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PRODUCTION OF SOYBEAN PLANTS FOR HYDROPONIC CULTIVATION FROM SEEDLING CUTTINGS IN A MEDIUM CONTAINING *RHIZOBIUM* INOCULUM DEPENDING ON VARIOUS CONCENTRATIONS OF NUTRIENT SOLUTION AND DIFFERENT NITROGEN SOURCES

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ABSTRACT

There has been an increasing interest in soybean cultivation in hydroponic systems. Because soybean plants can utilize nitrogen derived from biological N_2 fixation, the use of *Rhizobium* inoculated plants may eliminate or decrease the need for mineral nitrogen fertilization in hydroponic systems. Thus, the objective of this study was to establish effective methods for making inoculated soybean transplants for a substrate-based hydroponic system. Inoculated plants were effectively produced by rooting seedling stem cuttings in a medium containing a *Rhizobium* inoculant. We also investigated the effects of different nitrogen forms and subirrigated nutrient solution concentrations on the growth and root nodule formation of the inoculated plants. The application of a nitrogen-free nutrient solution had minimal positive effects on the growth and nodulation of the inoculated plants. Ammonium-fed plants lacked root nodules regardless of the ammonium concentration. Furthermore, a 50% nutrient solution combining with nitrate or urea as the nitrogen source did not promote root nodulation. Therefore, inoculated plants should be subirrigated with an approximately 25% nutrient solution supplemented with nitrate or urea to induce early growth and nodulation prior to transplantation.

Key words: ammonium, nitrate, urea, subirrigation, substrate hydroponic system

INTRODUCTION

Hydroponic cultivation enables growers to control environmental conditions, while also decreasing the amount of irrigation water required for plant growth, increasing productivity, and preventing infections (Palermo et al. 2012). Soybean (*Glycine max* (L.) Merr.) is traditionally grown as an open-field crop and hydroponically grown soybean plants have mainly been used to examine physiological characteristics rather than for commercial production (Paradiso et al. 2014a). However, space-oriented experiments have evaluated the adaptation of soybean plants to hydroponic systems under controlled environments and plant responses to changing climatic and cultural parameters for identifying the optimal cultivation protocol for food production in bio-regenerative life support systems. In Japan, there has recently been an increasing interest in applying hydroponic culture systems for growing soybean in the greenhouse, especially for the production of vegetable soybean (edamame), which involves harvesting immature seeds.

When grown in soil, some of the nitrogen taken up by soybean plants is derived from the symbiotic fixation of atmospheric N_2 by *Rhizobium* bacterial species in plant root nodules (Cooper & Scherer 2012; Ciampitti & Salvagiotti 2018). Because this N_2 fixation may provide plants with sufficient quantities of nitrogen, some studies have focused on the hydroponic cultivation of plants inoculated with *Rhizobium* species to avoid or decrease the need for mineral nitrogen fertilization (Shoji et al. 1991; Paradiso et al. 2014b, 2015; Kontopoulou et al. 2015). Paradiso et al. (2014b) reported that compared with nutrient film technique (NFT), soybean cultivation on rockwool positively influences root nodulation as well as plant growth and yield, without affecting the seed proximate composition. In such a substrate-based hydroponic system, the inoculation of transplants

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may be superior to the inoculation of the culture bed regarding the technology required and cost. Therefore, an effective method for inoculating plants grown in a substrate-based hydroponic system needs to be developed, but there is currently insufficient information to do so.

Soybean seeds are prone to imbibition injuries, which refer to the physical disruption of seed tissue caused by a too-rapid uptake of water, resulting in poor and unstable seedling emergence (Nakayama & Komatsu 2008; Sato et al. 2019). Because soybean seeds are very large and susceptible to imbibition injuries, sowing them directly in hydroponic substrates (e.g., rockwool cubes) is difficult. Therefore, we proposed that seeds should be sown and germinated in nursery soil without excessive amounts of water for the subsequent transplantation of healthy plants to hydroponic system.

