

Effect of Biostimulator *Chlorella fusca* on Improving Growth and Qualities of Chinese Chives and Spinach in Organic Farm

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This study was conducted to investigate the efficacy of freshwater alga, *Chlorella fusca* on the improvement of growth and qualities in organic spinach and Chinese chives farm. The average height of Chinese chives treated with the chlorella was 3.7 cm smaller than that of the untreated. The leaf width and fresh weight of Chinese chives treated with the chlorella was 0.5 mm wider and 30.3 g heavier than that of the untreated. The commercialization and yield of Chinese chives treated with the chlorella was 11.9% and 18.3%, respectively higher than that of the untreated. Also, the disease severity of gray mold disease of Chinese chives treated with the chlorella was reduced by more than 24.2% when compared with the untreated. The thickness and number of spinach leaves treated with chlorella was 27.9% and 41.8%, respectively higher than that of the untreated. The fresh weight and yield of the spinach treated with the chlorella was 63.6% and 31.5%, respectively higher than that of the untreated. Moreover, the mineral content of K, Ca, Mg, P, Fe, and Mn were recorded higher in the spinach treated with chlorella compared with that of untreated. The results indicated that the freshwater alga, *Chlorella fusca* is efficient and economical

biostimulant in improving plant growth and quality of Chinese chives and spinach in organic farm.

Keywords : Chinese chives, *Chlorella fusca*, growth promotion, quality, spinach

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Allium tuberosum, commonly called Chinese chives, is indigenous to mountainous areas in Korea and has a unique flavor and aroma that has long been a vegetable crop of Allium, such as garlics and onions (Yoo and Bae, 1993). Chinese chives are one of the most popular and widely used ingredients across Southeast Asia and have been used as a natural preservative for kimchi, which is one of the main side dish made from fermented vegetable in Korea (Hwang et al., 2001; Kim and Park, 1995). Chinese chives can have different nutritional and functional components, depending on their harvest time. Chinese chives are perennial crops that can be cultivated continuously for 5 to 6 years with only one sowing. They can be harvested about 7 to 8 times a year and have become increasingly economically valuable due to their antioxidant effect (Moon et al., 2003). Chinese chives are leafy vegetables that can grow in all seasons. Therefore, it is recommended to cultivate summer Chinese chives as a new source of revenue for organic farmers in Hongcheon, however the annual harvest has been becoming the biggest problem with risk of damage caused by soil borne pathogens, unbalance of nutrients and accumulation of salt, etc. for the farmers.

Spinach (*Spinacia oleracea* L.) is a winter vegetable with small leaves and red color in the basal part of leaves and root of Korean indigenous species but introduced species are characterized by large leaves (Chung and Youn, 1996;

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Park et al., 1998). In recent years, spinach has been growing in a range of 6,600-7,800 ha in South Korea. During the winter season, spinach is produced in southern areas such as Pohang, Namhae, Donghae and Shinan in Korea (Kim et al., 2009). It contains carotene, ascorbic acid and minerals, which are the precursors of vitamin A, calcium, iron and soft fibrin. The general consumption of spinach is steadily increasing as it is widely known as one of the most nutrient-rich vegetable foods (Kim et al., 2012; Kim, 2016).

Chlorella is one kind of microalgae that contains a large amount of various functional materials such as crude protein 50-60%, carbohydrate 15-20%, crude lipid 12-18%, and chlorophyll. In addition to the bio-energy industry, it is used in various fields such as animal husbandry and agriculture (Kang et al., 2004). Chlorophycean members including *Chlorella* were also being explored as biofertilizer as they are rich in carbohydrates, proteins, lipids, and growth hormones. Among the microalgae, *Chlorella vulgaris* (Dineshkumar et al., 2017; Faheed and Abd El Fattah, 2008; Özdemir et al., 2016), *Chlorella pyrenoidosa* (Abd Elhafiz et al., 2015), *Acutoseamua dimorphus* (Garcia-Gonzalez and Sommerfeld, 2016), *Spirulina platensis* (Anitha et al., 2016; Dineshkumar et al., 2017) are considered as competent candidates, which was able to increase the growth and yield of plant.

