HortScience 59(6):865–872. 2024. https://doi.org/10.21273/HORTSCI17726-24

Effects of the Mixed Seeding Rate of Milkvetch on Fertilizer Substrate, Growth, and Carotenoid Levels of Baby Leaf Vegetables in Vertical Indoor Farming

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Keywords. baby leaf crops, co-culture, companion seeding, plant factory, sowing density, substrate fertilization

Abstract. **During this study, an indoor experiment was conducted to determine the effect of mixed seeding rates of legumes used as green manure on the substrate fertilizer, growth characteristics, and bioactive compounds of baby leaf vegetables. The mixed seeding treatment was designed for milkvetch (*Astragalus sinicus* L.), tatsoi (*Barassica rapa* L.), kale (*Brassica oleracea* var. *sabellica* L.), and spinach (*Spinacia oleracea* L.) using five rates for each. Accordingly, a total of 15 treatments (3 baby leaf species × 5 mixed seeding rates) were constructed using a randomized complete block design with three replications for each treatment. During the baby leaf vegetable harvest, we evaluated the macronutrient levels (nitrogen, phosphorus, and potassium) in the substrate as well as the growth parameters and carotenoid contents. The substrate in the treatment mixed with milkvetch showed significantly higher levels of nitrogen, phosphorus, and potassium compared with those of tatsoi and kale sown alone ($P \leq 0.05$). However, there were no significant differences in macronutrients observed in substrate-sown spinach with or without the milkvetch mixture. The growth and carotenoid levels of each baby leafy vegetable sown alone were significantly higher than those of each baby leafy vegetable sown with the mixed seeding treatment ($P \leq 0.05$). Sowing the milkvetch–vegetable mixtures did not result in a significant increase in the growth and carotenoid levels of the three baby vegetables. The results showed that planting milkvetch with tatsoi and kale had a significant impact on substrate fertilization. However, regarding short-term vertical indoor farming, the growth and carotenoid responses of the three greens may be different. Nonetheless, we still believe that the combined interactions of legumes can provide long-term benefits by enhancing the biological functionality of the growing medium for balanced indoor agriculture production.**

Whole vegetables and multi-leafed vegetables have been mainly used for raw consumption; however, there has been a recent increase in the use of other types of vegetables, such as baby leaf vegetables. These baby leaf greens are grown for a period of 20 to 40 d from sowing to harvest. When the shoots reach a height of ∼10 cm, they are ready for harvest. One of the advantages of baby leaf vegetables is that they are coreless and completely edible. Additionally, they can be quickly and easily processed (Pignata et al. 2020). As a result, the market for ready-to-eat baby leaf vegetables is rapidly expanding. Consumers are attracted to these products because they are convenient and appealing. Furthermore, they are rich in health-beneficial bioactive compounds (Saini et al. 2016). However, bioactive compounds play a role in protecting tender buds and sprouts from predators as well as from toxic elements and pollution.

Additionally, the content and composition of bioactive compounds in plants can be influenced by cultivation and management conditions (Ju et al. 2022). Specifically, growth environments play a significant role in determining the level of bioactivity in vegetables and can potentially impact their health-promoting and sensory attributes (Matysiak et al. 2021). However, previous studies have primarily focused on abiotic factors such as light, temperature, humidity, cultivation period, and growth regulators (Bermejo and Munne-Bosch 2023; Matysiak et al. 2021; Pignata et al. 2020).

The growing interest in mixed seeding is driven by the developing awareness of the environmental degradation caused by high chemical inputs. Ways of reducing modern agriculture's excessive reliance on fertilizers, which are primarily manufactured using fossil energy, are sought after (Matysiak et al. 2021). Mixed seeding with legumes is mainly applied for various purposes, including increasing yields, controlling weeds, and maintaining soil moisture during forage cultivation (Erkovan and Tan 2009; Lafreniere and Drapeau 2011; Tan and Serin 2004). Additionally, including leguminous species has been proposed as a means of increasing sustainability with low maintenance because of the ability of legumes to biologically fix atmospheric nitrogen (McCurdy et al. 2013).

Green manures can also improve soil fertility and aeration by reducing soil bulk density and increasing soil microbial and enzymatic activities (Fan et al. 2023). Milkvetch (*Astragalus sinicus*) is the most common green manure legume that is often grown through mixed seeding in water-limited climates, where available soil moisture is a key factor associated with the establishment of new plants and also increases the nutritional value (Wang et al. 2023). The effects of replacing chemical fertilizers with milkvetch are closely related to the amount of green manure incorporated. Therefore, the application of a suitable amount of milkvetch is more appropriate (Asagi and Ueno 2009). Accordingly, because of improved production, quality, and ecosystem service provision in the field (rather than indoors), most studies have focused on forage yields through legume–cereal mixed cropping or sowing (Acar et al. 2010; Fujita et al. 1992; Jungers et al. 2015; McCurdy et al. 2013; Tan and Serin 2004).

With the increasing popularity of ready-to-eat baby leaf vegetables, there has been a growing interest in detailed bioactive components, such as carotenoids and lutein (Pignata et al. 2020). It has been reported that chia sprouts have the highest carotenoid content compared with those of seeds or germinating seeds (Bermejo and Munne-Bosch 2023). Most baby leafy vegetables studied contain high levels of violaxanthin, lutein, and beta-carotene (Saini et al. 2016). However, there has been insufficient research of growth and bioactive compounds when mixed sowing with legumes is used.

Most plants interact with multiple organisms, and more than 80% engage symbiotic relationships with mycorrhizal fungi (O'Neill et al. 2023). In addition to the innovative

Received for publication 22 Jan 2024. Accepted for publication 5 Apr 2024.
Published online 20 May 2024.
This paper was supported by KonKuk University in 2023.
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practice of mixed seeding with legumes in indoor vertical farming, the use of biotic factors in cultivation methods is also deemed essential for enhancing the presence of bioactive compounds in baby leaf vegetables. Intercropping systems play a crucial role in improving both crop quality and yield as well as environmental quality by maximizing the efficient use of space, light, water, and soil nutrients compared with monocropping systems (Xue et al. 2016).

Therefore, this study aimed to evaluate the effects of mixed seeding rates on milkvetch. Varieties of milkvetch are widely used as green manure on fertilizer substrate. This study also assessed the growth and carotenoid levels of three baby leafy vegetables (tatsoi, kale, and spinach).