Transplanting seedlings directly from nursery soil into hydroponic substrates or beds may be possible but there is a risk of contamination by various microorganisms, even if the roots are washed carefully to remove the attached soil. Additionally, washing roots is also a time-consuming and labor-intensive process. Therefore, one can assume that transplants should be raised from seedling stem cuttings that only requires planting the cut stem in the hydroponic substrates, as in the hydroponic production of rose. This process saves labor because of the lack of a root-washing step and results in the production of less contaminated transplants.

Accordingly, we hypothesized that inoculated soybean plants can be effectively produced via the rooting of seedling stem cuttings in a medium containing a *Rhizobium* inoculant. An investigation of the effects of various environmental factors on root growth and nodulation revealed that clean silica sand should be used as the rooting medium instead of rockwool cubes because it can be easily eliminated from the root system as well as it is characterized by very low nutrient levels. Furthermore, there have been many investigations on the relationship between nitrogen fertilization and nodulation (Dogra & Dudeja 1993; Bhangu & Virk 2019). Therefore, in this study, we examined the effects of different nitrogen forms and nutrient solution concentrations on the growth and root nodule formation of soybean rooted cuttings planted in silica sand containing a *Rhizobium* inoculant.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of soybean cultivar Tambaguro were sown in 30 × 40 cm plastic trays filled with commercially available nursery soil (Type S; Yanmar, Japan). The trays were then covered with wet newspapers until the seedlings emerged. All the trays were placed in an incubator set at 25 °C, in darkness. After emergence (3 days after sowing), seedlings were transferred to a greenhouse at the Experimental Agricultural Facility of the University of Shiga Prefecture. At the primary leaf stage (8 days after sowing), uniformly growing seedlings were selected from the trays and their hypocotyls were cut 4 cm below the cotyledonary node.

The hypocotyledonous stems of the cuttings were individually inserted into polyvinyl chloride (PVC) tubes (45 mm tall and 56 mm internal diameter), containing 100 ml silica sand (No. 4F; Toyo Matelan, Japan) mixed with 200 mg commercial *Rhizobium* inoculant, which contained rhizobia mixed with peat (Mamezo; Tokachi Federation of Agricultural Cooperative Association, Japan). The bottom of each PVC tube was covered with root-resistant and water-permeable sheet (BKS9812; Toyobo, Japan) to enable capillary watering without collapsing the rooting medium (silica sand with a *Rhizobium* inoculant). Ten PVC tubes with cuttings were placed on one tray for subirrigation from the bottom side of each PVC tube with a capped 2 dm^3 plastic bottle that refilled with a fresh solution as necessary. The plastic bottle had a small hole (8 × 8 mm) at the base about 2 mm from the bottom to maintain a relatively constant water level of about 1 cm depth in the tray (Fig. 1). Plants were subirrigated with tap water for the first week and then with the respective nutrient solutions for another 2 weeks.

Experiment 1. Applicability of a nitrogen-free nutrient solution

Seeds were sown as described above on May 27, 2016, and seedling stem cuttings were inserted into the rooting medium on June 4, 2016. Plants were cultivated for 3 weeks under natural sunlight without shading in a greenhouse, and irrigations with the nutrient solution were initiated after the first week. The treatments consisted of a nitrogen-free Enshi nutrient solution diluted to six concentrations (0, 10, 25, 50, 75, and 100% of full-strength).

The full-strength complete Enshi nutrient solution contained the following levels of salts per 1,000 dm^3 tap water: 950 g $Ca(NO_3)_2 \cdot 4 H_2O$, 810 g KNO_3, 500 g $MgSO_4 \cdot 7 H_2O$, 155 g $NH_4H_2PO_4$, 3 g H_3BO_3, 2 g $ZnSO_4 \cdot 7 H_2O$, 2 g $MnSO_4 \cdot 4 H_2O$, 0.05 g $CuSO_4 \cdot 5 H_2O$, 0.02 g Na_2MoO_4, and 25 g NaFe-EDTA. In the nitrogen-free solution, $Ca(NO_3)_2 \cdot 4 H_2O$, KNO_3, and $NH_4H_2PO_4$ were substituted with $CaCl_2 \cdot 2 H_2O$, K_2SO_4, and KH_2PO_4. Each solution was prepared by mixing the full-strength solution and tap water (the 0% solution consisted of only tap water). As normal growth control, additional seedling stem cuttings grown in rooting medium without the *Rhizobium* inoculant were similarly irrigated with half-strength complete Enshi nutrient solution. All the nutrient solutions were adjusted to pH 6.0 with H_2SO_4 before use.