The field application of microalgae was applied as a biofertilizer to variable crops, tomato (Garcia-Gonzalez and Sommerfeld, 2016; Özdemir et al., 2016), cucumber and eggplant (Abd El hafiz et al., 2015), lettuce (Abd El hafiz et al., 2015; Faheed and Abd El Fattah, 2008), okra (Agwa et al., 2017), spinach (Cassan et al., 1992; El-din and Hassan, 2016; Fan et al., 2013), rice (Abd El hafiz et al., 2015), wheat (Renuka et al., 2016; Shaaban, 2001), maize (Dineshkumar et al., 2017), grape (Abd El Moniem and Abd Allah, 2008), mango (El-Sharony et al., 2015), and orange (Amro, 2015). The application of algae has been shown to improve biomass, quality and yield in overall described fruit and vegetable crops. Moreover, the brown alga extracts have been widely used in agriculture and horticulture as a complement to biofertilizers because of their beneficial effects on crop production, nutrient uptake, stress resistance, and the quality of products after harvest (Abdel-Hafeez, 2005; Craigie 2011; Fan et al., 2013; Fornes et al., 2002; Khan et al., 2009; Norrie and Hiltz, 1999).

The purpose of this study is to investigate the effects of freshwater alga *Chlorella fusca* as new functional biomaterials on the growth and qualities of organic Chinese chives and spinach.

Materials and Methods

Plant preparation. In order to investigate the efficacy of green algae, *C. fusca* on the improvement of growth and qualities in organic spinach and Chinese chives. Spinach was selected as an experimental plant. The spinach variety used as *Spinacia oleracea* cv. Matis in this study. Its roots are lustrous, and its leaf shape is rather round with dark green and relatively stable. It is a semi-long type, which means growing slowly and not growing in height. Chinese chives are called “Super Greenbelt,” a preferred farming type during summer season in Gangwon province.

They are carried out in a greenhouse of organic spinach and Chinese chives farms in Samcheok and Hongcheon, Gangwon Province. Chinese chive was sown two years ago and the experiment started in the third year of cultivation. Spinach seeded directly into the farm field in mid-November 2016. The managements of nutrient, disease and insect pest required for each of the crops are based on the organic cultivation method of practice in the farms. The bases of spinach and Chinese chive were fertilized with 2,000 kg/10a and 4,000 kg/10a of fully composted dairy compost, respectively. Pest and disease management was conducted once or twice per a month for prevention using organic agricultural materials, EM and neem extract for each crop. EM was used as biocontrol agent for plant disease at one time per two weeks. Neem extract included 2% azadiractin and 1% citronella oil was applied to control of insect two times per one month.

The Chinese chive were grown in a greenhouse with a circular water curtain under natural light, 65-75% relative humidity and 12.8-31.2°C. The spinach was grown in a normal type greenhouse under natural light, 60-72% relative humidity and 19.7-32.5°C.

Preparation of freshwater algae. To culture and treat chlorella, we used *Chlorella fusca*, which was isolated and purely cultured in our laboratory. The culture of *C. fusca* is prepared by adding 5 ml/l of chlorella exclusive culture medium to 4 l of commercial mineral water, using ‘Chlorella farmer's self-light incubator’ designed by National Institute of Agricultural Sciences at Hongcheon and Samcheok Agricultural Technology Center. After inoculation, irradiate artificial LED (R:B=2:1) light combined red (710 nm) and blue (470 nm) light with more than 2,000 lux or sunlight at 28-30°C and continuously blowing air over 5 l/min by using home bubble generator. After culturing chlorella for 5 to 7 days, it was confirmed by using light microscope (Leica, DM5500B, Japan) with hemocytometer (Marien-

feld, German) that the cell concentration of chlorella was 1.5×10^7 cells/ml or more and used in this experiment.

Experimental design and treatment of freshwater algae.

Five or seven days old chlorella, *C. fusca* culture solution (1.5×10^7 cells/ml) was diluted with tap water to a concentration of 0.4% (v/v) and the control was sprayed with water, followed by leaf application and soil irrigation at intervals of two weeks using a high-pressure atomizer in tested two farms in Samcheok and Hongcheon. Plots of 660 m² were arranged as a randomized complete block with three replicates per treatment.

