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Development of an N-Free Culture Solution for Cultivation of Nodulated Soybean with Less pH Fluctuation by the Addition of Potassium Bicarbonate

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Abstract: Soybean plants can grow by solely depending on fixed N_2 through their root nodules, a symbiotic organ with rhizobia. In this study, when nodulated soybeans were cultivated using hydroponics and an N-free culture solution, the pH rapidly decreased to 4.0, which may be harmful for root growth and nutrient absorption. Therefore, we prepared a new N-free culture solution characterized by less pH fluctuation due to the addition of potassium bicarbonate. A total of 1–2 mM sodium bicarbonate optimized the pH between 6 and 7. However, the solution pH increased to 8–9 during soybean cultivation when 5–20 mM of sodium bicarbonate was applied. The addition of potassium bicarbonate did not affect the dry weight of each organ. The evapotranspiration rate of the plants with bicarbonate on the 37th day after planting was higher than that of the control without bicarbonate. When the K_2SO_4 was replaced by $KHCO_3$, the pH just after preparation ranged between 6.3 and 6.5, while that after cultivation for two days ranged frp, about 6.1 to 6.5. It was found that more than half of the bicarbonate remained in the culture solution after 3 days of cultivation. The optimum P concentrations for the modified culture solution were found to be 50 and 100 μM, while the P concentrations over 150 μM reduced the plant growth and led to yellowing in the lower leaves.

Keywords: culture solution; pH; potassium bicarbonate; soybean

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1. Introduction

Hydroponic culture is essential for research on plant nutrition and plant physiology because the compositions of nutrients can be more easily controlled in this culture compared with soil culture. In addition, the composition of nutrients can be easily altered by changing the solution. Recently, plant cultivation through hydroponics for agricultural production has become popular especially for vegetables such as tomato, cucumber, and pepper and leaf vegetables [1]. The global hydroponics crop market was estimated to be USD 37.7 billion in 2022 and is expected to reach USD 53.4 billion by 2027 [2]. Hydroponics provides faster crop growth and higher yields compared to other methods. Further, hydroponics avoids the risk of soil-borne diseases without requiring the application of pesticides [3]. Tomatoes are the largest hydroponically cultivated global crop, owing to their fast cultivation rate and their lower water requirements compared to regular tomatoes grown in the soil [3]. Indoor farmers use rock wool, perlite, or coconut coir as growing materials for tomato hydroponic cultivation. It is estimated that the European and Asia-Pacific regions will cultivate the largest quantities of tomatoes using the hydroponic method by 2028 [3].

Historically, plant cultivation through hydroponics was developed independently by Sacks and Knop around 1860 [4]. They used solutions containing the nutrient elements N, P, K, Ca, Mg, S, and Fe, which were recognized as the essential elements for plants at that time. During the 20th century, other essential elements, the micronutrients Mn, B, Zn, Cu, Mo, Cl, and Ni, were discovered [5]. Hoagland and Arnon prepared two types of culture

solutions with B, Mn, Zn, Cu, and Mo; one contained N only in the form of NO_3^-, and the other used both NH_4^+ and NO_3^- to prevent pH fluctuation during cultivation.

In addition to the concentrations of nutrients, the pH of a culture solution affects plant growth, especially root growth and nutrition absorption, so the solution pH should be kept within an adequate range. Generally, plants grow well at pH 5.5–6.5, but pH 5.0–5.5 is optimum for rice cultivation. The pH of a culture solution tends to decrease when N is supplied in the form of NH_4^+, while the pH increases when NO_3^- is the sole N source [5]. The pH of a culture solution needs to be adjusted through the addition of HCl or NaOH when the pH shifts out of the optimum pH range, but this is time-consuming and involves heavy labor. Ikarashi [6] used a culture solution containing NO_3^-:NH_4^+ (4:1) to minimize the pH changes during cultivation.

Many studies have noted that pH affects plant growth in hydroponics. Islam et al. [7] reported that regarding the optimum pH levels of six plant species, namely ginger, cassava, maize, wheat, green bean, and tomato, these species grew in a continuously flowing nutrient solution with seven constant pH values that ranged from 3.3 to 8.5. All of these species achieved maximum or near-maximum growth in the pH range of 5.5–6.5. The roots of all the species at pH 3.3 and green bean at pH 4.0 exhibited hydrogen ion injury. Further, Kawasaki and Moritsugu [8] used an automated pH adjustment device to maintain pH values ranging from 3.6 to 6.6, which varied by $\pm$0.1, for the cultivation of barley, rice, tomato, corn, and cucumber plants. The N forms with 5 mM NH_4^+ and 5 mM NO_3^- were compared. The growth of the plants was vigorous within the pH range of 5.4 to 6.0. Roosta and Rezaei [9] grew rose within a pH range of 4.5–8.0; the plant growth was good at pH 4.5 and 6.5 but bad at 8. Moreover, the concentrations of chlorophyll, P, Fe, Mn, and Cu were the highest at pH 6.5 and the lowest at pH 8.0.

Concerning the effect of pH on leguminous plants, Vargas and Graham [10] investigated seven cultivars of bean (*Phaseolus vulgaris*) in a sand culture using a culture solution with pH values of 4.5, 5.0, 5.5, and 6.0. The number and fresh weights of the nodules decreased with decreasing pH value. Harper and Nicholas [11] used an ion-exchange column to maintain the pH of a culture solution for nodulated soybean cultivation. By circulating the culture solution through the column using aeration, the solution pH fluctuated in a magnitude around 0.5–0.9 of the initial pH value of 6.5 for 2 weeks. They used a cation-exchange resin Amberlite IRC 50 in which the ratio of Ca^{2+}:H^+ was equilibrated at 8:2. This apparatus was able to maintain the pH of the culture solution at around 6.5 for 2 weeks.

