducible in the same way as previously observed for RSL.

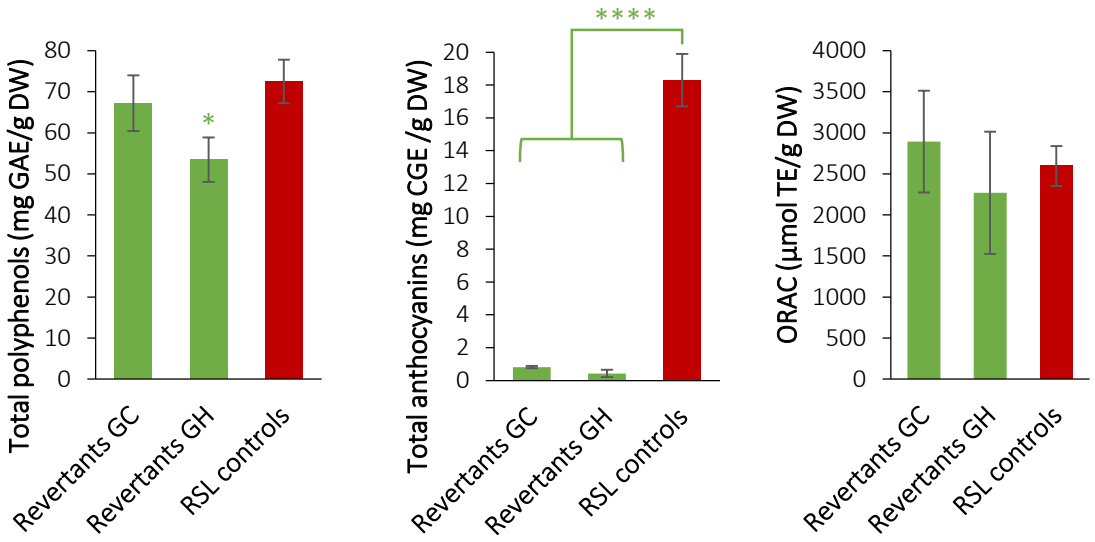



**Fig. 31. Phytochemical content of 'RSL NAR' green revertant plants.** n = 5 batches of 5 revertant plants and n = 2 control plants. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside equivalents (CGE), and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Significant differences between the RSL control and both revertant groups were analyzed by one-way ANOVA followed by a Dunnett test (* $p \leq 0.05$, **** $p \leq 0.0001$). DW, dry weight basis; GC, growth chamber with high light intensity conditions; GH, greenhouse with moderate light intensity conditions.

| | Total polyphenol (mg/g) | | Total anthocyanin (mg/g) | | ORAC (µmol/g) | |
|---|---|---|---|---|---|---|
| | DW | FW | DW | FW | DW | FW |
| Revertants GC | 67.2 ± 6.8 | 6.0 ± 0.6 | 0.8 ± 0.1 | 0.1 ± 0.0 | 2849 ± 621 | 258 ± 55 |
| Revertants GH | 53.5 ± 5.4 | 4.8 ± 0.5 | 0.4 ± 0.2 | 0.0 ± 0.0 | 2269 ± 745 | 202 ± 66 |
| RSL controls | 72.5 ± 5.3 | 6.5 ± 0.5 | 18.3 ± 1.6 | 1.8 ± 0.2 | 2595 ± 242 | 231 ± 22 |

**Table 10. Phytochemical content of 'RSL NAR' green revertant plants.** n = 5 batches of 5 revertant plants and n = 2 control plants. Data were pooled and are presented as the mean ± SD. Total polyphenols are reported as gallic acid equivalents (GAE), total anthocyanins are reported as cyanidin 3-glucoside (C3G) equivalents, and ORAC antioxidant capacity is reported as Trolox equivalents (TE). Fresh weights estimated from dry weights assuming a water content in lettuce of 91.9%. DW, dry weight basis; FW, fresh weight basis; GC, growth chamber with high light intensity conditions; GH, greenhouse with moderate light intensity conditions.

levels were close to zero for all revertant plants, as expected, and no significant differences were found between the ORAC antioxidant capacity of any of the revertant groups and that of the red 'RSL NAR' control group.