Disease control efficacy of chlorella against gray mold.

To investigate the control effect of 0.4% *C. fusca* against gray mold, an experiment was performed in a Chinese chive growing farmer's greenhouse in Hongcheon, Gangwon Province. The Chinese chive plants were infected naturally with *Botrytis squamosa*. Plots of 330 m² were arranged as a randomized complete block with three replicates per treatment. Disease incidence was determined by counting the number of diseased plants among 50 plants per treatment at middle harvesting season after the fifth treatment and then the control efficacy was calculated using the following formula:

Control efficacy (%) = $100 - (100 \times \text{disease incidence of treatment} / \text{disease incidence of control})$

Analysis of mineral content in plant. The composition and content of mineral content from spinach leaves harvested from chlorella treated plants were investigated according to A.O.A.C. (1990). In order to analyze the content of eight inorganic components including calcium and potassium, 0.5 g of Chinese cabbage sample was weighed, and 20 ml

of decomposition liquid (HNO₃:H₂SO₄:HClO₄=10:1:4) was added, followed by decomposition at high temperature and filtration. The filtrate was analyzed using ICP (inductively coupled plasma, Heraeus, DE/Vario-EL, GBC Integra XL).

Estimation of plant growth characteristics. Plants were harvested at a middle harvesting season from planting, 20 plants of spinach and Chinese chive were randomly chosen from the different treatments and their growth was measured based on plant height (cm), leaf width (mm), leaf thickness (mm), leaf number per plant, fresh weight (g) of plant, total yield according to the method described by Park et al. (1998) and Yoo and Bae (1993). The marketable value of Chinese chive was calculated using the following formula:

Marketable value (%) = $100 - (100 \times \text{Percentage of injured or diseased plant} / \text{Percentage of healthy plant})$

Statistical analysis. Experiments were conducted using a randomized complete block design with 3 replicates of each treatment. Statistical analysis was performed using the SPSS (Statistical Package for the Social Science, ver. 12.0) software. Pairwise comparisons between treatments were performed using the least significant difference (LSD) test.

Results

Growth promoting and quality enhancing of Chinese chives treated with algae. The effects of 0.4% *C. fusca* on the growth and qualities of winter Chinese chives were investigated in four organic farms in Hongcheon County. As a result, there were some differences between farms; however, the data showed very similar trends between the treatments regardless the farms tested. For example, the average

Table 1. Improving of growth and marketable value of the winter season organic Chinese chives treated with 0.4% *Chlorella fusca* (CF) in Hongcheon in 2017

| Farm | Treatment | Height (cm) | Leaf width (mm) | Fresh weight (g) | Marketable value (%) |
|-------|-------------------|--------------|-----------------|------------------|----------------------|
| Farm1 | 0.4% CF | 35.0 ± 0.0 | 5.6 ± 0.06* | 115.0 ± 1.0** | 93.3 ± 2.9* |
| | Untreated control | 38.0 ± 0.6* | 5.2 ± 0.7 | 85.7 ± 1.2 | 82.0 ± 2.0 |
| Farm2 | 0.4% CF | 31.0 ± 0.1 | 5.6 ± 0.03* | 115.7 ± 0.6** | 93.0 ± 1.7** |
| | Untreated control | 35.7 ± 0.6* | 5.1 ± 0.06 | 85.7 ± 1.2 | 83.0 ± 1.0 |
| Farm3 | 0.4% CF | 32.7 ± 0.6 | 5.8 ± 0.1** | 112.3 ± 1.6** | 90.0 ± 0.0** |
| | Untreated control | 36.0 ± 0.1** | 5.0 ± 0.06 | 82.3 ± 0.6 | 76.7 ± 1.2 |
| Farm4 | 0.4% CF | 35.0 ± 0.1 | 5.6 ± 0.06* | 116.0 ± 1.0** | 93.0 ± 2.9* |
| | Untreated control | 38.7 ± 0.6** | 5.3 ± 0.03 | 84.3 ± 2.1 | 80.3 ± 2.2 |

Data are expressed as means ± S.D (n = 20).

*Correlation is significant at $P < 0.05$, **Correlation is significant at $P < 0.001$.