Materials and Methods

Experimental design, plant materials, and growing conditions. The indoor vertical farming experiment was performed at the Green Environmental Control Laboratory of KonKuk University. Baby leaf vegetables, namely tatsoi (*Barassica rapa* L.), kale (*Brassica oleracea* var. *sabellica* L.), and spinach (*Spinacia oleracea* L.), were chosen because of their widespread consumption as leaf salads and similar germination rates (Sharma et al. 2022). As a legume known for its ability to enhance soil fertility, milkvetch (*Astragalus sinicus* L.) was selected as a green manure (Wang et al. 2023). Milkvetch is commonly grown as a nitrogen-fixing green manure crop, but it also possesses edible leaves, making it advantageous for harvesting alongside baby leaf crops. These seeds obtained were from Aramhara Seed Product Co., Ltd. (Seoul, Korea).

Three separate experiments were established for tatsoi, kale, and spinach, with each having the following experimental variants: V1, 18 milkvetch seeds + 0 baby leaf crop seeds; V2, 12 milkvetch seeds + 6 baby leaf crop seeds; V3, 9 milkvetch seeds + 9 baby leaf crop seeds; V4, 6 milkvetch seeds + 12 baby leaf crop seeds; and V5, 0 milkvetch seeds + 18 baby leaf crop seeds. A total of 15 treatments (3 baby leaf species × 5 mixed seeding rates) were constructed as a randomized complete block design with three replications for each treatment.

Seeds were sowed in polypropylene plastic pots (0.6 L) at five different mixing rates. Each pot contained 15 g of perlite (New Perl-Shine No. 3; Green Fire Chemicals Co., Ltd., Gyeongbuk, Korea) and 65 g of commercial horticultural substrates (Hanpanseung; Samhwa Greentech Co., Ltd., Gyeonggi-do, Korea). These substrates primarily consisted of artificial soils comprising 74% cocopeat, 15% vermiculite, 5% perlite, 0.158% fertilizer, 0.002% humectant, and no commercial inoculum source.

After sowing the seeds, the small plastic pots were watered with mist water (100 mL) and covered with pot caps to maintain high humidity levels. Once germination occurred, the cap was removed to allow the plants to grow into baby leaf vegetables. The plants were cultivated in a light-emitting diode cultivation chamber (Mareuda Co., Ltd., Gwangju, Korea) with three vertically installed shelves with a measurement of 150 cm (length) × 70 cm (width) × 35 cm (height). They were grown for a period of 27 d with day/night temperatures of 25 °C /21 °C, relative humidity of ∼65%, and light/dark cycle of 16 h/8 h. Light intensity in the chamber was set at 300 μmol/m/s (Fig. 1).

Macronutrients in substrate and growth characteristics. After completing the growth experiment, we used a portable soil quality meter (SOIL6CH; Testauction Co., Seoul, Korea)

Fig. 1. Experimental setup of the light-emitting diode (LED) cultivation chamber. Vertical indoor farming experiments involved testing five different seeding mixture rates of milkvetch and three leafy vegetables in an automatic operational setting.

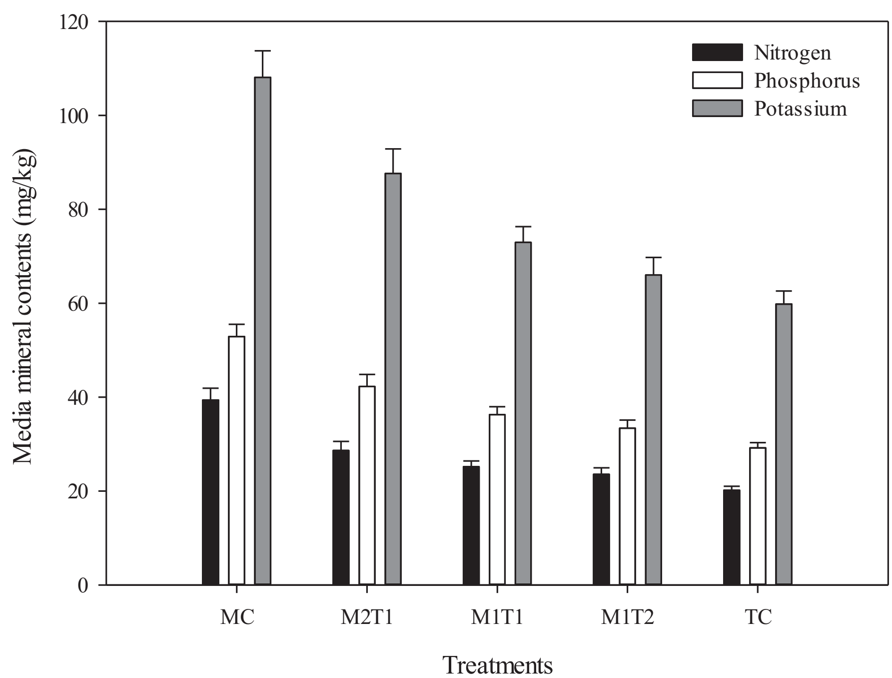

Fig. 2. Effects of mixed seeding rates of milkvetch (*Astragalus sinicus*) and tatsoi (*Brassica rapa*) on the macronutrient content of the substrate in vertical indoor farming. Different letters indicate a significant difference among treatments at $P \leq 0.05$ according to Duncan's multiple range test. The bars represent the standard error (±*SE*), with n = 9 at 27 d after sowing, either alone or in a mixed combination. MC = 18 milkvetch seeds + 0 tatsoi seeds; M2T1 = 12 milkvetch seeds + 6 tatsoi seeds; M1T1 = 9 milkvetch seeds + 9 tatsoi seeds; M1T2 = 6 milkvetch seeds + 12 tatsoi seeds; TC = 0 milkvetch seeds + 18 tatsoi seeds.

to measure the macronutrients (nitrate, phosphorus, and potassium) present in the substrate of each leafy vegetable. Additionally, we evaluated various parameters of nine representative plants per treatment 27 d after mixed sowing. These parameters included leaf height, leaf length, leaf width, leaf shape index, leaf area, number of leaves, and shoot fresh and dry weights. To measure the leaf height, length, and width, we simply used a ruler. Specifically, the leaf height was measured as the length from the upper surface of the growing medium to the tip of the shoot. Furthermore, we calculated the leaf shape index of the third leaf from the basal part by dividing the leaf length by the leaf width using the following formula:

$$\text{Leaf shape index (LSI)} = \text{leaf length}/\text{leaf width}$$

The leaves were counted manually, and the fresh weights of shoots were measured using a digital scale (IP65; A&D Co., Tokyo, Japan). The dry weight of the shoot was determined by drying it in an oven at 80 °C until a constant weight was reached; then, the weight was measured again.