The addition of buffer reagents has been used as an alternative method for maintaining a solution pH. Imsande and Ralston [12] used 1–2 mM 2-(N-morpholino)ethanesulfonic acid (MES) plus 0.1 mM phosphate for a nodulated soybean culture because a culture solution with 1 mM phosphate is toxic to a soybean plant that derives its nitrogen solely from dinitrogen fixation. Soybean plants with an N-free culture solution with 0.1 mM phosphate and 1–2 mM MES achieved excellent growth and a high rate of nitrogen-fixation activity. Five days after the change in medium, the change in the pH was $-$2.0 when using the solution without MES, while this ranged from $-$0.7 to $-$1.4 when the MES solution was used. On the other hand, the addition of MES to a hydroponic culture of clover (*Trifolium repense*) decreased its growth and nitrogen-fixation activity [13]. MES also decreased *Arabidopsis*'s root growth [14]. Nicholas and Harper [15] reported that the pH of a 4.0 mM MES buffered nutrient solution decreased from 6.5 to below 4.0 in 5 days with 25- to 30-day-old plants.

In this research, we used sodium bicarbonate or potassium bicarbonate for the buffering reagent of an N-free culture solution to prevent decreases in pH when cultivating nodulated soybean plants. Buffering by bicarbonate is used for pH adjustment in animal blood and CO_2 discharge [16]. HCO_3^- is a major form of inorganic C in seawater and accounts for 90% of inorganic C [17]. On the other hand, the accumulation of bicarbonate in soil causes high pH values and Fe deficiency in crops, especially in calcareous soils in arid and semiarid regions [18]. Tang and Thomson [19] investigated the effects of the addition of 5 mM $KHCO_3$ in 14 species of leguminous plants, and almost all plants showed decreases in their shoot growth, nodulation, and shoot N concentration.

2. Materials and Methods

2.1. Plant Cultivation

Seeds of soybean (*Glycine max* [L.] Merr., cv. Williams) were soaked in 70% ethanol for 30 sec and sterilized with 0.5% sodium hypochlorite solution for 5 min. Then, the seeds were thoroughly washed with tap water, inoculated with a suspension of *Bradyrhizobium diazoefficience* (USDA110), and planted in a vermiculite bed. At 5 DAP (days after planting), a plant seedling was transplanted into 800 mL of nitrogen-free nutrient solution [20] in a 900 mL glass bottle covered with aluminum foil with continuous aeration. Plants were cultivated in a biophotochamber (LH-350S, Nippon Medical & Chemical Instruments Co. Ltd., Osaka, Japan) under 28 °C day/18 °C night temperatures, 55% relative humidity, and a photon flux density of 228 μmol m^{-2} s^{-1} with a 16 h photoperiod and an 8 h dark period.

2.2. Composition of Culture Solution

Table 1A shows the original nutrient solution we used for soybean cultivation. The composition of this solution was based on the solution used in Dr. Harper's laboratory at Illinois University [21,22]. We used a half strength of the original solution [20], because some Japanese cultivars showed leaf burn, possibly due to excess nutrient symptoms. When we used a half-strength solution, no such symptoms were observed. For Stock 1 of the modified solution (Table 1B), 625 μM of K_2SO_4 and 12.6 μM of KCl in original composition (Table 1A) were replaced by 1250 μM of $KHCO_3$. Stocks 2 (Ca), 3 (Mg), and 4 (Fe) were the same. Stock 5 (micronutrient) concentrations were the same except for Cu. A culture solution was prepared in a bucket. A total of 10 L of distilled water was put in the bucket, and 20 mL of Stock 1 was added and mixed well. Then, 10 mL of Stocks 2, 3, 4, and 5 were added and mixed well each time. The initial pH was around 6–7, so the pH of the solution was not adjusted.

Table 1. Compositions of original culture solution (A) and modified culture solution with bicarbonate (B). Stock solutions were separated into five bottles with either 500-fold (Stock 1) or 1000-fold (Stocks 2–5) composition increases compared to the original solution.

	(A) Original Composition		
Stock (Enrichment)	**Compound**	**Molar Concentration (μM)**	**Weight Concentration (mg/L)**
1 (×500)	K_2HPO_4	49	8.5
	K_2SO_4	625	109
	KCl	12.6	0.936
2 (×1000)	$CaCl_2 \cdot 2H_2O$	1,250	184
3 (×1000)	$MgSO_4 \cdot 7H_2O$	500	123
4 (×1000)	Fe-EDTA·$3H_2O$	50	21.05
5 (×1000)	H_3BO_3	5.94	0.367
	$CuSO_4 \cdot 5H_2O$	0.13	0.032
	$MnSO_4 \cdot 5H_2O$	0.772	0.186
	$ZnSO_4 \cdot 7H_2O$	0.5	0.144
	$CoCl_2 \cdot 6H_2O$	0.181	0.043
	$(NH_4)_6Mo_7O_{24} \cdot 7H_2O$	0.0032	0.004
	$NiCl_2 \cdot 6H_2O$	0.013	0.003

Table 1. *Cont.*

	(B) Modified Composition with Bicarbonate		
Stock (Enrichment)	**Compound**	**Molar Concentration (μM)**	**Weight Concentration (mg/L)**
1 (×500)	K_2HPO_4	50	8.71
	$KHCO_3$	1250	125
2 (×1000)	$CaCl_2 \cdot 2H_2O$	1250	184
3 (×1000)	$MgSO_4 \cdot 7H_2O$	500	123
4 (×1000)	Fe-EDTA·$3H_2O$	50	21.05
5 (×1000)	H_3BO_3	5.94	0.367
	$CuSO_4 \cdot 5H_2O$	1.26	0.315
	$MnSO_4 \cdot 5H_2O$	0.772	0.186
	$ZnSO_4 \cdot 7H_2O$	0.5	0.144
	$CoCl_2 \cdot 6H_2O$	0.181	0.043
	$(NH_4)_6Mo_7O_{24} \cdot 7H_2O$	0.0032	0.004
	$NiCl_2 \cdot 6H_2O$	0.013	0.003

2.3. Measurement of pH

The pH of the culture solution was measured just after preparation of the culture solution as well as after plant cultivation. An aliquot of the solution was used for measurement with a pH meter (LAQUA twin B-71X, Horiba Ltd., Kyoto, Japan). The pH meter was calibrated at 6.86 and 4.01 using standard pH buffers.