UPLC/UV quantification of the major chemical components normally present in RSL **(Fig. 1)** revealed levels of hydroxycinnamic acids and flavonols in the revertants equal or higher to those found in the red 'RSL NAR' controls **(Fig. 32, Table 11)**. No significant difference was found between the chlorogenic acid levels in the revertant plants grown in the greenhouse under moderate light intensity and those in the 'RSL NAR' red controls grown in the growth chamber under high light intensity; however a significant ($p \leq 0.01$) increase was seen in the revertant plants grown in the growth chamber under high intensity light. The same trend was observed for the second major component quercetin 3-malonyl glucoside. Levels of the normally abundant cyanidin 3-malonyl glucoside were close to zero in all green revertant samples, as expected from the results obtained by measuring total anthocyanin content.

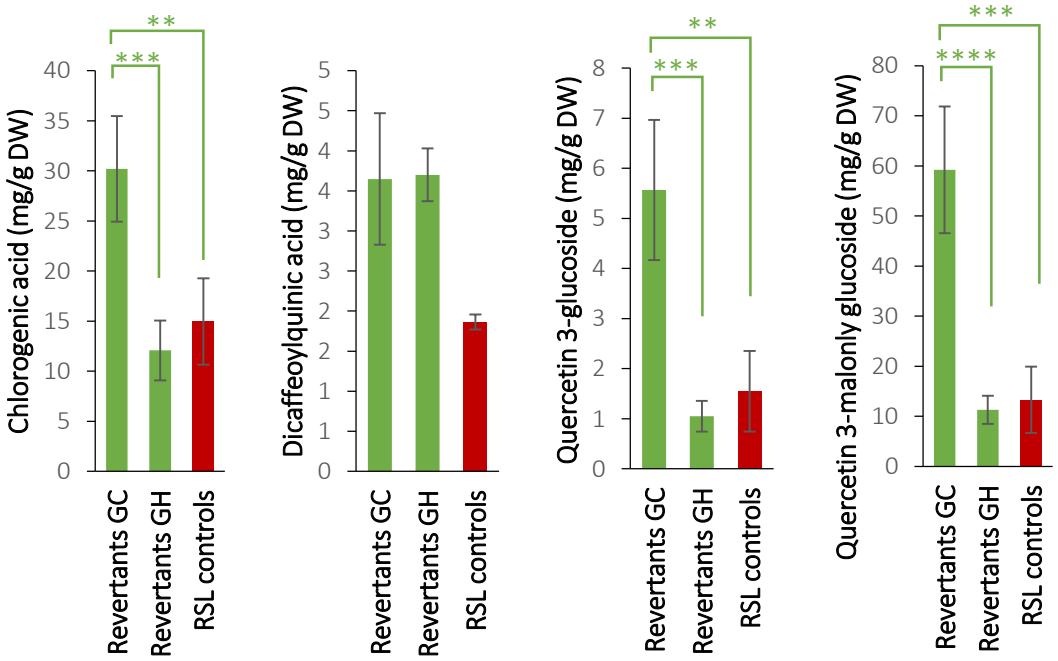



**Fig. 32. Major phytochemical components of 'RSL NAR' green revertant plants.** n = 5 batches of 5 revertant plants and n = 2 control plants. Data were pooled and are presented as the mean ± SD. All results are expressed as mg per gram of dry weight. . Quercetin 3-malonyl glucoside values estimated using quercetin 3-glucoside as standard compound. All values estimated using UV spectroscopy. Significant differences between the RSL control and each green revertant group were analyzed by one-way ANOVA followed by a Dunnett test (** $p \leq 0.01$, *** $p \leq 0.01$, **** $p \leq 0.0001$). DW, dry weight basis; GC, growth chamber with high light intensity conditions; GH, greenhouse with moderate light intensity conditions.