Extraction and quantification of carotenoids. The main carotenoid found in baby leaf vegetables is lutein; however, zeaxanthin, β-cryptoxanthin, and β-carotene have also been reported (Bermejo and Munne-Bosch 2023). According to previous research, baby leaf vegetables were harvested 27 d after sowing, when their bioactive compound values are optimized (Ju et al. 2021). To obtain representative samples of each leaf vegetable, 100 g of each treatment was randomly collected in three replicates. The plant samples were washed with deionized water, dewatered, transported to the laboratory, and minced immediately. Carotenoids were extracted and quantified following the method of Ju et al. (2021), with some modifications.

All extractions were performed under low light conditions to prevent carotenoid degradation. Within 2 d of collection, plant samples were processed. Carotenoids were extracted from independent samples were extracted and aliquoted into 1-g portions in freezer vials, which were stored at −80 °C for further analyses. Each 1-g sample was placed in a 50-mL amber glass vial, and 5 mL of methanol was added. The mixture was vortexed for 30 s. Then, the plant samples and methanol mixture were incubated at room temperature for 1 h and homogenized in an ice bath for 30 s. After homogenization, the samples were centrifuged at 3000 rpm for 5 min at 4 °C, and the supernatant was transferred to a new 50-mL volumetric flask. The extraction process was repeated four times by adding 10 mL of tetrahydrofuran and then vortexing and centrifuging until the samples became colorless.

The tetrahydrofuran layers were combined with the methanol layer, and the total volume was adjusted to 50 mL. Then, 1 mL of extract was obtained, dried under nitrogen, and resuspended in 100 μL of ethanol. Another 1 mL of extract was filtered through a 0.45-μm membrane syringe filter (Whatman Plc., Kent, England) and transferred to an amber ultraperformance liquid chromatography vial for analysis on the same day.

Carotenoids were analyzed using an ultraperformance liquid chromatography (ACQUITY UPLC I-Class; Waters Co., Milford, MA, USA) system. The system was equipped with a BEH C18 column (1.7 μm; 2.1 mm × 50 mm; Waters Co.), a binary pump delivery system, an autosampler, and a photodiode array detector. The mobile phase A consisted of acetonitrile/

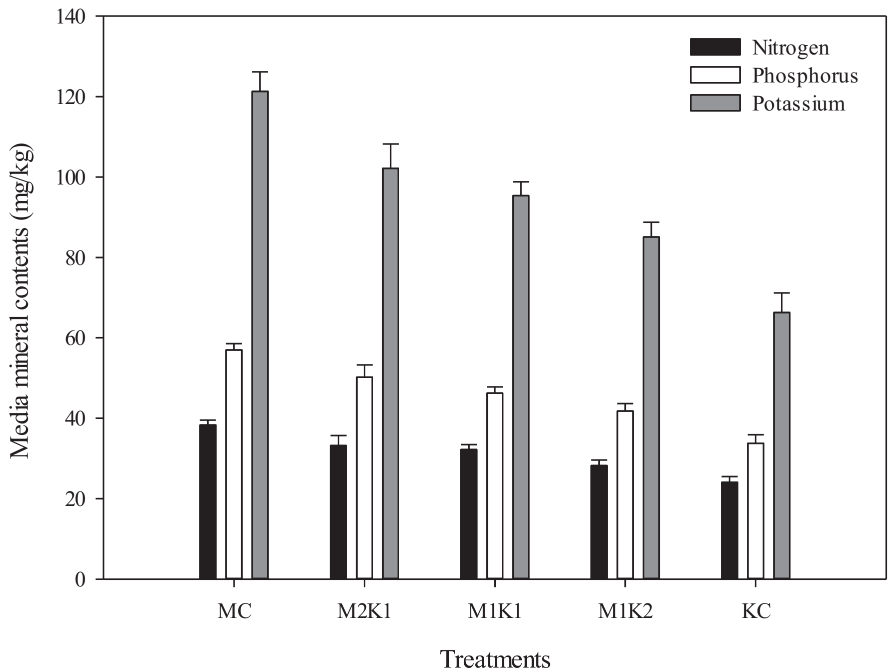

Fig. 3. Effects of mixed seeding rates of milkvetch (*Astragalus sinicus*) and kale (*Brassica oleracea*) on the macronutrient content of the substrate in vertical indoor farming. Different letters indicate a significant difference among treatments at $P \leq 0.05$ according to Duncan's multiple range test. The bars represent the standard error (±*SE*), with n = 9 at 27 d after sowing, either alone or in a mixed combination. MC = 18 milkvetch seeds + 0 kale seeds; M2K1 = 12 milkvetch seeds + 6 kale seeds; M1K1 = 9 milkvetch seeds + 9 kale seeds; M1K2 = 6 milkvetch seeds + 12 kale seeds; KC = 0 milkvetch seeds + 18 kale seeds.