2.4. Determination of Bicarbonate Concentration

The concentration of bicarbonate in the culture solution was determined by the modified method of Mishima et al. [23], in which the spectrum changes in bromocresol green (BCG) by pH were measured. The procedure was as follows. A total of 0.5 mL of 0.1 mM HCl and 19 μL of 0.04% (*w*/*v*) BCG solution was placed in a 1.5 mL microtube. Then, 30 μL of the culture solution or the standard solution was mixed and kept for 5 min. A total of 200 μL of the reaction mixture was transferred to a microplate well (96 wells with flat bottoms), and the optical absorptions at 445 nm and 616 nm were measured by a microplate reader (SSH-1000, Corona Electric Co., Ltd., Ibaraki, Japan). The standard curve was obtained using 0, 20, 40, 60, 80, 100, and 120 mg/L $NaHCO_3$ solutions, wherein the concentration of $NaHCO_3$ was plotted on the horizontal axis, while the ratios of the absorbance (616 nm/445 nm) were plotted on the vertical axis.

2.5. pH Changes through the Addition of Various $NaHCO_3$ Concentrations

At first, an experiment that measured the effects of the addition of high concentrations (0, 2, 5, 10, 20 mM) of $NaHCO_3$ on the pH changes in the culture solution cultivated between 20 and 24 DAP was conducted. Then, a similar experiment that measured the effects of low concentrations (0, 0.5, 1.0, 1.5, 2.0 mM) of $NaHCO_3$ on the pH changes in the culture solution cultivated between 32 and 36 DAP was conducted.

2.6. Effects of the Addition of $NaHCO_3$ or $KHCO_3$ on Evapotranspiration and Plant Growth

The effects of the addition of $NaHCO_3$ or $KHCO_3$ to the culture solution on the plant growth parameters, such as the evapotranspiration and chlorophyll contents of each leaf as well as the dry weights of the plants, were compared with control plants without bicarbonate. The 19 DAP soybean plants were cultivated with either the original solution (Table 1A) or the solutions to which 1 mM $NaHCO_3$ or $KHCO_3$ had been added. Evapotranspiration was measured by weighing the culture solution in the glass bottle before and after cultivation. The plants were harvested on 37 DAP, and the chlorophyll

contents (SPAD values) of each leaf were measured using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Japan, Inc., Tokyo, Japan). The parts of each plant were separated into the leaves, stems plus petioles, roots, and other and were dried to a constant weight in a ventilation oven at 80 °C.

2.7. Effects of P Concentrations on Soybean Growth

To determine the concentration of P suitable for a new culture solution with bicarbonate, soybean plants were cultivated with KH_2PO_4 concentrations of 50 μM (P50), 100 μM (P100), 150 μM (P150), 200 μM (P200), and 400 μM (P400). Soybean seeds were inoculated and germinated using the same method described in Section 2.1. The seedlings on 7 DAP were transplanted into the glass bottle with a culture solution of 50 μM K_2HPO_4. At 33 DAP, plants were subjected to KH_2PO_4 concentrations of 50, 100, 150, 200, and 400 μM for 14 days. The culture solution was changed every 3 days. Plants were harvested on 47 DAP; dried in a ventilation oven at 80 °C; separated into roots, nodules, stems plus petioles, healthy leaves, damaged leaves, and young immature leaves; and weighed. The roots, nodules, stems, healthy leaves, and damaged leaves were ground into fine powder, and 20 mg of dry powder was placed in a 1.5 mL plastic tube and extracted with 1 mL of 1% HCl solution by shaking the tube for 15 min. Then, the extract and precipitate were separated by centrifugation. The extract was dried using a centrifuge drier, and the precipitate was dissolved in 1 mL of water and then diluted to 50 mL using distilled water. Concentrations of anions and cations were determined by ion chromatography (IC-2010, Tosoh Techno-System, Inc., Tokyo, Japan) using cation column (TSKgel superIC-Anion) or anion column (TSKgel superIC-Cation). The concentrations of P in the precipitate were determined after digestion by the Kjeldahl digestion and molybdenum blue method [24].

2.8. Statistics

Experiments were conducted with 4 biological replications. Plants were cultivated using a random arrangement in a growth chamber. Statistical significance by Tukey's test or Student's *t*-test was determined using the statistical analysis program of Biomedical Statistics, Graduate School of Medicine, Osaka University.

3. Results

3.1. Changes through the Addition of Various $NaHCO_3$ Concentrations

Figure 1 shows the effects of the addition of 0, 2, 5, 10, and 20 mM $NaCO_3$ on the pH of the culture solution. The plants at 20 DAP were treated for 4 days. The culture solution was changed on day 0 (20 DAP) and was not changed thereafter. The pH of the culture solution without bicarbonate (0 mM) was initially 6.0 but decreased to nearly 4.0 on day 2 of the treatment period and reached 3.7 on day 3. The initial pH values increased as the $NaHCO_3$ concentration increased: at 2, 5, 10, and 20 mM $NaHCO_3$, these values were 6.3, 7.0, 7.6, and 8.0, respectively. Under the 2 mM $NaHCO_3$ treatment, the pH values ranged between 6.0 and 7.0 until day 4 of the treatment. However, under the higher $NaHCO_3$ concentrations, the pH of the culture solution increased by about +1.0 from day 0 to day 2, reaching pH values ranging between 8.0 and 9.0; the pH then remained constant from day 2 to day 4. The measurements were repeated from 24 to 28 DAP and from 28 to 32 DAP, and similar results were observed, although the decrease in pH under the 0 mM treatment became faster (data not shown).