Experiment 2. Applicability of a nitrogen-containing nutrient solution

The sowing and cutting dates were June 20 and 28, 2016, respectively. The cultural management of the cuttings was the same as in Experiment 1, except nitrogen-containing nutrient solutions were used (Table 1). Plants were irrigated with Enshi nutrient solutions at four concentrations (0, 10, 25, and 50% of full-strength), with nitrogen source as nitrate (NO_3-N), ammonium (NH_4-N), or urea (Urea-N). Half-strength nutrient solutions were prepared for each nitrogen source and diluted with tap water. Similar to Experiment 1, uninoculated plants cultivated with half-strength complete Enshi nutrient solution were served as normal growth control. All the nutrient solutions were adjusted to pH 6.0 with H_2SO_4 before use.

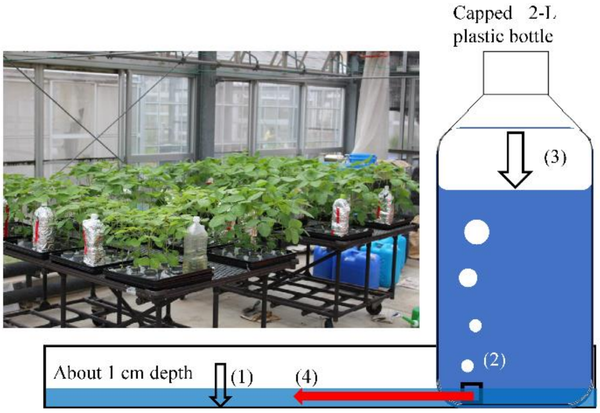

Fig. 1. Overview of a subirrigation system. If the water level in the tray decreases because water is absorbed by plants (1), air enters the plastic bottle through a small hole (2), after which the water level in the bottle decreases as the liquid exits (3), resulting in an increase in the water level in the tray for a depth of about 1 cm (4). After the water level in the tray is restored, air stops entering the bottle and the water level in the bottle stops decreasing

Table 1. Composition of standard and modified Enshi nutrient solution with different nitrogen form at a constant total nitrogen of 17.3 mM

Enshi nutrient solution	Chemical composition (mM)								
	$Ca(NO_3)_2$	KNO_3	$NaNO_3$	NH_4Cl	$NH_4H_2PO_4$	KH_2PO_4	K_2SO_4	$CaCl_2$	$MgSO_4$
Standard	8	8	0	0	1.3	0	0	0	2
Modified									
NO_3-N 100%	8	6.7	2.6	0	0	1.3	0	0	2
NH_4-N 100%	0	0	0	16	1.3	0	4	2	2
Urea-N 100%	0	0	0	0	0	1.3	3.35	4	2

Enshi nutrient solution	Nutrient composition (mM)									
	NH_4^+	NO_3^-	Urea-N	PO_4^{3-}	K^+	Ca^{2+}	Mg^{2+}	Na^+	SO_4^{2-}	Cl^-
Standard	1.3	16.0	0	1.3	8	4	2	0	2	0
Modified										
NO_3-N 100%	0	17.3	0	1.3	8	4	2	2.6	2	0
NH_4-N 100%	17.3	0	0	1.3	8	4	2	0	6	20
Urea-N 100%	0	0	17.3	1.3	8	4	2	0	5.35	8

Data collection and analysis

At 3 weeks after cutting, the following parameters were measured for 10 individuals per treatment: main stem length, number of trifoliate leaves, green leaf color intensity and number of nodules per root system. The green leaf color intensity of fully expanded second trifoliate leaves was measured with the SPAD-502 chlorophyll meter (Minolta Camera, Japan). After removing the rooting medium, the number of nodules ≥ 2 mm in diameter was determined. Roots and shoot were separated and dried in an oven at 60 °C, after which the constant dry weight was recorded. Differences in the values of the samples (n = 10) were analyzed with the Tukey-Kramer multiple comparison test following an ANOVA.

