Plant anti-aging: Delayed flower and leaf senescence in Erinus alpinus treated with cell-free Chlorella cultivation medium

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ABSTRACT
Plant tissues naturally senesce over time. Attempts to improve plant robustness and increase longevity have involved genetic modification, application of synthetic chemicals, and use of beneficial microbes. Recently, culture supernatant from a microalga 
Chlorella fusca
was found to prime innate immunity against
Pseudomonas syringae
in
Arabidopsis thaliana
. However, the capacity of Chlorella culture supernatants to prevent or delay aging in higher plants has not been elucidated. In this study, roots of the ornamental flowering plant
E. alpinus
L. were drenched with cell-free supernatants from three Chlorella species. Flower and leaf senescence in
E. alpinus
was significantly reduced and delayed with all three Chlorella supernatants. Investigations of the mode of action underlying delayed senescence showed that the Chlorella supernatants did not act as a chemical trigger to elicit plant immunity or as a growth-promoting fertilizer in
E. alpinus
. The mechanisms underlying the anti-aging effects remain undetermined, and several possible hypotheses are discussed. Several Chlorella species are industrially cultivated, and disposal of cell-free supernatant can be economically and environmentally challenging. This study provides a novel method for extending plant lifespan through use of Chlorella supernatant and discusses the potential of using industrial waste supernatants in agriculture and horticulture to reduce reliance on chemical pesticides and genetic modification.

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Introduction
The ability to prolong plant development and delay age-related phenotypes such as grain maturation in cereals and flower turnover in ornamental plants is economically important in crop science and critical in fundamental plant research.1,2 Previous research showed that senescence of plant tissues and organs was a key indicator of plant aging.1,2 Recent studies focused on leaf and flower senescence due to their importance in influencing yield and nutritional value in agricultural crops.3,4 Several studies have examined the molecular basis of senescence with the aim of understanding the mechanisms governing crop senescence and using the findings to improve crop yield and biomass.1,4 Plant hormones such as cytokinin, ethylene, and abscisic acid were shown to play important roles in plant senescence through their responses to various environmental factors.1 Knowledge regarding the molecular underpinnings of plant senescence have facilitated attempts to manipulate senescence in crop plants through genetic engineering approaches.5 However, many jurisdictions prohibit the widespread release of genetically modified plants, leading researchers to employ alternative approaches for modulation of plant aging.

Application of synthetic chemicals to alter plant aging is one alternative to genetic modification. For example, the phytohormone ethylene is a known facilitator of senescence. Ethylene production inhibitors such as naphthaleneacetic acid, 1-methylcyclopropene, silver thiosulfate, cyclic olefin norbornadiene, and L-a-(aminoethoxyvinyl)-glycine were effective in delaying leaf and flower senescence.6–10 Similarly, application of a synthetic auxin derivative, 2,4-dichlorophenoxyacetic acid, delayed senescence-related fruit drop in
Citrus species.11 Finally, flower senescence was delayed after application of the protein synthesis inhibitor cycloheximide.2,3 Nevertheless, synthetic chemical inhibitors of plant senescence can have undesirable side effects such as damage to plants, human toxicity, and environmental damage.7,8 One alternative approach is to use beneficial microbes rather than potentially harmful chemical inhibitors to help delay plant senescence.12–16

Several beneficial bacteria were previously shown to modulate a plant senescence-related phytohormone ethylene production in plants.13,15 The endophytic
Pseudomonas
species
P. putida
GR12–2, P. fluorescens
YsS6, and
P. migulae
8R6 were found to produce 1-amino-1-cyclopropane-1-carboxylic acid (ACC) deaminase, which degraded the ethylene precursor ACC into ammonia and a-ketobutyrate and thus delayed flower senescence through reducing endogenous ethylene contents.12,13,17 Treatment with
Enterobacter cloacae
also delayed flower senescence via a reduction in ethylene...
production, in this case due to a decrease in ethylene biosynthesis gene expression. The plant growth-promoting rhizobacterium *Bacillus pumilus* S1r1 delayed foliar senescence in maize via enhancement of nitrogen fixation. Finally, inoculation with *Pseudomonas fulva* and *Escherichia coli* K12 significantly prolonged flower duration in *Zinnia elegans*. Taken together several beneficial microbes and their metabolites were shown to be effective in delaying senescence in small-scale greenhouse trials via decreases in plant hormone production. However, microalgae-mediated delayed senescence of plant has not been reported.