The concentrations of $NaHCO_3$ over 5 mM were not adequate. The effects of the lower concentrations of $NaHCO_3$ were evaluated (Figure 2). Under the 0 mM $NaHCO_3$ concentration, the pH of the culture solution decreased rapidly to 3.6 on day 2, then remained constant. When the culture solution with 0.5 mM $NaHCO_3$ was used, the pH did not change on day 1 but decreased to 4.3 on day 3. The pH values ranged between 6 and 7 under the 1 mM $NaHCO_3$ concentration. When 1.5 mM or 2.0 mM $NaHCO_3$ was used, the pH tended to increase over the initial 2 days. From the results obtained, it is clear that 1 mM $NaHCO_3$ was the optimum concentration under our cultivation conditions for maintaining the pH of the solution.

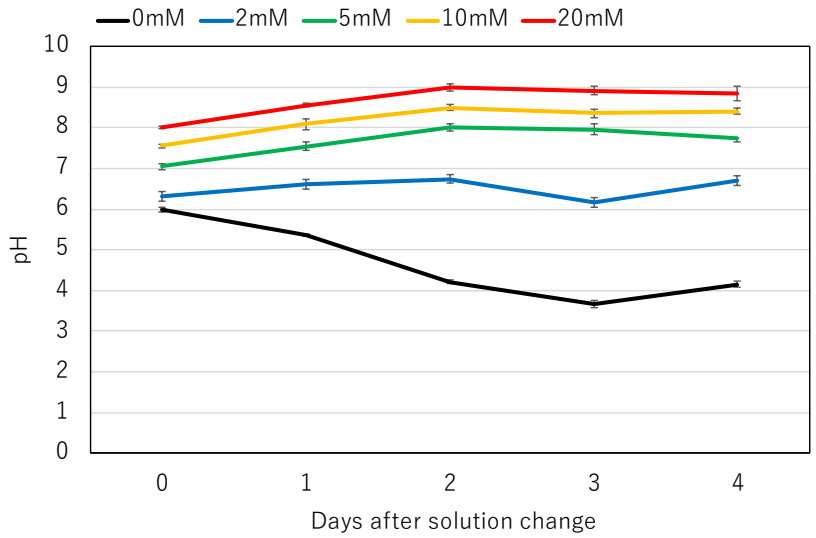

Figure 1. Effects of addition of 0, 2, 5, 10, and 20 mM $NaHCO_3$ on pH of the culture solution. Average ± standard error; 20 DAP plants were used.

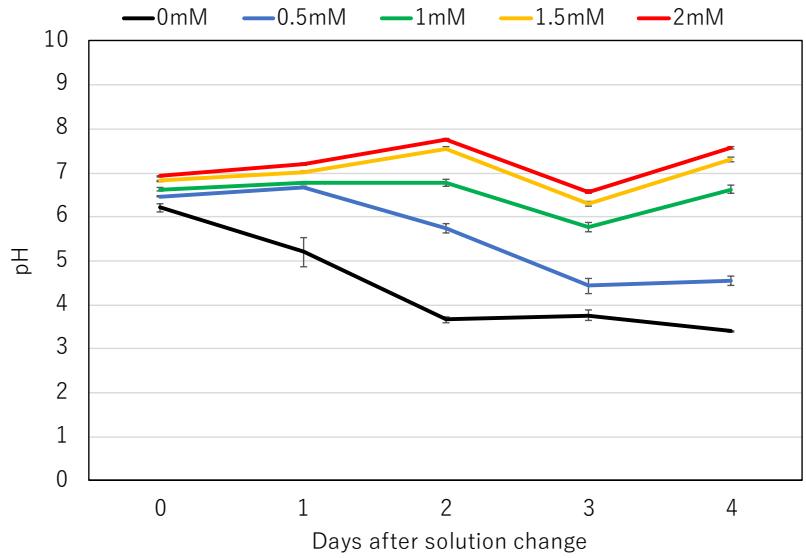

Figure 2. Effects of addition of 0, 0.5, 1.0, 1.5, and 2.0 mM $NaHCO_3$ on pH of the culture solution. Average ± standard error; 32 DAP plants were used.

3.2. Effects of the Additions of $NaHCO_3$ or $KHCO_3$ on Evapotranspiration and Plant Growth

From the results in Figures 1 and 2, it can be seen that the culture solution with 1–2 mM $NaHCO_3$ was able to maintain the pH of the culture solution to near a neutral level. However, there is a possibility that the addition of Na or HCO_3^- may be toxic to or have detrimental effects on soybean growth. Therefore, long-term cultivation from 19 to 37 DAP with 1 mM $NaHCO_3$ or $KHCO_3$ was conducted, and the effects of this on the evapotranspiration (Figure 3) and chlorophyll contents (Table 2) of each plant and the dry weight of each plant part (Figure 4) were compared with those of the original solution without bicarbonate.

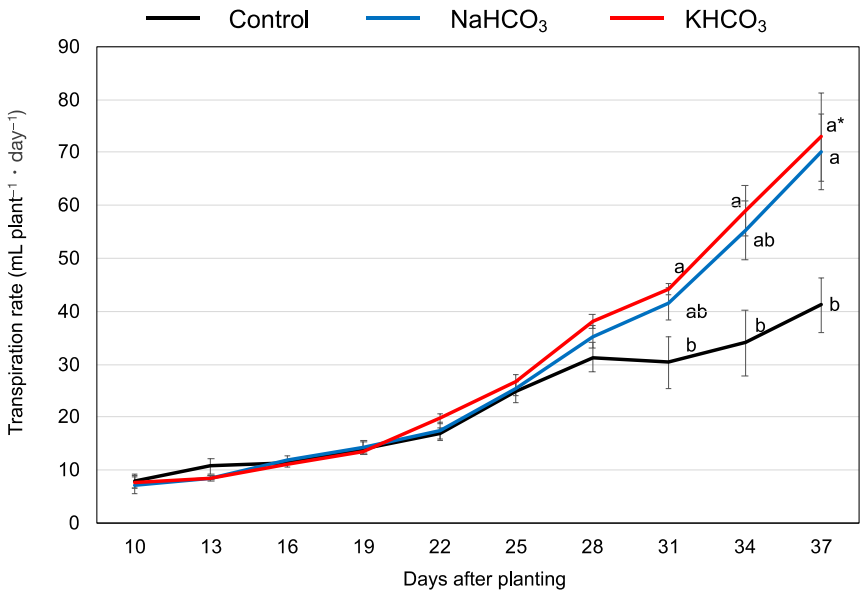

Figure 3. Effect of the addition of 1.0 mM $NaHCO_3$ or $KHCO_3$ at 19 DAP on the transpiration rate. Average ± standard error. Different letters indicate a significant difference in the values among treatments.