RESULTS

Experiment 1. Applicability of a nitrogen-free nutrient solution

The rate of adventitious rooting of cuttings was as high as 100% without any auxin treatment. In all the rooting media with an inoculant, nodulated adventitious roots arose from the base of the cuttings (Fig. 2). Nodules on the adventitious roots were mainly distributed at the neighboring stem base, although some formed on the adventitious roots apart from the stem or the surface of the rooting medium.

The growth of the rooted cuttings varied, but the highest values for the recorded shoot parameters were obtained for the normal growth control samples (Fig. 3 & 4A–D). The main stem length, number of trifoliate leaves, green leaf color intensity and shoot dry weight were significantly greater for the uninoculated plants supplied with nitrogen than for the inoculated plants not supplied with nitrogen. Regarding the nitrogen-free treatments, there were only small differences in the main stem length and number of trifoliate leaves among the treatments. The lowest and highest shoot dry weights resulted from the treatments with 0% and 25% dilutions, respectively, whereas they were not significantly different among plants of the other dilutions. However, the green leaf color intensity decreased substantially with increasing nutrient solution concentrations. Leaves turned yellow from the base towards the top of the plants, which was reflected in the green leaf color intensity.

In contrast to shoot growth, root growth was not significantly promoted by the application of the half-strength complete Enshi nutrient solution (Fig. 4E). Inoculated plants treated with the nitrogen-free nutrient solution, except the 0% dilution (i.e., tap water), tended to develop more adventitious roots than the uninoculated control plants, with the highest root dry weight for 25% dilution. Because semi-aseptic culture conditions were provided with the combination of silica sand and cuttings, uninoculated control plants never formed root nodules (Fig. 4F). When inoculated, the number of nodules per root system was higher for the 0% and 10% dilutions, and decreased with increasing nutrient solution concentrations.

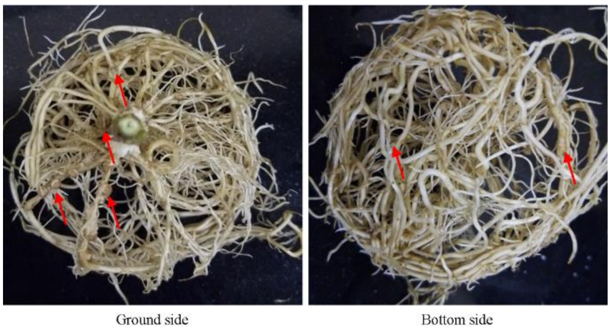

Fig. 2. Spatial distribution of adventitious roots and root nodules on an inoculated soybean plant at 3 weeks after cutting. Some of the nodules are indicated with red arrows

Fig. 3. Differences in the shoots and roots of the inoculated soybean plants treated with various concentrations of the nitrogen-free nutrient solution. Plants were photographed at 3 weeks after cutting. The control plants at both ends were irrigated with either tap water (0%) or the complete nutrient solution without a *Rhizobium* inoculant (Cont.)