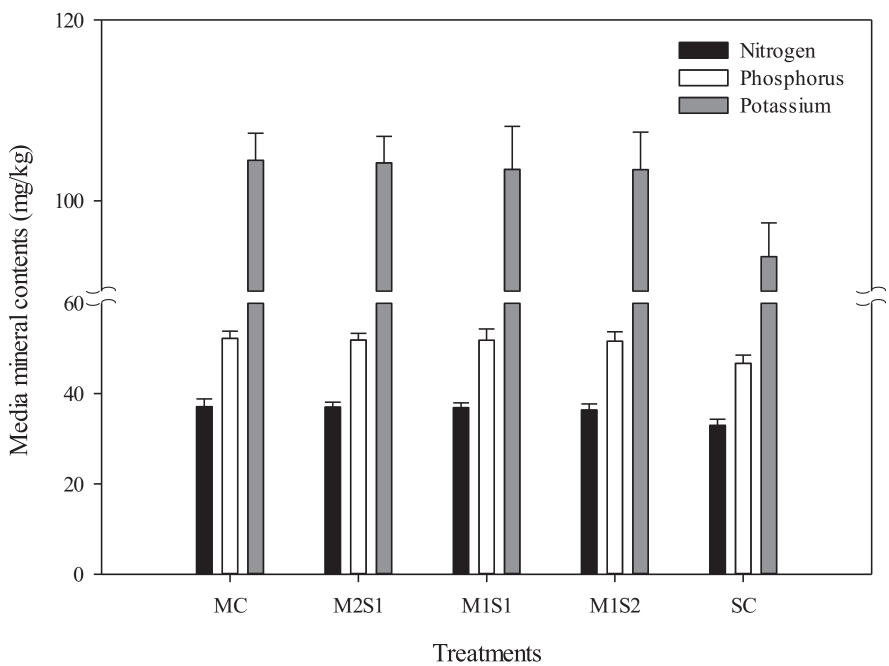

Fig. 4. Effects of mixed seeding rates of milkvetch (*Astragalus sinicus*) and spinach (*Spinacia oleracea*) on the macronutrient content of the substrate in vertical indoor farming. Different letters indicate a significant difference among treatments at $P \leq 0.05$ according to Duncan's multiple range test. The bars represent standard error (±*SE*), with n = 9 at 27 d after sowing, either alone or in a mixed combination. MC = 18 milkvetch seeds + 0 spinach seeds; M2S1 = 12 milkvetch seeds + 6 spinach seeds; M1S1 = 9 milkvetch seeds + 9 spinach seeds; M1S2 = 6 milkvetch seeds + 12 spinach seeds; SC = 0 milkvetch seeds + 18 spinach seeds.

methanol (7:3, volume/volume), while mobile phase B was water. Each sample was injected into the BEH C18 column (1.7 μm, 2.1 mm × 50 mm) and scanned at a detection wavelength of 450 nm. Carotenoids were quantified using standard curves, and each peak was confirmed by retention time and its unique spectrum. The interassay coefficient of variation (*CV*) was less than 4% (n = 10), and the intra-assay *CV* was also less than 4% (n = 10).

Statistical analysis. A one-way analysis of variance was performed to compare the means among treatments using mixed seeding rates. Duncan's multiple range test ($P \leq 0.05$) was used for this comparison. All statistical analyses were conducted using SPSS statistical software version 18.0 (SPSS Inc., Chicago, IL, USA). Graphs were also created using SigmaPlot (Systat, San Jose, CA, USA).

Results and Discussion

Changes in macronutrients of substrates grown using a seed mixture of legumes and vegetables. The macronutrient contents of the substrates showed significant ($P \leq 0.05$) variation depending on the mixed seeding rate of milkvetch (*Astragalus sinicus*) and tatsoi (*Barassica rapa*). As the mixed seed rate of tatsoi increased, the nitrogen content gradually decreased from 39.37 mg/kg to 20.19 mg/kg. The phosphorus value was slightly higher than that of nitrogen; however, as the seed rate of tatsoi increased, it gradually decreased from 52.89 mg/kg to 29.19 mg/kg.

The potassium content in the substrate was highest at 108.11 mg/kg in the milkvetch alone (MC) treatment and lowest at 59.81 mg/kg in the tatsoi alone (TC) treatment (Fig. 2). In general, as the rate of milkvetch seeds in mixed seeding increased, so did the macronutrient value of the substrate.

As the seed rate of milkvetch increased, the macronutrients in substrates grown as a mixture of milkvetch with kale also increased. When the kale seed rate increased, the nitrogen content of the substrate decreased from 38.29 mg/kg to 24.07 mg/kg. Similarly, the phosphorus content of the treatment in which only milkvetch was sown (MC) was 56.6 mg/kg, whereas the treatment in which only kale was sown (KC) had a phosphorus content of 33.77 mg/kg. The phosphorus content was significantly higher in the MC treatment than in the KC treatment ($P \leq 0.05$). The potassium content was 121.29 mg/kg in the MC substrate and 66.33 mg/kg in the KC substrate. The potassium content was also significantly higher in the MC treatment than in the KC treatment ($P \leq 0.05$) (Fig. 3).

In substrate sown alone or in a mixture of milkvetch and spinach, as the amount of spinach sown increased, the levels of nitrogen, phosphorus, and potassium tended to decrease. However, there was no statistically significant difference in the rates between treatments (Fig. 4). When milkvetch and spinach (compared with tatsoi and kale) were sown together, there was no significant impact ($P \leq 0.05$) on the macronutrients of the substrate.

Table 1. Growth characteristics of tatsoi (*Brassica rapa*) were examined using a mixed seeding rate with milkvetch (*Astragalus sinicus*) in vertical indoor farming after 27 d of seeding.

Treatment[i]	Leaf ht (mm)	Leaf shape index[ii]	Leaf area (cm^2)	Number of leaves	Fresh shoot wt (g/plant)	Dry shoot wt (g/plant)
MC[iii]	—	—	—	—	—	—
M2T1	89.93 b[i]	1.18 a	31.63 ab	1.18 a	1.321 b	0.110 b
M1T1	91.93 b	1.21 a	30.67 b	1.21 a	1.886 a	0.141 ab
M1T2	100.22 a	1.16 a	32.59 a	1.16 a	1.909 a	0.158 a
TC	103.30 a	1.25 a	27.67 c	1.25 a	1.706 ab	0.171 a

[i] Different letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$ (n = 9).
[ii] The leaf shape index is the leaf length/leaf width.
[iii] MC = 18 milkvetch seeds + 0 tatsoi seeds; M2T1 = 12 milkvetch seeds 1 6 tatsoi seeds; M1T1 = 9 milkvetch seeds + 9 tatsoi seeds; M1T2 = 6 milkvetch seeds + 12 tatsoi seeds; TC = 0 milkvetch seeds + 18 tatsoi seeds.