Table 2. Leaf color index (SPAD) values of each leaf of 37 DAP soybean treated with 1.0 mM $NaHCO_3$ or $KHCO_3$. Average ± standard error, n = 3, * significant error by Student's *t*-test compared to control treatment ($p < 0.05$).

	Control	**$NaHCO_3$**	**$KHCO_3$**
Primary Leaves	37.0 (1.3)	40.3 (1.4)	41.7 (0.7) *
1st Leaves	31.8 (5.6)	40.3 (1.0)	40.2 (0.8)
2nd Leaves	26.7 (6.5)	38.9 (0.4)	38.1 (0.9)
3rd Leaves	28.1 (8.0)	41.4 (1.0)	41.1 (1.0)
4th Leaves	28.7 (6.0)	37.5 (1.0)	36.5 (1.3)
5th Leaves	31.3 (3.0)	34.3 (1.3)	33.5 (1.8)
6th Leaves	31.5 (0.8)	31.0 (1.1)	30.3 (1.4)
7th Leaves	26.0 (1.5)	29.5 (1.1)	28.8 (0.9)

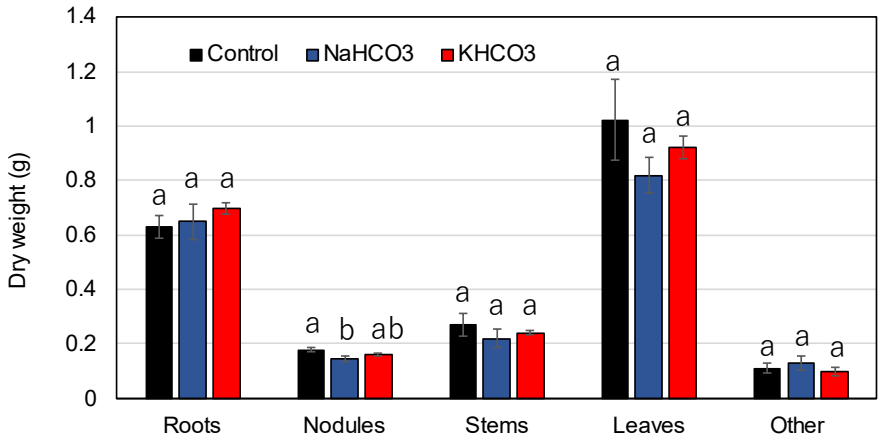

Figure 4. Effect of the addition of 1.0 mM $NaHCO_3$ or $KHCO_3$ on the dry weight of each part at 37 DAP. Average ± standard error. Different letters indicate a significant difference in the values among treatments.

The daily evapotranspiration rates became higher during cultivation with the bicarbonate treatments, possibly due to the growth of the roots and leaves. After the treatments imposed on 19 DAP, there was no significant difference among the treatments until 28 DAP. However, from 31 to 37 DAP, the evapotranspiration rates under the $NaHCO_3$ and $KHCO_3$ treatments were higher than those under the control without bicarbonate.

The dry weight of the roots, stems, leaves, and other parts did not significantly differ among the treatments, including the control. However, the nodule dry weight of the plants under the $NaHCO_3$ treatment was significantly lower than the control plants but not the $KHCO_3$ treatment plants. Although the root dry weight did not significantly differ among the treatments (Figure 4), the evapotranspiration rates were significantly higher under the 1.0 mM $NaHCO_3$ and $KHCO_3$ treatments compared to those under the control treatment. This might have been due to the low pH damage that occurred in the control roots. The color of the roots was white in the $NaHCO_3$- and $KHCO_3$-treated plants but was brownish in the control plants' roots.

The leaf color values of the primary leaves and the lower first to fifth leaves were higher under the $NaHCO_3$ and $KHCO_3$ treatments than those under the control treatment. Because all the plants depended only on N_2 fixation, the higher leaf color values achieved under the $NaHCO_3$ and $KHCO_3$ treatments might have been due to the higher N_2 fixation activities these treatments produced by stabilizing the medium pH.

Figure 5 shows the changes in the pH values of the solution with 1.0 mM $NaHCO_3$ or $KHCO_3$ compared with the control. The plants were cultivated using the above solution, and the changes in pH were monitored every day for 3 days from 12 to 15 DAP. Although young plants were used in this experiment, the pH of the control solution decreased from the initial pH of 6.2 to 5.0 by day 3. On the other hand, the initial pH of the solution with 1.0 mM $NaHCO_3$ or $KHCO_3$ was about 7.2 and tended to increase to 7.6 by day 3. The pH values did not differ between the $NaHCO_3$ and $KHCO_3$ treatments, indicating that buffering activities depend only on HCO_3 and not on cations.

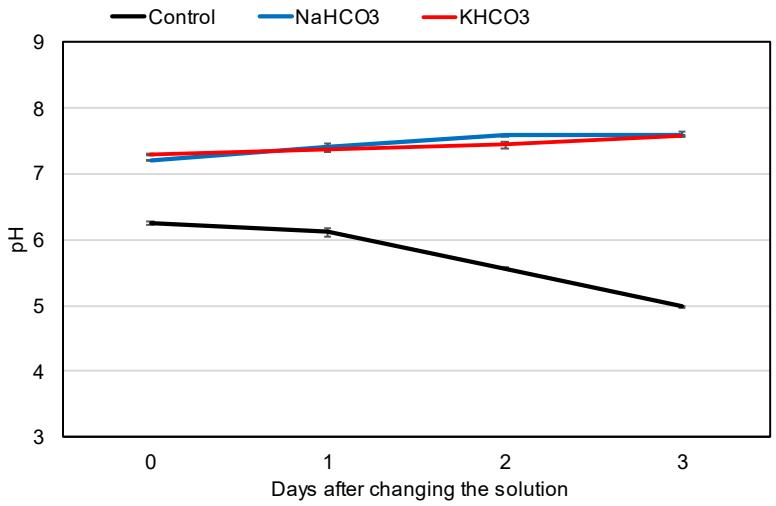

Figure 5. Effect of the addition of 1.0 mM $NaHCO_3$ or $KHCO_3$ on pH of the culture solution. Plants at 12–15 DAP were used. Average ± standard error.