Table 2. Effect of green algae, 0.4% *Chlorella fusca* (CF) on improving of yield of the winter season Chinese chives in five organic farms at Hongcheon

| Treatment | Yield of Chinese chive (kg/10a) | | | | | |
|-------------------|---------------------------------|---------------|---------------|---------------|---------------|--------------|
| | Farm 1 | Farm 2 | Farm 3 | Farm 4 | Average | Total |
| 0.4% CF | 2,270 ± 37.1* | 1,780 ± 26.5* | 1,660 ± 10.0* | 1,980 ± 26.5* | 1,923 ± 15.6* | 7,690 ± 62.4 |
| Untreated control | 2,090 ± 26.5 | 1,620 ± 36.1 | 1,570 ± 34.6 | 1,220 ± 40.0 | 1,625 ± 18.0 | 6,500 ± 72.1 |

Data are expressed as means ± S.D (n = 20).

*Correlation is significant at $P < 0.05$.

height of chlorella treated plants was 3.7 cm smaller than that of untreated (Table 1). The width of Chinese chives leaves was 0.5 mm wider, and the fresh weight of chlorella treated Chinese chives was 30.3 g heavier than that of untreated control (Table 1). In particular, the profitability of chlorella-treated Chinese chives was 11.9% higher than that of the untreated (Table 1). To conclude, based on the results, 0.4% *C. fusca* treatment on the Chinese chives reveals overgrowth inhibition, growth enhancement, and profitability. The effects of 0.4% *C. fusca* treatment on the yield of winter Chinese chives were investigated, and the average yield of Chinese chives treated with chlorella (1,923 kg/180 m²) was 18.3% higher than that of untreated control (1,625 kg/180 m²) (Table 2).

Suppression of gray mold in Chinese chive treated with algae.

The effects of chlorella treatment on the control of gray mold caused by *Botrytis squamosa* were investigated from late July to late August in organic Chinese chives in 2017. Four tested organic Chinese chives farms were treated with 0.4% *C. fusca* with more than 1.5×10^7 cells/ml with two weeks intervals by the foliar spray and soil irrigation. As a result, on the leaves of the untreated Chinese chives, small white lesions were formed, but the lesions of Chinese chives treated with 0.4% *C. fusca* treated had much less lesions. The occurrence of gray mold disease of Chinese chive treated with the chlorella was significantly reduced by over 24.2% compared to its occurrence in untreated Chinese chives farms (Fig. 1).

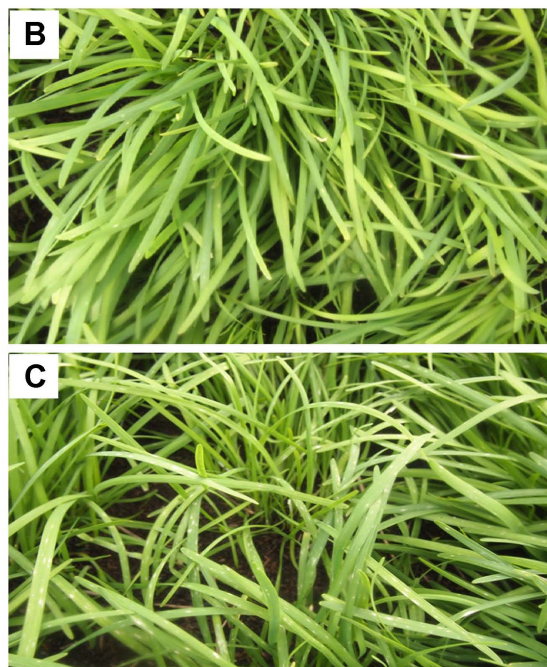
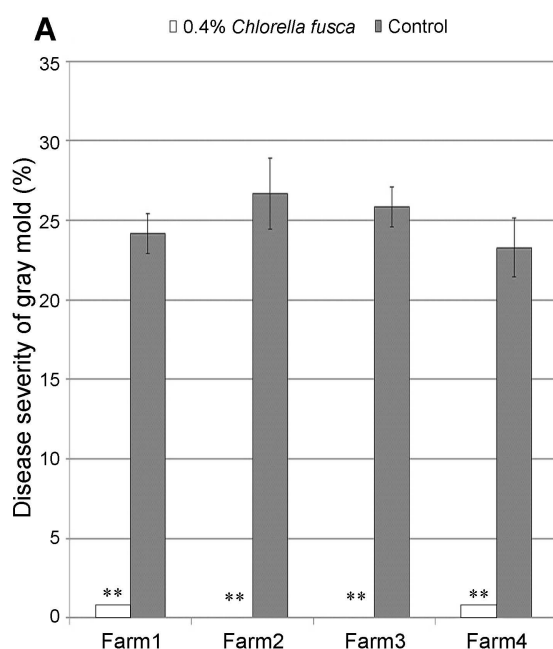