Changes in the individual macronutrient levels in the mixed seeding substrate were similar between the treatment comprising milkvetch and tatsoi and the treatment comprising milkvetch and kale. A higher seed rate of leafy greens, compared with that of milkvetch, led to a decrease in the nitrates, phosphorus, and potassium contents of the substrate. These changes were particularly significant for tatsoi and kale. Excluding the mixed sowing with spinach, in the mixed sowing of tatsoi and kale, the macronutrient content of the substrate was highest when milkvetch was sowed alone. Under phosphorus-deficient conditions, milkvetch secretes large quantities of organic acids through its root system. These molecules can interact with ligand groups on mineral surfaces, thereby increasing the availability of anionic nutrients such as phosphorus (Huang et al. 2003). During a study conducted by Fan et al. (2023), compared with conventional fertilization, using milkvetch as a treatment significantly improved phosphorus fertilizer recovery efficiency by 23.1% to 36.4%. Additionally, the agronomic efficiency of nitrogen, phosphorus, and potassium fertilizers increased by 16.1% to 21.1%, 13.9% to 19.6%, and 15.7% to 21.4%, respectively, during 5 to 12 years. Milkvetch is capable of mobilizing soil macronutrients through various root mechanisms, resulting in the secretion of solubilizing compounds in the rhizosphere. The release of flavonoids by legume roots helps to promote the growth of arbuscular mycorrhizal fungi symbiosis, which enhances nitrogen and phosphorus nutrition in the soil. The rhizobium of milkvetch belongs to the rhizobial species *Mesorhizobium huakuii* and is not a permanent member of the soil microflora. Rhizobia have a significant impact on the number and weight of nodules as well as nitrogenase activity, morphology, and yield. There are positive correlations between milkvetch and nodules and between milkvetch and soil nitrogen content (Liu et al. 2020). When milkvetch is added to soils, it contributes a substantial amount of organic matter, which can enhance fertilizer solubility (Wang et al. 2023).

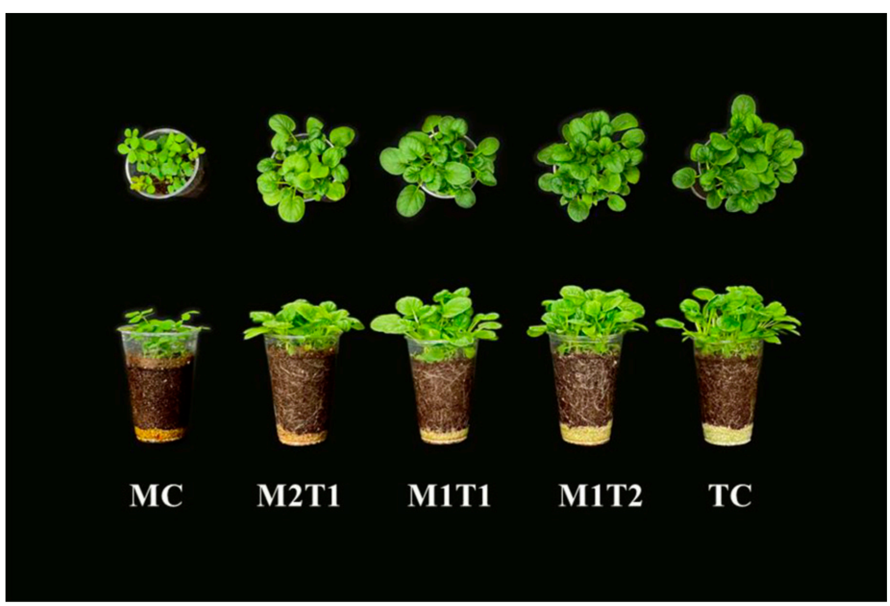

Fig. 5. Effects of the mixed seeding rates of milkvetch (*Astragalus sinicus*) with tatsoi (*Brassica rapa*) on growth in vertical indoor farming. MC = 18 milkvetch seeds + 0 tatsoi seeds; M2T1 = 12 milkvetch seeds + 6 tatsoi seeds; M1T1 = 9 milkvetch seeds + 9 tatsoi seeds; M1T2 = 6 milkvetch seeds + 12 tatsoi seeds; TC = 0 milkvetch seeds + 18 tatsoi seeds.

Additionally, the difference in the nitrogen fixation capability of milkvetch may be influenced by the physicochemical properties of the long-term planting field in its place of origin (Liu et al. 2020). The availability of high substrate macronutrients and the response to short-term application of milkvetch on accumulation in mixed sowings with milkvetch and spinach were deemed independent. Although these findings may differ depending on the companion seeding species, they clearly demonstrate that legumes can enhance the macronutrient content of the substrate in vertical indoor farming. However, it was challenging to observe nodules on milkvetch roots within 30 d after sowing in indoor vertical farming. The most likely cause for this is that the proprietary plant's rhizobium strains have not been applied to the field environment for a long period of time, resulting in milkvetch generally having limited nodule growth and nitrogen fixation (Liu et al. 2020). However, incorporating milkvetch may result in saving the application of inorganic fertilizer, thus improving the sustainability of these crop production systems. This work specifically focuses on the effects of the milkvetch seeding rate with baby leaf crops on macronutrient changes in substrates. The effects of the nodule shape, formation time, color, and distribution, whether they are single or plural nodules, and relevant environmental conditions that affect nodule formation by rhizobia have not been investigated. Therefore, further research is needed to verify these factors.

Growth characteristics of the three baby leaf vegetables. As the sowing rate of milkvetch (*Astragalus sinicus*) increased, the height of tatsoi (*Barssica rapa*) leaves decreased significantly ($P \leq 0.05$) compared with that when sown alone. However, in the M2T1 (milkvetch:tatsoi = 2:1) treatment, the leaf area of tatsoi was significantly higher than that of tatsoi grown through single sowing. There was no significant difference ($P \leq 0.05$) in the leaf shape and number of leaves of tatsoi. The fresh weight of the tatsoi shoot grown in the M1T2 treatment (milkvetch: tatsoi = 1:2) was the highest compared with that of the other treatments. The shoot dry weight of tatsoi was highest in the single sowing (TC) treatment, but it showed no significant difference when compared with the M1T2 treatment (Table 1, Fig. 5).