The modified culture solution is shown in Table 1B. The 1.25 mM K (0.625 mM K_2SO_4) used in the original solution (Table 1A) was replaced by 1.25 mM $KHCO_3$. The soybean plants were cultivated with the modified solution, and the culture solution was changed at 23, 25, 27, and 29 DAP. The initial pH and the pH of the culture solution after cultivation were measured (Table 3). Further, the changes in the HCO_3^- concentration of the solution were also determined. The initial pH values ranged between 6.33 and 6.48, and the final pH values ranged between 6.15 and 6.35. The initial concentration of HCO_3^- ranged between 1.13 and 1.35 mM, and the final concentration decreased to a range between 0.87 and 0.97 mM but was not depleted during cultivation for 2 days.

Table 3. Changes in pH and HCO_3^- concentration before and after changing culture solution during soybean cultivation. Average (standard error); n = 4.

	pH		**HCO_3^- Concentration (mM)**	
DAP	before	after	before	after
21~23	6.38 (0.09)	6.45 (0.10)	1.19 (0.07)	0.97 (0.09)
23~25	6.35 (0.10)	6.35 (0.13)	1.18 (0.07)	0.91 (0.03)
25~27	6.33 (0.10)	6.18 (0.10)	1.35 (0.04)	0.87 (0.00)
27~29	6.48 (0.03)	6.15 (0.06)	1.13 (0.00)	0.71 (0.06)

3.3. Evaluation of the Appropriate P Concentrations in a New Culture Solution with $KHCO_3$ for Plant Growth and Evaluation of the Concentrations of Major Anions and Cations

The plants were cultivated with the modified culture solution (Table 1B) with 50 µM K_2HPO_4. At 33 DAP, the plants were subjected to different P conditions, namely P50, P100, P150, P200, and P400, for 14 days. Figure 6 shows photographs of shoots, roots, and nodules separated at harvest at 47 DAP after 14 days of P treatments. The root size was smaller in the plants under the P200 and P400 treatments than in those under the P50 and P100 treatments. Further, the leaf size was smaller in the plants under the P200 and P400 treatments than in those under the P50 and P100 treatments. The smaller leaves at the lower position showed a yellow color, so we classified these as damaged leaves.

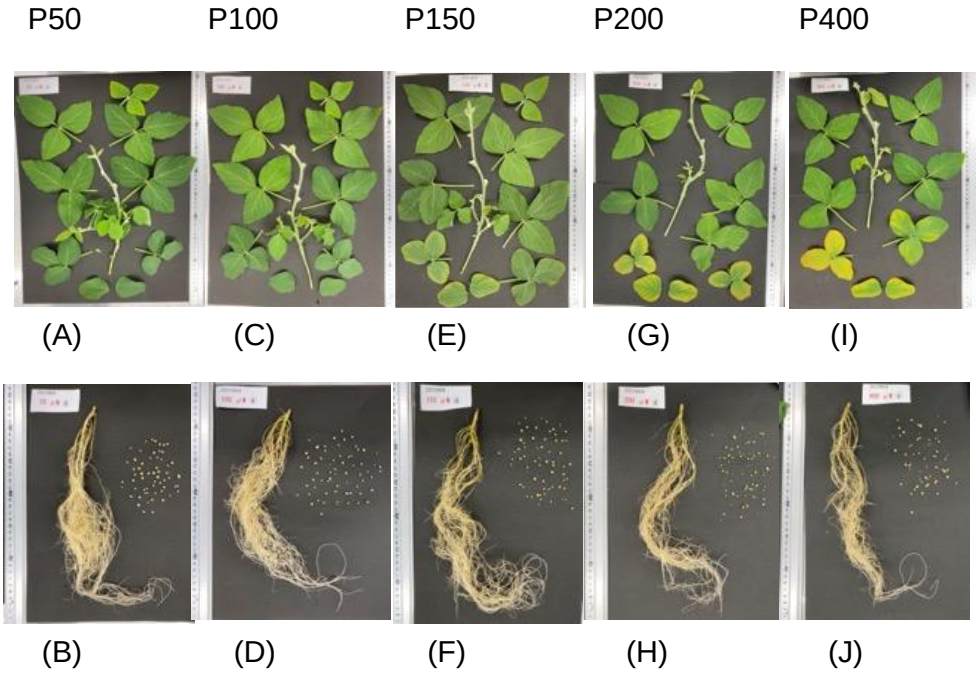

Figure 6. Photographs of soybean plants in the new culture solution with $KHCO_3$ harvested on 47 DAP. P concentrations of 50 (**A**,**B**), 100 (**C**,**D**), 150 (**E**,**F**), 200 (**G**,**H**), and 400 µM (**I**,**J**) were applied from 33 to 47 DAP.

The total plant dry weight decreased as the concentration of P in the solution increased; it was significantly higher in P50 and P100 than in P400 (Figure 7). The same was true for the roots, nodules, healthy leaves, and immature leaves. Damaged leaves appeared under the P150, P200, and P400 treatments. These results suggest that P50 and P100 were suitable concentrations for soybean growth under the experimental conditions, while P concentrations higher than 150 µM may cause harmful effects on the growth of roots, nodules, and leaves. Moreover, the leaf colors displayed excess symptoms above 150 µM K_2HPO_4.