*Erinus alpinus* L., commonly known as ‘Alpine-Liver balsam’ in USA and Europe and ‘Fairy Star’ in Korea, is an ornamental plant species in the Plantaginaceae family. Because *E. alpinus* L. has a short lifespan, delaying leaf and flower senescence is highly desirable for both growers and consumers. The objective of this study was to develop methods to delay plant aging, and hence reduce leaf and flower senescence in *E. alpinus*. To address this, *E. alpinus* was treated with cell-free supernatant from three *Chlorella* species to determine whether this treatment would delay aging. In this study, we evaluated effect of three *Chlorella* spp. including *Chlorella fusca* CHK0059, *Chlorella* sp. ABC001, and *Chlorella* sp. HS2 on delaying plant aging. *C. fusca* CHK0059 was previously shown to elicit plant innate immune responses against *P. syringa* pv. *tomato* in *Arabidopsis thaliana*. *Chlorella* spp. ABC001 and HS2 were not previously applied to higher plants but demonstrated high efficiency growth characteristics suitable for biofuel production. Root-drenching of three *Chlorella* supernatant significantly delayed flower and leaf senescence of *E. alpinus* through different function immunity-eliciting chemical or fertilizer. This is the first report of the novel plant anti-aging strategy using cell-free supernatant of microalgae *Chlorella*.

**Materials and methods**

**Preparation of Chlorella supernatant and *E. alpinus* plant**

*C. fusca* strain CHK0059 was obtained from Dr. Chang-Ki Shim at the Rural Development Administration, Wanju, South Korea, and *Chlorella* spp. ABC001 and HS2 were obtained from Prof. Yong-Keun Chang at the Korea Advanced Institute of Science and Technology (KAIST), Daejeon, South Korea. Three *Chlorella* species were cultivated under mixotrophic condition as described previously. Supernatants were harvested from 107 cells/ml mixotrophic *Chlorella* cultures using centrifugation at 4000 x g for 10 min. To avoid contamination with *Chlorella* and bacterial cells, the supernatant of three *Chlorella* species were filtered using a 0.45 µm syringe filter (Figure 1a).

*E. alpinus* L. planted in flower pots (diameter 110 mm, height 100 mm) was obtained from a commercial grower (Nakwon Garden Shop, Daejeon, South Korea). *E. alpinus* was placed in the KRIBB greenhouse facility in Daejeon, South Korea. The temperature in greenhouse was controlled at 20–22°C. For the quality control of flower plant, before the treatment of *Chlorella* supernatant, *E. alpinus* was adapted to KRIBB greenhouse conditions for a week.

**Treatment of Chlorella supernatant**

To examine delaying senescence by *Chlorella* supernatant, *E. alpinus* higher than 20 cm-long were selected. The roots of *E. alpinus* were drenched with 20 ml of filtrated supernatant, 0.5 mM BTH, and BG11 broth medium with 1 g/L glucose, and 1 g/pot Osmocote® fertilizer (ICL Specialty Fertilizers; nitrogen 10%, phosphorus pentoxide 5%, potassium oxide 20%, magnesium oxide 2%, calcium oxide 2.5%, iron 0.35%, manganese 0.07%, copper 0.01%, zinc 0.03%,

![Figure 1](image_url). Procedures of the plant anti-aging experiment with *Chlorella* cell-free supernatant. *Chlorella* supernatant isolation procedure and its application protocol. (a) Preparation of *Chlorella* supernatant- Cell-free supernatants of *Chlorella fusca* HSK0059, *Chlorella* sp. ABC001, and *Chlorella* sp. HS2 were prepared from *Chlorella* cultures at 107 cells/ml by centrifugation 4000 rpm for 10 min followed by filtration with 0.45 µm syringe filter. (b) Treatment of *Chlorella* supernatant – The root systems of *E. alpinus* were drenched with 20 ml filtered solution of the *Chlorella* supernatant, BG11 growth medium, and 0.5 mM BTH, at every five days for 25 days. The 1 g/pot Osmocote® fertilizer was treated in a single application for 25 days. (c) Assessment of flower and leaf senescence – The percentage of senescent plants was measured for 10 week post treatments. Plants exhibiting withered leaves and flowers in more than 80% of shoots were defined as senescent and the overall percentage of senescent plants was determined.
boron 0.01%, and molybdenum 0.009%). The filtrated supernatant, 0.5 mM BTH, and BG11 broth medium were treated 5 times (every five days) for 25 days. The solid Osmocote® fertilizer was not treated five times but one time at 1st application of other treatments (Figure 1a).