Fig. 1. Control effect (A) and symptoms of gray mold disease caused by *Botrytis squamosa* on *Chlorella fusca* treated (B) and untreated (C) Chinese chives leaves after treated with 0.4% *C. fusca* at two weeks intervals in winter season organic farms in Hongcheon.

*Correlation is significant at $P < 0.05$, **Correlation is significant at $P < 0.01$.

Table 3. Improving of plant growth and yield of summer season organic spinach treated with 0.4% *Chlorella fusca* (CF) in Samcheok

| Treatment | Leaf thickness (mm) | Leaf number | Fresh weigh (g) | Yield (kg/10a) |
|-------------------|---------------------|--------------|-----------------|----------------|
| 0.4% CF | 1.97 ± 0.11** | 35.6 ± 1.4** | 102.1 ± 5.5** | 473.3 ± 24.9** |
| Untreated control | 1.54 ± 0.09 | 25.1 ± 1.3 | 62.4 ± 4.9 | 360.0 ± 32.7 |

Data are expressed as means ± S.D (n = 20).

*Correlation is significant at $P < 0.05$, **Correlation is significant at $P < 0.01$.

Growth promoting and quality enhancing of spinach treated with algae.

The effects of 0.4% *C. fusca* on the growth and yield of summer spinach were investigated with two weeks intervals in organic spinach cultivation area in Samcheok. The thickness of spinach leaves treated with chlorella (1.97 ± 0.11 mm) was 27.9% higher than that of the untreated (1.54 ± 0.09 mm). Also, the number of spinach leaves treated with chlorella (35.6 ± 1.4) was 41.8% higher than that of the untreated (25.1 ± 1.3). The fresh weight of the spinach treated with chlorella (102.1 ± 5.5 g) was 63.6% higher than that of the untreated (62.4 ± 4.9 g). The amount of spinach treated with chlorella (473.3 ± 24.9 kg/10a) was improved by 31.5% compared to un-

treated plant (360.0 ± 32.7 kg/10a). Based on the above results, the growth of summer spinach was improved by 0.4% *C. fusca* treatment with increased yields (Table 3, Fig. 2).

Freshwater algae affected mineral contents of spinach.

The effects of chlorella treatment on the mineral content of spinach leaves were also investigated. The mineral contents of K, Ca, Mg, P, Fe, and Mn were higher in the spinach leaves of chlorella treatments than the untreated ones (Table 4). However, the contents of Na and Zn in the spinach leaves of chlorella treated group were significantly lower than that of the untreated (Table 4). In both treatments, Cu in heavy metals was detected at 0.9 ± 0.03 mg/kg FW.