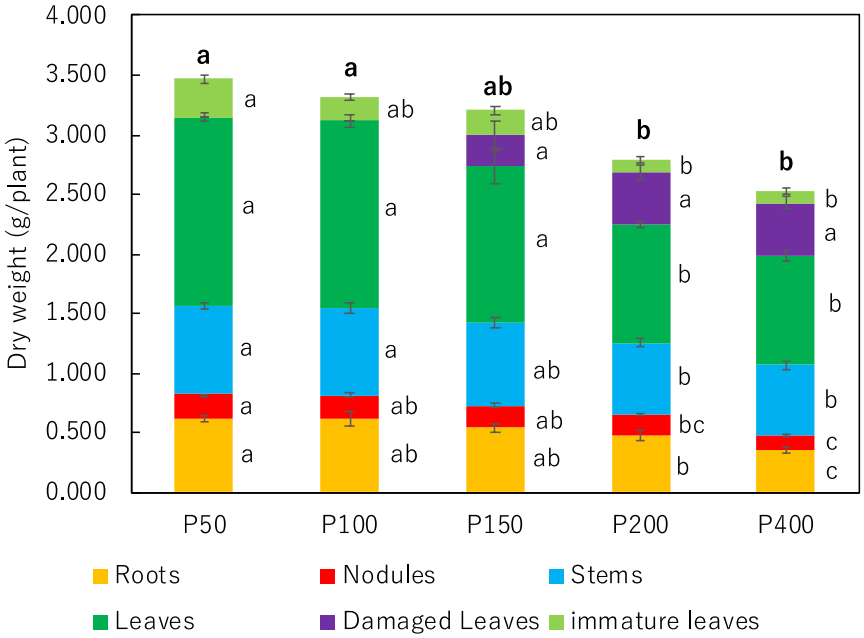

Figure 7. Effect of the concentrations of P on the dry weight of each part of soybean plants in the new culture solution with $KHCO_3$ on 47 DAP. P concentrations of 50, 100, 150, 200, and 400 μM were applied from 33 to 47 DAP. Average ± standard error. Different letters at the top of the colored bars indicate a significant difference in the total DW. Different letters beside the column indicate a significant difference in each organ.

Further, the concentrations of anions and cations in the 1% HCl extract of each organ were measured (Figure 8). Through 1% HCl extraction, most of the inorganic anions, namely PO_4, SO_4, and Cl, and the cations K, Ca, and Mg were found to be extractable. Organic P, such as P in the form of nucleic acids, phospholipids, etc., remained in the residue. When the P concentration in the medium was increased, the concentration of inorganic PO_4 became significantly higher in all the organs. The P concentration in the roots under the P200 and P400 treatments was about 10 times higher than that under the P50 treatment. Further, the concentration of P in the damaged leaves was about twice that of the healthy leaves under the P200 and P400 treatments. Conversely, the concentrations of SO_4 in the roots were significantly lower under the P400, P200 and P150 treatments than those under P50 and P100. Further, the concentrations of SO_4 in the nodules, stems, and healthy leaves did not differ among the various P treatments. The Cl concentrations in the roots and nodules decreased as the P concentration increased in the culture solution, but the Cl concentrations in the shoots were not affected by the various P treatments. Further, the concentrations of organic P in the residue did not significantly differ among the various P treatments. The average concentrations of the organic P in the plant parts were as follows: roots (1.12), nodules (1.52), stems (0.96), healthy leaves (1.19) and damaged leaves (1.66 mmole g^{-1}DW).

The concentrations of K, Ca, and Mg were not significantly affected by the P concentration used in the culture solution. On the other hand, the Ca concentration in the damaged leaves was about twofold higher than that in the healthy leaves ($p < 0.01$).