Leaf height is commonly used to characterize baby leaf vegetables; the typical harvestable leaf height is usually approximately 10 cm (Saini et al. 2016). The decrease in leaf height observed when milkvetch is mixed with the crop may be attributed to the competition between the two plants for the amount of light needed for growth. This suggests that mixed sowing could potentially limit the expansion of leaf height. Mutual shading caused by taller cereals significantly decreases both the biological nitrogen fixation and yield of associated legumes in mixed legume–cereal cropping systems (Fujita et al. 1992). Therefore, differences in the co-culture effect may be attributed to factors such as crop cultivar, growth duration, or soil type because these elements can influence the outcome of interactions among mixed seeding plants.

Table 2. Growth characteristics of kale (*Brassica oleracea*) according to the mixed seeding rate with milkvetch (*Astragalus sinicus*) under vertical indoor farming after 27 d of seeding.

Treatment[i]	Leaf ht (mm)	Leaf shape index[ii]	Leaf area (cm^2)	Number of leaves	Fresh shoot wt (g/plant)	Dry shoot wt (g/plant)
MC[iii]	—	—	—	—	—	—
M2K1	64.48 b[i]	1.30 a	8.49 b	5.22 b	0.774 b	0.097 a
M1K1	65.67 b	1.32 a	8.55 b	5.37 ab	0.901 ab	0.102 a
M1K2	67.67 b	1.32 a	8.96 ab	5.41 ab	1.017 a	0.111 a
KC	75.19 a	1.37 a	10.03 a	5.70 a	1.120 a	0.137 a

[i] Different letters indicate significant differences among treatments according to Duncan's multiple range test at $P < 0.05$ (n = 9).
[ii] The leaf shape index is the leaf length/leaf width.
[iii] MC = 18 milkvetch seeds + 0 kale seeds; M2K1 = 12 milkvetch seeds + 6 kale seeds; M1K1 = 9 milkvetch seeds + 9 kale seeds; M1K2 = 6 milkvetch seeds + 12 kale seeds; KC = 0 milkvetch seeds + 18 kale seeds.

The height of kale leaves was highest when sown alone (KC) and tended to decrease as the milkvetch sowing rate increased. There was no significant ($P \leq 0.05$) difference in the leaf shape index of kale depending on the mixed seeding rate. Both the leaf area and number of leaves of kale were highest when kale was sown alone, and they decreased as the sowing rate of milkvetch increased. In mixed sowing, as the milkvetch seeding rate increased, the shoot fresh weight of kale decreased, but there was no significant ($P \leq 0.05$) difference between KC (a single kale sowing) and M1K2 (a mixed sowing of milkvetch and kale at a ratio of 1:2). With the increase in the milkvetch seeding rate, the shoot dry weight of kale showed a numerical decrease, but there was no significant difference compared with that of the kale seeding treatment alone (Table 2, Fig. 6). Similarly, as the milkvetch sowing rate decreased, compared with kale, the leaf shape index remained similar, but there was a tendency for the leaf height, leaf area, and number of leaves to increase. However, there was no significant difference in the shoot fresh weight and shoot dry weight of kale between KC and M1K2.

These results suggest that competition for light between the same species may have been equal, even with the presence of milkvetch in the mixed sowing. However, as the proportion of kale increased, the seeding interval became narrower. It is worth noting that there was no significant difference in the shoot dry weight. Therefore, from a long-term perspective, it seems that single sowing of kale may not be the only condition that positively affects growth. Interspecific facilitation refers to the positive impact of the individuals from different species on each other. This can happen when sparingly soluble inorganic nutrients are indirectly mobilized, or when nutrients are transferred through common mycorrhizal networks that connect different co-cultivated crop plant species (Xue et al. 2016). However, this study showed that despite the significant increase in nitrogen, phosphorus, and potassium fertilizers in the

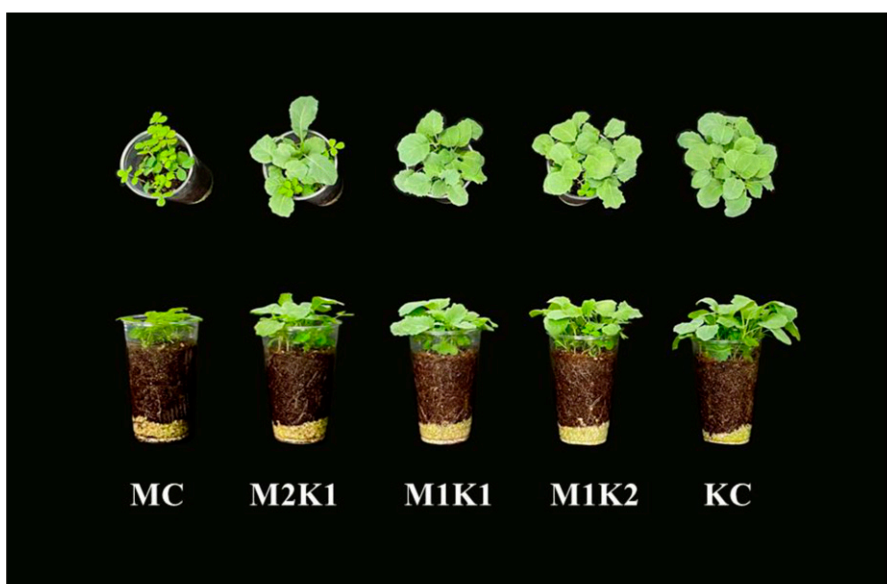

Fig. 6. Effects of the mixed seeding rates of milkvetch (*Astragalus sinicus*) with kale (*Brassica oleracea*) on growth in vertical indoor farming. MC = 18 milkvetch seeds + 0 kale seeds; M2K1 = 12 milkvetch seeds + 6 kale seeds; M1K1 = 9 milkvetch seeds + 9 kale seeds; M1K2 = 6 milkvetch seeds + 12 kale seeds; KC = 0 milkvetch seeds + 18 kale seeds.

substrate used for mixed sowing with milkvetch, there were no positive effects on kale growth parameters attributable to interspecific competition rather than facilitation.

As the sowing rate of milkvetch (*Astragalus sinicus*) increased, there was a tendency for the leaf height and leaf area of spinach (*Spinacia oleracea*) to decrease significantly ($P \leq 0.05$). The height, leaf shape index, and number of leaves of spinach were observed in the M1S2 treatment (milkvetch:spinach = 1:2), but there was no significant difference among treatments when the mixed seeding rate was considered. Although there was no statistically significant difference, the fresh and dry weights of spinach shoots decreased as the mixed sowing rate of milkvetch increased (Table 3, Fig. 7).