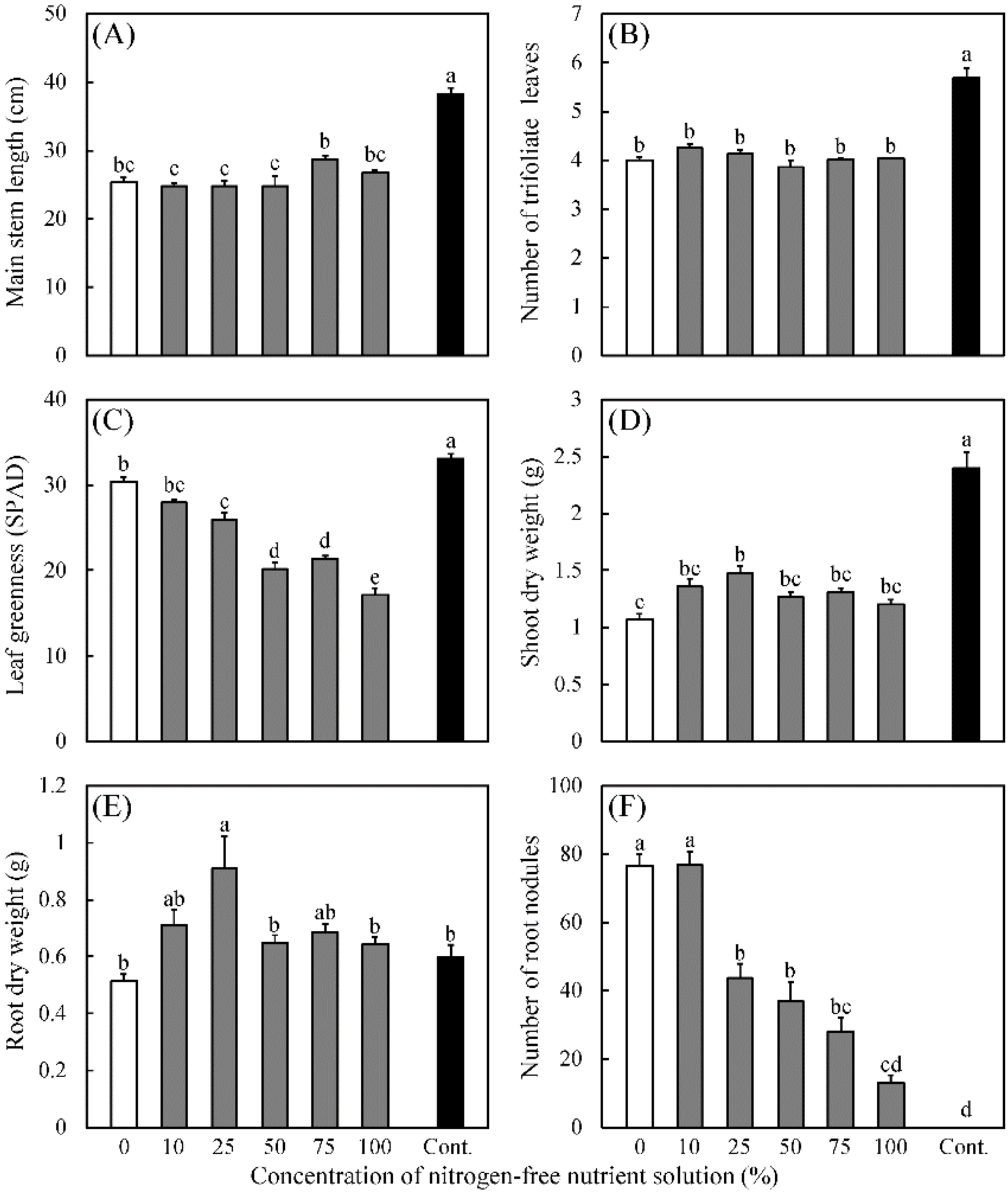

Fig. 4. Effects of the various nitrogen-free nutrient solution concentrations on the main stem length (A), number of trifoliate leaves (B), green leaf color intensity (C), shoot dry weight (D), root dry weight (E), and number of root nodules (F) of the inoculated soybean plants at 3 weeks after cutting. Values represent means ± S.E. (n = 10). Different letters above bars represent significant differences as determined with Tukey-Kramer's multiple range test ($p < 0.05$)

Experiment 2. Applicability of a nitrogen-containing nutrient solution

The nutrient solution had a considerable effect on plant growth in a concentration-dependent manner (Fig. 5). Specifically, the nutrient solution promoted growth when nitrate or urea was used as the nitrogen source. The ammonium fertilization inhibited shoot and root growth compared with the effects of the nitrate, urea and tap water treatments. The leaves of the ammonium-fed plants became chlorotic and/or necrotic, which was more pronounced in the upper plant parts. The severity of these symptoms increased with increasing nutrient concentrations.