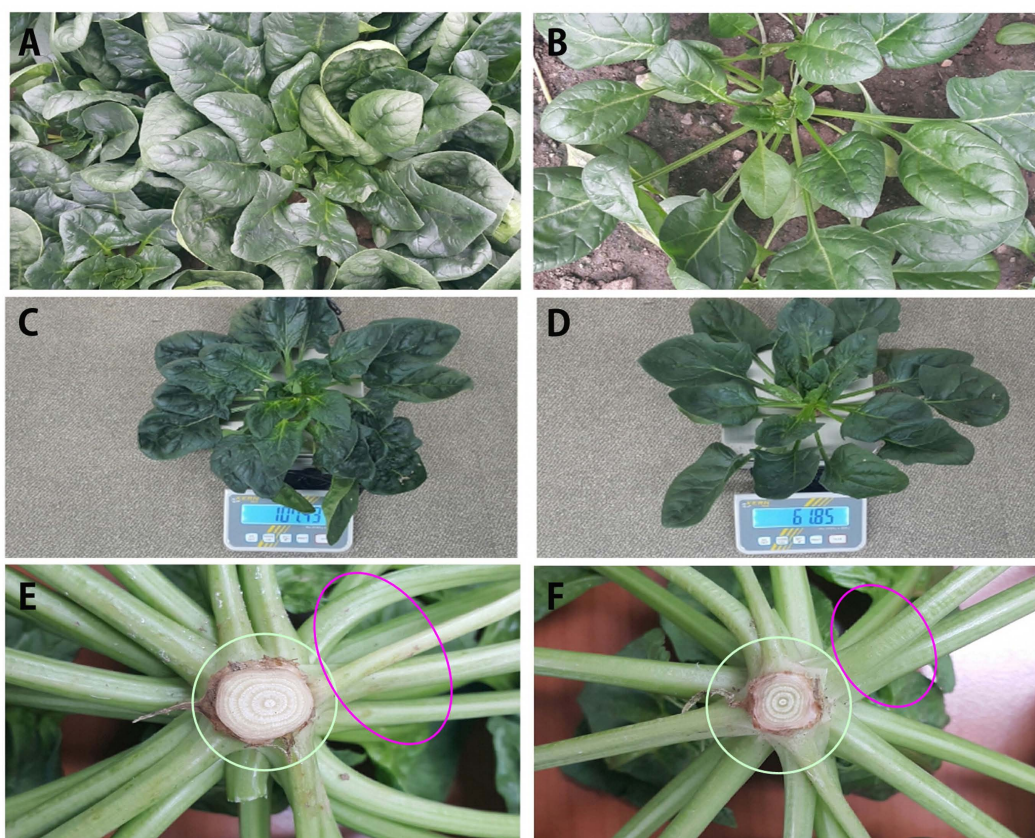


Fig. 2. Comparison of growth, weight and leaves number of 0.4% *Chlorella fusca* (A, C, E) and conventional (B, D, F) treated summer season spinach in 2017.

Table 4. Effect of green algae, 0.4% *Chlorella fusca* (CF) on the mineral content of spinach leaves in organic farm

| Treatment | Mineral content of spinach (mg/kg F.W.) | | | | | | | | | |
|-------------------|---|-----------------|--------------|---------------|-----------------|---------------|----------------|------------|---------------|--|
| | Na | Ca | Fe | Zn | Mg | Mn | K | Cu | P | |
| 0.4% CF | 718.5 ± 4.9 | 2208.9 ± 51.4** | 89.6 ± 0.8** | 7.6 ± 0.02 | 2012.2 ± 53.9** | 15.1 ± 0.21** | 9848.1 ± 5.2** | 0.9 ± 0.03 | 910.7 ± 2.0** | |
| Untreated control | 1325.4 ± 7.6** | 993.6 ± 1.9 | 21.8 ± 0.4 | 10.8 ± 0.04** | 1456.0 ± 7.7 | 4.2 ± 0.02 | 7630.9 ± 7.5 | 0.9 ± 0.03 | 676.3 ± 2.4 | |

Data are expressed as means ± S.D (n = 10).

*Correlation is significant at $P < 0.05$, **Correlation is significant at $P < 0.01$.

Discussion

The results of this study showed that the Chinese chives treated with 0.4% chlorella showed less plant height than the untreated plants but promotes volumetric growth, resulting in increasing leaf thickness. The commercialization yield of Chinese chives treated with chlorella was higher than that of the untreated.

de Mulé et al. (1999) reported that blue-green algae producing plant growth promoting regulators which is said to resemble gibberellin and auxin, vitamins, amino acid, polypeptide, antibacterial and antifungal substances that exert phytopathogen biocontrol in rice seedling.

The dry biomass of microalgae obtained from *Acutodesmus dimorphus* was able to increase the growth promotion and production of flowers in Roma tomato plants (Garcia-Gonzalez and Sommerfeld, 2016). Lettuce seedlings treated with *C. vulgaris* were positively affected with increased fresh and fry weight (Faheed and Abd-El Fattah, 2008). A greater number of leaves with bigger surface area were observed in soybean seedlings irrigated with *C. pyrenoidosa* (Dubey and Dubey, 2010).