Measurement of plant senescence

Aboveground senescence of *E. alpinus* was assessed for 10 weeks after 5th treatment. Plants exhibiting withered leaves and flowers in more than 80% of shoots were defined as senescent and the overall percentage of senescent plants was determined. The 12 plants for a treatment were measured the 'percentage of senescent plant (%)'. The treatments were randomly arranged among blocks with three replications. The experiment was performed three times.

Statistical analysis

Data were analyzed by analysis of variance using JMP 4.0 software (SAS Institute Inc., Cary, NC, USA). Significant treatment effects were determined on the basis of the magnitude of the F-value (p < .05). When a significant F-value was obtained, the separation of means was analyzed by determination of Fisher’s protected least significant difference at p < .05.

Results

**Delaying plant senescence by cell-free *Chlorella* supernatant**

To examine delayed plant senescence by *Chlorella* supernatant, 20 mL supernatant was drench-applied to the root systems of *E. alpinus* plants (Figure 1). Supernatants from three *Chlorella* strains were used. The overall percentage of senescent plants was determined weekly for 10 weeks after treatment with *Chlorella* spp. supernatants (Figures 2a–b). Treatment of *E. alpinus* plants with supernatant from *C. fusca* CHK0059 delayed senescence onset by two weeks and significantly reduced the percentage of senescent plants by 8.2- and 5.0-fold at 6 and 7 weeks after 5th treatment, respectively, compared to plants treated with culture medium only. The percentage of senescent plants was also lower 8 weeks after treatment, but this was not statistically significant (Figures 2a–b). Treatment with supernatants from *Chlorella* spp. ABC001 and HS2 delayed the onset of foliar senescence in *E. alpinus* by an additional two weeks compared to *C. fusca* CHK0059 supernatant (Figures 2a–b). Treatment with supernatants from *Chlorella* spp. ABC001 and HS2 reduced the percentage of *E. alpinus* plants that were senescent to below 10% until 8 weeks after treatment. *E. alpinus* senescence was significantly lower than in the medium-only control, even after 9 weeks, with senescence percentages 5.9- and 4.7-fold lower for *Chlorella* spp. ABC001 and HS2 supernatants, respectively (Figures 2a–b). The lowest *E. alpinus* senescence rate was detected with supernatant from *Chlorella* HS2, with the percentage of senescent plants remaining below 20% for the full 10 weeks trial period (Figure 2b). These results indicated that root-drenching of three *Chlorella* supernatants delayed plant senescence in *E. alpinus*.

**Elucidating mode of action underlying delayed senescence by *Chlorella* supernatant**

Treatment with *C. fusca* CHK0059 was previously shown to enhance plant growth and immunity via modulation of stress hormone signaling, including salicylic acid, jasmonic acid, and ethylene signaling. In particular, modulation of stress hormones by plant-growth-promoting rhizobacteria led to delayed plant senescence. Therefore, we hypothesized that *Chlorella* supernatants delayed senescence by regulating plant growth promotion or plant immunity via modulation of hormone signaling. To examine this hypothesis, *E. alpinus* root systems were treated with 0.5 mM benzothiadiazole (BTH) or fertilizer. BTH, a synthetic plant immune trigger, was previously reported to affect leaf and flower senescence during constant activation of plant immunity. Fertilizer as a plant growth stimulant was applied to assess the effect of plant growth promotion on plant senescence. Roots were treated with 0.5 mM BTH or with 1 g Osmocote® fertilizer per plant pot (Figures 2c–d). Compared with treatment with BG11 medium alone, neither BTH treatment nor fertilizer application significantly altered senescence of *E. alpinus* during the 10-week trial period (Figures 2c–d). Thus, constant activation of plant immunity and growth stimulation with nutrient supply did not affect senescence in *E. alpinus,* suggesting that the delayed leaf and flower senescence observed in plants treated with *Chlorella* supernatant was caused by the two known mechanisms on higher plant such as growth promotion and plant immunity activation.