As the rate of mixed milkvetch seeds increased, the height and leaf area of spinach decreased. However, there was no statistical difference in the leaf shape index, number of leaves, and shoot fresh and dry weights of spinach based on the mixed seeding rate. In general, researchers have suggested that if a companion crop is planted, then it should be sown at low densities because different species compete for light.

It has also been reported that the use of companion crops at higher seeding rates is detrimental to the density and productivity of the main plant (Tan and Serin 2004; Wang et al. 2023). Therefore, when sowing a mixture of milkvetch and spinach in vertical indoor farming, it is expected that reducing the milkvetch rate will improve the growth of spinach. When there is more competition for light than for substrate nutrients, shorter spinach may be at a disadvantage compared with milkvetch. Moreover, allelopathic interactions may vary depending on the cultivar, and different cultivars may exhibit distinct leaf phenotypes (Pierce and Nattrass 2023). Therefore, it is important to consider these factors when choosing co-cultivated plants for mixed sowing in indoor vertical farming.

Carotenoid contents of the three baby leaf vegetables. The zeaxanthin content of tatsoi sown alone (TC) was highest, followed by those sown with M1T2 (milkvetch:tatsoi = 1:2), M2T1 (milkvetch:tatsoi = 2:1), and M1T1 (milkvetch:tatsoi = 1:1). Similarly, lutein was highest in TC, and there was no clear trend in the lutein content of tatsoi when mixed with milkvetch. Consistent with these findings, β-cryptoxanthin and β-carotene were lower in tatsoi sown alone than when sown with tatsoi mixed with milkvetch (Table 4).

The carotenoid content of tatsoi mixed with milkvetch was lower than that of the single seeding treatment. However, the lutein content of baby leaves of tatsoi mixed with milkvetch was lower than that reported for salad rocket and komatsuna (0.054 and 0.068 mg/g fresh weight, respectively) regardless of the seeding rate. Additionally, the β-cryptoxanthin and β-carotene contents of tatsoi were lower than those reported by previous studies (0.093 and 0.075 mg/g fresh weight, respectively) (Xiao et al. 2015).

Table 3. Growth characteristics of spinach (*Spinacia oleracea*) according to the mixed seeding rate with milkvetch (*Astragalus sinicus*) in vertical indoor farming after 27 d of seeding.

Treatment[i]	Leaf ht (mm)	Leaf shape index[ii]	Leaf area (cm^2)	Number of leaves	Fresh shoot wt (g/plant)	Dry shoot wt (g/plant)
MC[iii]	—	—	—	—	—	—
M2S1	54.81 c[i]	2.74 a	2.12 c	5.48 a	0.624 a	0.036 a
M1S1	59.56 bc	3.33 a	3.02 bc	5.63 a	0.745 a	0.028 a
M1S2	64.04 b	3.68 a	3.43 b	5.74 a	0.797 a	0.044 a
SC	76.37 a	2.66 a	4.67 a	5.78 a	0.819 a	0.049 a

[i] Different letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$ (n = 9).
[ii] The leaf shape index is the leaf length/leaf width.
[iii] MC = 18 milkvetch seeds + 0 spinach seeds; M2S1 = 12 milkvetch seeds + 6 spinach seeds; M1S1 = 9 milkvetch seeds + 9 spinach seeds; M1S2 = 6 milkvetch seeds + 12 spinach seeds; SC = 0 milkvetch seeds + 18 spinach seeds.

As the seeding rate of milkvetch increased, the zeaxanthin content of kale mixed with milkvetch decreased. The difference between the lowest and highest contents was approximately two-fold. Similarly, the lutein content in kale was highest in the KC treatment (kale sown alone) and lowest in the M1K1 treatment (milkvetch:kale = 1:1). The lutein content of kale showed slight fluctuations or changes between sowing rates when mixed with milkvetch; however, no clear trend was observed. However, when the mixing rate of milkvetch seeds increased, the contents of β-cryptoxanthin and β-carotene also increased, except in the case of kale seeding alone (Table 5). In general, the carotenoid content in kale was highest in the single seeding treatment, and it was difficult to identify a clear trend based on the mixed seeding rate with milkvetch in vertical indoor farming.

We found the highest content of zeaxanthin (5.784 mg/100 g fresh weight) in the M1S2 (milkvetch:spinach = 2:1) treatment, which was approximately two-fold higher than that of spinach grown in the M2S1 (3.632 mg/100 g fresh weight) treatment. However, the change according to the milkvetch seeding rate was not clear. As the mixed sowing rate of milkvetch increased, the lutein, β-cryptoxanthin, and β-carotene contents of spinach tended to decrease. Therefore, we found that the carotenoid content could be maximized when spinach was sown alone rather than mixed with milkvetch (Table 6). However, it is worth noting that the carotenoid values in the M1S2 (milkvetch:spinach = 1:2) treatment and SC (spinach sowing alone) treatment showed similar carotenoid levels and good growth. This suggested that mixed seeding with milkvetch may still be beneficial. As reported previously, mixed cropping has a stimulatory effect on various crop parameters by improving the biochemical constituents of plants (Xu and Chang 2008). In the case of pea intercropped tea shoots, there was a decrease in the total polyphenols and catechins, whereas there was an increase in amino acids.

Correspondingly, genes related to amino acid metabolism and flavonoid biosynthesis

Fig. 7. Effects of the mixed seeding rates of milkvetch (*Astragalus sinicus*) with spinach (*Spinacia oleracea*) on growth in vertical indoor farming. MC = 18 milkvetch seeds + 0 spinach seeds; M2S1 = 12 milkvetch seeds + 6 spinach seeds; M1S1 = 9 milkvetch seeds + 9 spinach seeds; M1S2 = 06 milkvetch seeds + 12 spinach seeds; SC = 0 milkvetch seeds + 18 spinach seeds.

Table 4. Carotenoid contents in baby leaf of tatsoi (*Brassica rapa*) according to the mixed seeding rate with milkvetch (*Astragalus sinicus*) in vertical indoor farming after 27 d of seeding.