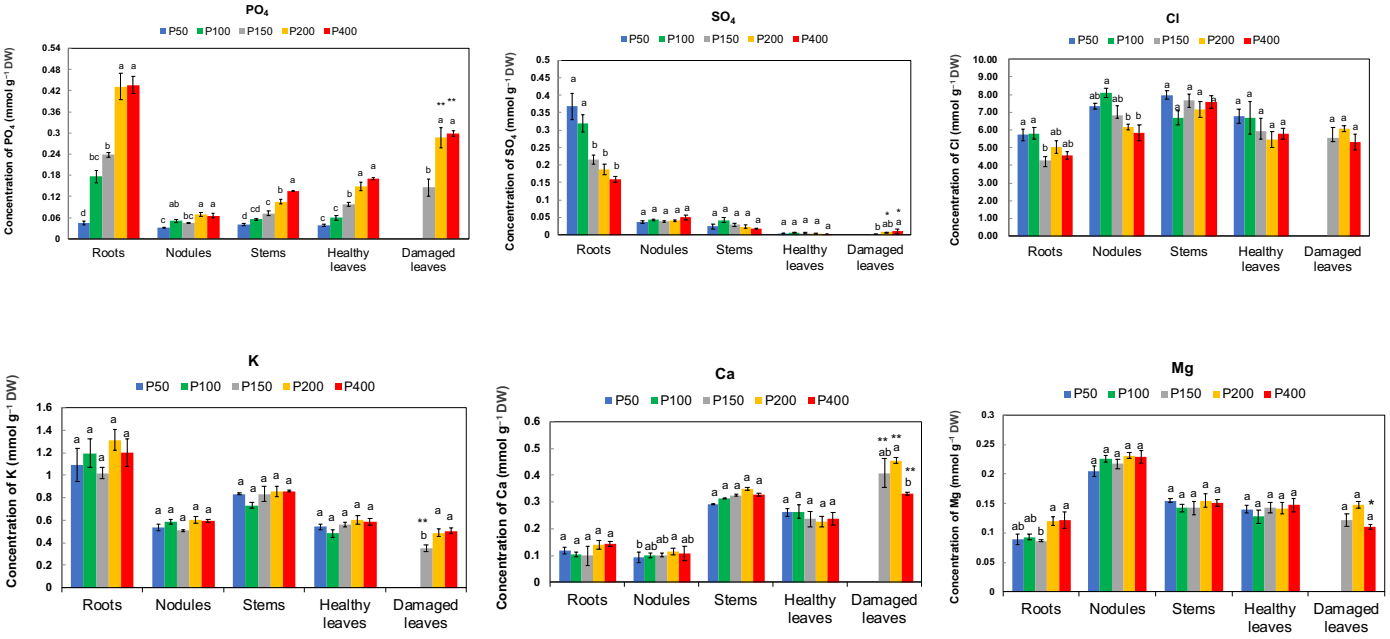

Figure 8. Effect of the concentrations of P on inorganic anions and cations of each part of soybean plants in the new culture solution with $KHCO_3$ on 47 DAP. Different P concentrations of 50, 100, 150, 200, and 400 μM were applied from 33 to 47 DAP. Average ± standard error. Different letters at the top of the colored bars indicate a significant difference by Tukey's test. * and ** on the column of damaged leaves indicate significant differences between healthy and damaged leaves at $0.05 > p > 0.01$ and $0.01 > p$, respectively.

4. Discussion

In hydroponics, a culture solution's pH is important for growing healthy plants. In this study, the addition of 1–2 mM $NaHCO_3$ to the original solution was effective in maintaining an adequate pH range during nodulated soybean cultivation. However, the use of $KHCO_3$ was better than $NaHCO_3$ for nodule growth (Figure 4), although the evapotranspiration rates (Figure 3) and chlorophyll concentrations (Table 2) were the same under the $KHCO_3$ and $NaHCO_3$ treatments. Therefore, we replaced the 0.625 mM K_2SO_4 in the original solution (Table 1A) with 1.25 mM $KHCO_3$ (Table 1B).

The decrease in pH observed during cultivation may have been primarily due to H^+ efflux from the roots [25]. H^+ efflux is known to decrease under low pH conditions. The pH decrease in the original solution stopped at around pH 3–4 in this experiment (Figures 1 and 2). It has been suggested that H^+ efflux may be related to an imbalance in the absorption of cations and anions [26,27]. In our study, the mechanism that stabilized pH by the addition of $KHCO_3$ may have operated through the neutralization of H^+ by bicarbonate (HCO_3^-), producing H_2CO_3 in the solution [28].

On the other hand, a high concentration of bicarbonate is harmful to soil and hydroponic cultures. Souri [29] reported that the use of calcium bicarbonate at high concentrations (5 mM) reduced plant growth, particularly root growth. In alkaline soils, especially in calcareous soil, high concentrations of $NaHCO_3$ or other bicarbonate salts accumulate, and plant cultivation is repressed by increased pH and an imbalance in the availabilities of nutrient elements, especially Fe [18]. In scientific studies, distilled water or deionized water is used to prepare culture solutions. However, when underground or river water is used for hydroponics in agricultural practice, the concentrations of bicarbonate that affect the solution pH need attention [30].

$KHCO_3$ can be used for hydroponics in not only scientific studies but also agricultural production. The main advantage of this method is its good buffering effect. Further, potassium bicarbonate is cheaper than other buffer reagents, such as MES. Additionally, $KHCO_3$ is safe for humans and the environment when discharged. Bicarbonate accounts

for 90% of the inorganic carbon in seawater [17], and it is related to pH buffering and the exclusion of CO_2 in animals [16]. Bicarbonate is a safe compound because it has been used as baking powder and in medicines such as antacids. Phosphate has pH-buffering capacity, but using a high concentration of phosphate in a culture solution is harmful for soybean growth [12,31].

In this study, the optimum concentrations of P in a modified culture solution were found to be 50 µM and 100 µM. P concentrations over 150 µM exhibited harmful effects on the roots, nodules, and leaf growth, with the lower leaves showing yellowing damage. The concentrations of organic P in the residue did not significantly differ among the various P treatments, suggesting that P deficiency did not occur at the level of 50 µM P at this stage. Foote and Howell [32] investigated the P tolerance and sensitivity of soybean varieties, and Lincoln plants exhibited decreased growth caused by P toxicity at P levels over 0.72 mM. Burnett et al. [33] studied the effects of P concentrations on hydroponically grown Scaevola plants, and the root and shoot dry mass, leaf area, and number of flowers of the plants reached their maximum values under the lowest level of P (0.65 mM) and decreased under higher P levels. Gremaud and Harper [21] used a culture solution with 100 µM P.

Phosphate availability is often limited in soil, and due to its low mobility, little information is available regarding the effect of excess P on plants [34]. In this study, as seen in Figure 8, the PO_4^{3-} concentration in the roots increased as the concentration of P increased in the culture solution, while SO_4^{2-} and Cl^- decreased. The high concentration of PO_4^{3-} itself, or the decrease in SO_4^{2-} or Cl^-, may have caused the growth retardation and leaf damage. Another possibility is that excess PO_4^{3-} may have decreased the soluble Ca concentration in the cells. The total cations, including Ca, were not affected by the P concentrations in the culture solution, while the Ca concentrations in the cytosol may have decreased under toxic P levels. Shukla et al. [34] reported that an excess P response is related to a modulation in ethylene biosynthesis and signaling, the metal ion deficiency response, and root development gene expressions.

5. Conclusions

In this study, a culture solution was modified by adding 1.25 mM $KHCO_3$ to an N-free solution in order to stabilize the pH of the culture solution for the cultivation of nodulated soybean plants. The soybean plants cultivated with the new solution exhibited healthy white roots, and their evapotranspiration rate was higher than that of the control plants without bicarbonate. This study's use of appropriate concentrations of bicarbonate is applicable for agricultural practices that use N in a given medium, although the concentrations and formulae used should be evaluated for different methods and crops. Bicarbonate ion is a major inorganic carbon and is used for pH-stat in animals. Therefore, the use and discharge of bicarbonate will not lead to harmful effects on human health and the environment. This study found that the optimum P concentrations for a modified culture solution were 50 and 100 µM, while the concentrations over 150 µM reduced the plant growth and led to lower leaf damage.

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