**Discussion**

Our results describe a novel strategy for delaying flower and leaf senescence using *Chlorella* culture supernatants. At present, industrial *Chlorella* growth is aimed at cell production, with culture supernatants discarded. Large amounts of supernatant can be produced during *Chlorella* cultivation, and disposal can be expensive and damaging to the environment. Agricultural application of waste *Chlorella* supernatants may address the economic and environmental concerns while providing plant growth benefits. Supernatant from the high-lipid producing strain *Chlorella* sp. HS2 was most effective in delaying senescence (Figure 2b). *Chlorella* sp. HS2 is industrially grown in outdoor cultivation systems for the production of biofuel, and supernatant for agricultural use could be produced simultaneously. Cell-free supernatants from *Chlorella* spp. cultivation could be easily applied alongside fertilizer or water in agriculture and horticulture using watering technologies such as drip-irrigation systems or sprinkler systems. However, we did not provide clear evidence of mode of action for anti-aging effect on flower and leaf of *E. alpinus*. Four hypotheses are proposed to explain the delayed senescence observed when *Chlorella* supernatants were applied to *E. alpinus* roots.
First, *Chlorella* supernatant might activate or prime antioxidant enzyme expression, leading to a reduction in reactive oxygen species (ROS) in *E. alpinus*. ROS act as internal triggers and signal molecules during plant senescence, and their breakdown can be catalyzed by antioxidant enzymes.\(^1\) \(^3\) \(^{30}\) *C. fusca* and its potential determinants were previously shown to activate antioxidant enzyme activity. Root drenching with *C. fusca* culture medium increased the activity of the antioxidant enzyme superoxide dismutase (SOD) in soybean sprouts.\(^{26}\) In addition, our previous research showed that *C. fusca* CHK0059-derived D-lactic acid primed the expression of two antioxidant enzyme genes, *alternative oxidase 1* (AOX1) and *cytochrome C oxidase subunit 2* (COX2), after flagellin 22 (flg22) treatment in *Arabidopsis* leaves.\(^{20}\) D-lactic acid antioxidant enzyme priming may be activated more strongly in response to flg22-triggered ROS production.\(^{20}\) This suggests that root drenching of *E. alpinus* with *Chlorella* supernatants might delay senescence by stimulating antioxidant enzymes and reducing the abundance of ROS.

Second, delayed senescence in *E. alpinus* may be linked to antioxidant compounds produced by *Chlorella* spp. rather than by *E. alpinus*. Of the three *Chlorella* supernatants tested, supernatant from *Chlorella* sp. HS2 was most effective in delaying plant aging over the full 10 week trial period (Figure 2b).
Chlorella spp. ABC001 and HS2 were previously found to be highly efficient strains for the production of beneficial compounds such as specific lipids and antioxidant pigments.\textsuperscript{21–25,31} Especially, Chlorella sp. HS2 accumulated high levels of the antioxidant pigments β-carotenoid and lutein.\textsuperscript{25,31} The antioxidant pigments produced by Chlorella sp. HS2 may reduce ROS levels during senescence in E. alpinus.

Third, activation of D-lactate dehydrogenase (D-LDH) in E. alpinus by D-lactic acid produced by Chlorella spp. could affect senescence of E. alpinus. D-lactic acid secreted by C. fusca CHK0059 was previously shown to activate expression of D-LDH upon flg22 treatment in Arabidopsis.\textsuperscript{30} In plants, D-LDH is involved in the detoxification of methylglyoxal (MG), a cytotoxic compound generated as a byproduct of glycolysis.\textsuperscript{32} MG can trigger abiotic stress tolerance mechanisms and can promote aging.\textsuperscript{33} The end product of MG detoxification is D-lactate, which is oxidized by D-LDH.\textsuperscript{32} Loss-of-function D-LDH mutants in Arabidopsis exhibited hypersensitivity to treatment with exogenous MG,\textsuperscript{34} demonstrating that D-LDH activation played an important role in reducing MG levels. Although similar mechanisms were not tested in E. alpinus, it is possible that treatment of E. alpinus with Chlorella supernatant may reduce MG levels during senescence via activation of D-LDH and MG detoxification.

Fourth, the role of Chlorella-derived plant hormone mimics cannot be excluded. Chlorella species can produce auxin and cytokinin-like molecules that were previously linked to leaf senescence.\textsuperscript{35,36} Our previous research did not find phytohormones in C. fusca supernatant,\textsuperscript{20} but evaluation of phytohormone levels in supernatants of Chlorella sp. ABC001 and HS2 is required.

Our study confirms that Chlorella culture supernatants contain anti-aging components that can delay leaf and flower senescence and proposes that supernatants could be retrieved from existing large-scale Chlorella cultivation systems and reused in agricultural field. This would provide the dual benefit of reducing the environmental and economic costs of Chlorella production waste while providing a longevity benefit when applied to the root systems of agricultural and horticultural plants. Improving plant robustness by application of Chlorella supernatants could also potentially reduce reliance on chemical pesticides and genetically modified plants, reducing environmental damage at the whole-ecosystem level.

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Disclosure statement

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