Treatment[i]	Zeaxanthin (mg/100 g FW)[ii]	Lutein (mg/100 g FW)	β-cryptoxanthin (mg/100 g FW)	β-carotene (mg/100 g FW)
MC[iii]	—	—	—	—
M2T1	2.666 ± 0.107[i]	4.584 ± 0.078	2.045 ± 0.021	7.029 ± 0.116
M1T1	2.160 ± 0.046	3.574 ± 0.069	1.566 ± 0.023	6.007 ± 0.129
M1T2	2.711 ± 0.031	4.478 ± 0.102	1.930 ± 0.066	7.116 ± 0.227
TC	2.837 ± 0.027	4.642 ± 0.025	2.073 ± 0.020	7.297 ± 0.200

[i] Samples analyzed using ultraperformance liquid chromatography (UPLC). Each value was calculated as the mean ± *SE* of triplicate experiments.
[ii] FW = fresh weight.
[iii] MC = 18 milkvetch seeds + 0 tatsoi seeds; M2T1 = 12 milkvetch seeds + 6 tatsoi seeds; M1T1 = 9 milkvetch seeds + 9 tatsoi seeds; M1T2 = 6 milkvetch seeds + 12 tatsoi seeds; TC = 0 milkvetch seeds + 18 tatsoi seeds.

Table 5. Carotenoid contents in baby leaf of kale (*Brassica oleracea*) according to the mixed seeding rate with milkvetch (*Astragalus sinicus*) in vertical indoor farming after 27 d of seeding.

Treatment[i]	Zeaxanthin (mg/100 g FW)[ii]	Lutein (mg/100 g FW)	β-cryptoxanthin (mg/100 g FW)	β-carotene (mg/100 g FW)
MC[iii]	—	—	—	—
M2K1	2.847 ± 0.458[i]	4.766 ± 0.744	2.254 ± 0.235	7.140 ± 0.980
M1K1	2.871 ± 0.039	4.734 ± 0.075	2.077 ± 0.020	6.819 ± 0.118
M1K2	2.456 ± 0.010	3.970 ± 0.061	1.834 ± 0.170	6.020 ± 0.069
KC	4.560 ± 0.246	4.798 ± 0.081	2.584 ± 0.030	9.174 ± 0.068

[i] Samples analyzed using ultraperformance liquid chromatography (UPLC). Each value was calculated as the mean ± *SE* of triplicate experiments.
[ii] FW = fresh weight.
[iii] MC = 18 milkvetch seeds + 0 kale seeds; M2K1 = 12 milkvetch seeds + 6 kale seeds; M1K1 = 9 milkvetch seeds + 9 kale seeds; M1K2 = 6 milkvetch seeds + 12 kale seeds; KC = 0 milkvetch seeds + 18 kale seeds.

were differentially expressed (Ma et al. 2022). However, in the present study, the effect of the short-term application of milkvetch on the carotenoid content of spinach was independent and thought to be more influenced by the light reception rate, which is determined by the seeding density rather than by macronutrients in the substrate.

Conclusions

This study aimed to assess the impact of different seeding rates of milkvetch (*Astragalus sinicus*), which is commonly used as green manure, on the fertilizer substrate, growth, and carotenoid levels of three baby leafy vegetables, tatsoi, kale, and spinach. The findings indicated that companion seeding with milkvetch had a significant influence on nearly all the macronutrients in the substrates used to grow tatsoi (*Brassica rapa*) and kale (*Brassica oleracea*).

With an increasing rate of milkvetch seeding, there were significant increases in nitrogen, phosphorus, and potassium. However, we observed that varying the seeding rate of milkvetch did not have any advantage in terms of macronutrients for substrate-grown spinach (*Spinacia oleracea*). The growth and carotenoid contents generally decreased when milkvetch was companion-seeded, but the extent of the reduction varied depending on the species and plant type. With a decreasing milkvetch seeding rate, the leaf height, leaf shape, leaf area, number of leaves, shoot fresh weight, and shoot dry weight of each of three species of the baby leaf vegetables studied increased incrementally. Additionally, increasing the companion seeding ratios of milkvetch resulted in decreases in zeaxanthin, lutein, β-cryptoxanthin, and β-carotene. These findings suggest that in substrate-grown tatsoi and kale, the seeding mixture rate of milkvetch significantly influenced the macronutrients. However, in indoor farming, the growth and carotenoid responses of greens during the short-term application of milkvetch could be independent.

Therefore, the inclusion of legume seeds in mixed seeding has a positive beneficial effect on increasing the macronutrients in the substrate up to a certain level. However, it is unclear how well the plants are able to absorb or use these nutrients. Nevertheless, the combined interactions between legume seeds and the growing medium can enhance biological processes and ultimately lead to sustainable and balanced indoor farming production in the long term. Further studies should also evaluate nitrogen fixation and transfer in milkvetch when it is sown together with legumes and investigate nutrient uptake and use efficiency in relation to leafy vegetables.

Table 6. Carotenoid contents in baby leaf of spinach (*Spinacia oleracea*) according to mixed seeding rates with milkvetch (*Astragalus sinicus*) under vertical indoor farming after 27 d of seeding.

Treatment[i]	Zeaxanthin (mg/100 g FW)[ii]	Lutein (mg/100 g FW)	β-cryptoxanthin (mg/100 g FW)	β-carotene (mg/100 g FW)
MC[iii]	—	—	—	—
M2S1	3.632 ± 0.052[i]	3.479 ± 0.025	1.707 ± 0.003	4.637 ± 0.046
M1S1	4.639 ± 0.142	4.252 ± 0.040	2.123 ± 0.040	7.507 ± 0.213
M1S2	5.784 ± 0.292	5.257 ± 0.222	2.677 ± 0.117	7.719 ± 0.159
SC	5.591 ± 0.165	5.364 ± 0.220	2.829 ± 0.023	8.257 ± 0.143

[i] Samples analyzed using ultraperformance liquid chromatography (UPLC). Each value was calculated as the mean ± *SE* of triplicate experiments.
[ii] FW = fresh weight.
[iii] MC = 18 milkvetch seeds + 0 spinach seeds; M2S1 = 12 milkvetch seeds + 6 spinach seeds; M1S1 = 9 milkvetch seeds + 9 spinach seeds; M1S2 = 6 milkvetch seeds + 12 spinach seeds; SC = 0 milkvetch seeds + 18 spinach seeds.

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