Özdemir et al. (2016) reported that tomato treated with *C. vulgaris* as a biofertilizer increased plant growth, yield and fruit quality such as dry weight, total soluble solid contents, titratable acidity and vitamin C.

Generally, Chinese chives are perennial crops, which once they are planted and can be harvested for 5 to 6 years; It is a crop with high risk of damage caused by soil borne pathogens due to unbalance of nutrients and accumulation of salts and watering for a long time in summer. From May to September 1993, when the average temperature was low and humidity was high in the Okcheon, Chungbuk province, the occurrence of gray mold was found to be 83% in Chinese chive farm. Leafy gray mold is the most problematic disease, which is largely influenced by rainfall, rainfall days and average temperature (Lee, 1996).

In the present study, the disease severity of gray mold of Chinese chives was reduced by treated with the chlorella, *C. fusca*. In our previous study, the leaf application of *C. vulgaris* showed that the suppression of decay rate of the ‘Selhyang’ and ‘Yoogbo’ strawberry with 63.8% and 74.4%, respectively (Kim et al., 2014).

Abd Elhafiz et al. (2015) reported that the control seedlings of cucumber had infections while disease infections were not induced in the treated seedlings with *C. vulgaris* and *C. pyrenoidosa*. Bileva (2013) reported that *C. vulgaris* irrigated grape vine induced resistance against nematode, *Xiphinema index*.

According to the results, the mineral content of K, Ca, Mg, P, Fe, and Mn were recorded higher in the spinach treated with freshwater algae, *C. fusca*. Similar results were achieved by Shaaban et al. (2001), found to have beneficial effects, such as increased crop yield, increased uptake of nutrient and resistance to stress conditions, when such algae applied to soil. However, the detected Cu content was very lower than the allowable standard value (6.23 mg/kg FW) set for agricultural products in Korea (Kim et al., 2001). Thus, it seems to be safe for distribution of agricultural products.

Iron may enhance photosynthetic activity and protein synthesis in leaves. Also, iron important role in biosynthesis of IAA and it is required for prevention of the abscission layer formation (Hacisalihoglu et al., 2003). *Spirulina platensis* has been used as biofortifying agent to enhance the iron status in *Amaranthus gangeticus* plant. Iron is an important dietary component and the bioavailable iron form should be high to overcome iron deficiency anemia which is more prevalent in India (Pregnant women 87% and Children 75%) (Kalpana et al., 2014).

Also, the obtained results of algae reported that the mechanisms effect of algae on cell metabolisms are mainly through the physiological action of major and minor nutrients, amino acid, vitamins, and also growth regulators affect cellular metabolism in treated plants leading to enhanced growth and crop yield (Abd El-Motty et al., 2010; Ördög et al., 2004; Strik et al., 2003).

Martínez Lozano et al. (1999) reported that the extract from algae applied to soil or foliage increased ash, protein and carbohydrate contents of potato. Zaccaro et al. (1999) reported that foliar application of biochemical organic substances, which supply macro and micro nutrients, of increased demand.

In our results, the thickness and number of spinach leaves treated with chlorella was higher than that of the untreated. Also, the fresh weight and yield of the spinach treated with the chlorella was higher than that of the untreated.

Although there are few cases of agricultural use of chlorella culture and extracts, it has been reported that the auxin and cytokinin, which are plant growth regulators, increase when the chlorella indoor culture conditions are adjusted to light and dark (Stirk et al., 2014).

Cassan et al. (1992) reported that foliar sprays of the extracts of blue green increased the fresh weight of spinach (cv. Monstrueux De Viroflay and cv. Polka) leaves by 12-15%. Our present result is in conformity with the results of Cassan et al. (1992).

In conclusion, this study provides that the use of *Chlorella fusca* as a biostimulant to enhance growth and yield

of Chinese chive and spinach in Korea. The study opens the possibility of utilizing freshwater algae, *C. fusca* as a potential source of biofertilizer in organic farming. Further studies on evaluation of mode of action of *C. fusca* are imperative to conclude its use and to recommend to the safety organic crop productions.

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