| Compound (mg/g DW) | Revertants GC | Revertants GH | RSL controls |
|---|---|---|---|
| Chlorogenic acid | 30.2 ± 5.3 | 12.1 ± 3.0 | 15.0 ± 4.3 |
| Dicaffeoylquinic acid | 3.6 ± 0.8 | 3.7 ± 0.3 | 1.9 ± 0.1 |
| Quercetin 3-glucoside | 5.6 ± 1.4 | 1.1 ± 0.3 | 1.5 ± 0.8 |
| Quercetin 3-malonyl glucoside | 59.2 ± 12.6 | 11.3 ± 2.8 | 13.3 ± 6.6 |
| Cyanidin 3-glucoside | Undetectable | Undetectable | 1.2 ± 0.3 |
| Cyanidin 3-malonyl glucoside | 0.3 ± 0.1 | 0.1 ± 0.1 | 20.7 ± 1.7 |

**Table 11. Major phytochemical components of 'RSL NAR' green revertant plants.** n = 5 batches of 5 revertant plants and n = 2 control plants. Data were pooled and are presented as the mean ± SD. All results are expressed as mg per gram of dry weight. Quercetin 3-malonyl glucoside and cyanidin 3-malonyl glucoside values estimated using quercetin 3-glucoside and cyanidin 3-glucoside as standard compounds, respectively. All values estimated using UV spectroscopy. DW, dry weight basis; GC, growth chamber with high light intensity conditions; GH, greenhouse with moderate light intensity conditions.

## Discussion

The results from the phytochemical quantification seem consistent with the values obtained from the spectrophotometric assays. The decrease in monomeric anthocyanins expected and observed in the green revertant plants seems to be, as hypothesized, compensated for by an accumulation of colorless anthocyanin precursors – mainly those originally most abundant in RSL: chlorogenic acid and quercetin 3-malonyl glucoside. This strong accumulation effectively restores the total polyphenol levels to those of red 'RSL NAR' in the case of revertants grown under high intensity light conditions, and brings them to a slightly lower level in the case of revertants grown under moderate light intensity conditions, for which the accumulation is lower. This differential increase of the early pathway precursors observed under different light conditions implies that their biosynthesis is strongly mediated by light intensity in a manner similar to the original RSL, which is consistent with most of the UV inducible genes, such as PAL, CHS, F3H or DFR, being located in the upstream half of the pathway **(Fig. 3)**[24,27,32].

Aside from its green high polyphenol phenotype, a second characteristic of interest

was observed from the phenotypical analysis of the green revertant plants. Previous studies have shown the strong influence of light source on 'RSL NFR' polyphenol content, with total polyphenols in plants grown under high light intensity (8.4 ± 0.5 mg GAE/g FW) showing a 1.9-fold increase over plants grown under moderate light intensity (4.5 ± 0.3 mg GAE/g FW) (Table 6). However, 'RSL NAR' green revertants grown under similar high light intensity (6.0 ± 0.6 mg GAE/g FW) show a 1.2-fold increase over green revertants grown under moderate light intensity (4.8 ± 0.5 mg GAE/g FW) (Table 10). We hypothesize that the changes to the anthocyanin biosynthetic pathway in the green revertants have induced lower sensitivity to the light conditions required for polyphenol production. This trait is extremely interesting from an agronomic point of view, given that greenhouses are often made of materials with low UV transmittance, and that supplemental high intensity fluorescent lighting can be prohibitively expensive from a large-scale commercial perspective – which has led to significantly lower phenolic content in RSL produced by greenhouse lettuce growers, when compared to field grown RSL. A high polyphenol lettuce variety with decreased sensitivity to light conditions for polyphenol production would therefore allow greenhouse growers to obtain lettuce plants with levels of polyphenols only slightly lower than the levels of lettuce plants grown in the field.

From a genetic point of view, the striking variegated phenotype of the green revertants, with the observed high variability in number, size and position of the red sectors, as well as the identification of one plant among a hundred $M_2$ screened with the original dark red phenotype, or a "re-revertant" – all seem to be indicators of an unstable mutation and bear a lot of similarity to some somatic variegation phenotypes caused by

transposable elements. Many of these have been described to affect the anthocyanin biosynthetic pathway specifically, as is the case of the R locus and the *Ac/Ds* system described by Barbara McClintock in maize[70], the variegated *pallida* flower phenotype mediated by Tam3 in *Antirrhinum majus*[71], or a variety of transposon systems underlying variegated patterns in *Petunia* flowers[72–74]. Given that there are also some reports of the presence of transposable element sequences in the lettuce genome[75], it could be hypothesized that an endogenous transposon could affect anthocyanin biosynthesis. Southern blot analysis and/or querying the DNA sequence of the green revertant with motifs associated to different transposon element families would allow confirmation or rejection of this hypothesis. If the hypothesis were confirmed, this would be, to our knowledge, the first report of endogenous transposon activity in lettuce, and might constitute a valuable tool for generating genetic variability in lettuce without the use of external mutagens.

According to this hypothesis, the original, stable, dark red phenotype in RSL could be due to the transposons naturally present in its genome being silenced, perhaps by an epigenetics mechanism such as methylation, and the parent green revertant identified among untreated plants could be the product of a spontaneous activation of one the silenced transposable elements, perhaps by an error during genome methylation. There are reports of ultraviolet radiation increasing reactivation rates of certain types of transposable elements in maize[76], so the light conditions under which the original RSL parents were being grown, which included a UV intensity of $0.7 \pm 0.1$ mol/m$^2$d, might have played a role in transposon reactivation. The activated transposable element would then

excise and insert itself either into an allele of any of the anthocyanin biosynthesis late pathway key genes or into a pathway regulatory element, leading to changes in gene expression levels and resulting in the observed variegated phenotype. The pattern of the variegation in the plant would therefore be determined by the timing and rate of excision/insertion of the transposon during development. This new methylation pattern could be passed on to the progeny, leading to an active transposon and different variegation patterns in each daughter plant. Should the original methylation pattern be spontaneously, the transposon would again become inactive and the original dark red phenotype would be restored, explaining the "re-revertant" plant found in the $M_2$.

Aside from the genetic phenomenon underlying the phenotype, estimation of the overall frequency of green revertants as screening continues, as well as genomic and transcriptomic studies of these plants, will all provide valuable information regarding the stability of the red color trait and the regulation of the anthocyanin biosynthetic pathway.

## CONCLUDING REMARKS

The present research resulted in production of a green high polyphenol non-transgenic new variety of romaine lettuce. Given the observed transmission of the green high polyphenol trait to at least 99% of the $M_2$ generation, and having produced a collection of approximately 10000 $M_2$ seeds with a moderate to high germination rate, the objective of the present research has been fully accomplished – it is now a matter of field studies under commercial conditions, licensing and marketing until this Green Super Lettuce, or GSL, can join its big sister RSL on the supermarket shelves. Phytochemical analysis of the more recently identified 'RSL NFR' green revertant plant will be carried out in the near future and 'RSL NFR' $M_2$ screening efforts will also continue, so whenever a green 'RSL NFR' with a heritable high polyphenol trait is eventually identified as well, both romaine and loose leaf varieties of GSL could be marketed jointly.

As well as green high polyphenol lettuce, a working system for simple and efficient large-scale production of mutagenized lettuce, as well as a large collection of mutagenized RSL $M_2$ seeds potentially able to express recessive mutations, have both been produced as a result of this research. These resources will help develop more new lettuce varieties with enhanced content in minerals or vitamins, with efforts towards high iron, calcium and vitamin B12 currently underway. Aside from the commercial perspective, work to confirm the presence of endogenous transposon activity in lettuce for the first time is ongoing, and a new medium for high shoot induction efficiency in lettuce tissue culture has been defined, which may allow endogenous mutagenesis as well as better regeneration success after lettuce transformation – both valuable additions to the field of lettuce research.

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