The composition of the nutrient solution applied to the normal growth control was almost the same as that of the 50% dilution with nitrate as the sole nitrogen source, with the exception that the former contained a small amount of ammonium. Therefore, the shoot growth parameters were very similar for these two treatments, regardless of whether an inoculant was included in the rooting medium (Fig. 6A–D). The main stem length, number of trifoliate leaves, and shoot dry weight tended to increase with increasing concentrations from 0% to 50% for the plants treated with nitrate, whereas the stem length peaked with 25% dilution. These tendencies were also observed for the plants treated with urea, but there were no significant differences in the number of trifoliate leaves and shoot dry weight for the concentrations between 10% and 50%. For both the nitrate and urea treatments, the leaves from plants with the 0–25% dilutions were significantly less green than the leaves from the control plants, but the green leaf color intensity was the same for the plants treated with 50% dilution and the control plants at 5% significance level. At the same concentration, the shoot dry weight of the urea-fed plants was lower than that of the nitrate-fed plants, especially at higher concentrations. Regarding the ammonium treatment, there were no significant increases in the main stem length and number of trifoliate leaves for dilutions up to 50%. Furthermore, ammonium levels exceeding 25% significantly decreased the green leaf color intensity and shoot dry weight, with values lower than those of the plants treated with tap water.

The root dry weights of the plants exposed to the no-nutrient treatment and the control plants were not significantly different (Fig. 6E). The positive effects of nitrate were observed for concentrations up to 25%, with a maximum weight of 0.81 g, which was approximately 28.5% higher than that resulting from the 0% treatment. The urea fertilization did not promote root growth, and the root dry weight was lower for the urea-fed plants than for the nitrate-fed plants at all concentrations. Both the nitrate- and urea-treated plants yielded more root dry matter than the ammonium-treated plants. Increases in the ammonium concentration of the nutrient solution resulted in a linear decrease in the root dry weight.

The number of root nodules on the adventitious roots was highest for the plants treated with the 10% nitrate-based solution, followed by the plants that underwent the 25% nitrate, 10% urea, and 25% urea treatments (Fig. 6F). Fewer root nodules were detected following the control (i.e., tap water) and 50% nitrate treatments. The 50% urea fertilization significantly inhibited root nodule formation. In addition to the uninoculated control plants, all ammonium-fed plants lacked root nodules, although they were inoculated similarly to the plants that underwent the nitrate and urea treatments.

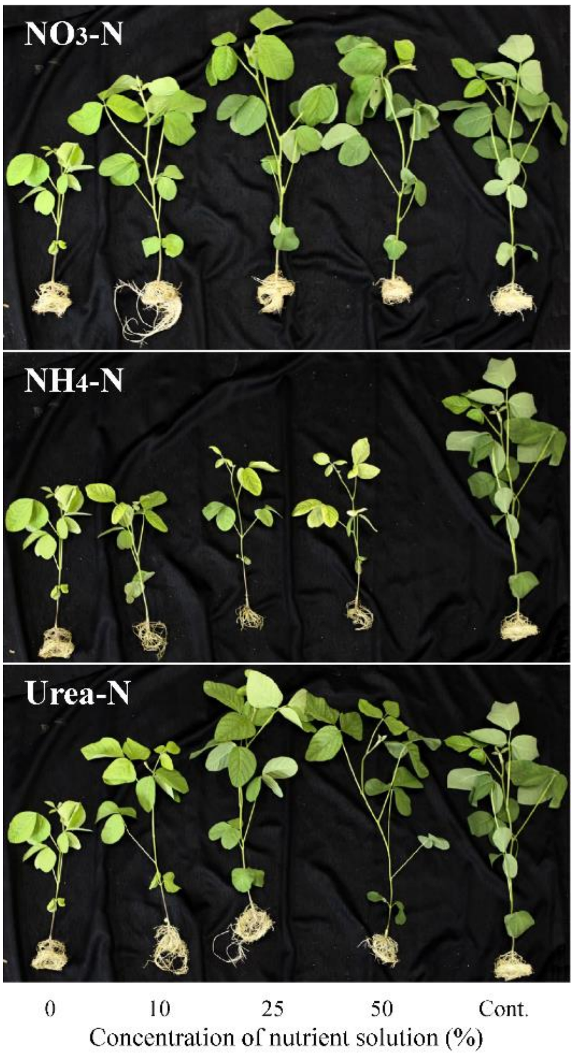

Fig. 5. Differences in the shoots and roots of inoculated soybean plants treated with various nitrogen forms and nutrient solution concentrations. Plants were photographed at 3 weeks after cutting. For the three images presenting the effects of different nitrogen forms, the control plants at both ends were same and irrigated with tap water (0%) or the complete nutrient solution without a *Rhizobium* inoculant (Cont.)

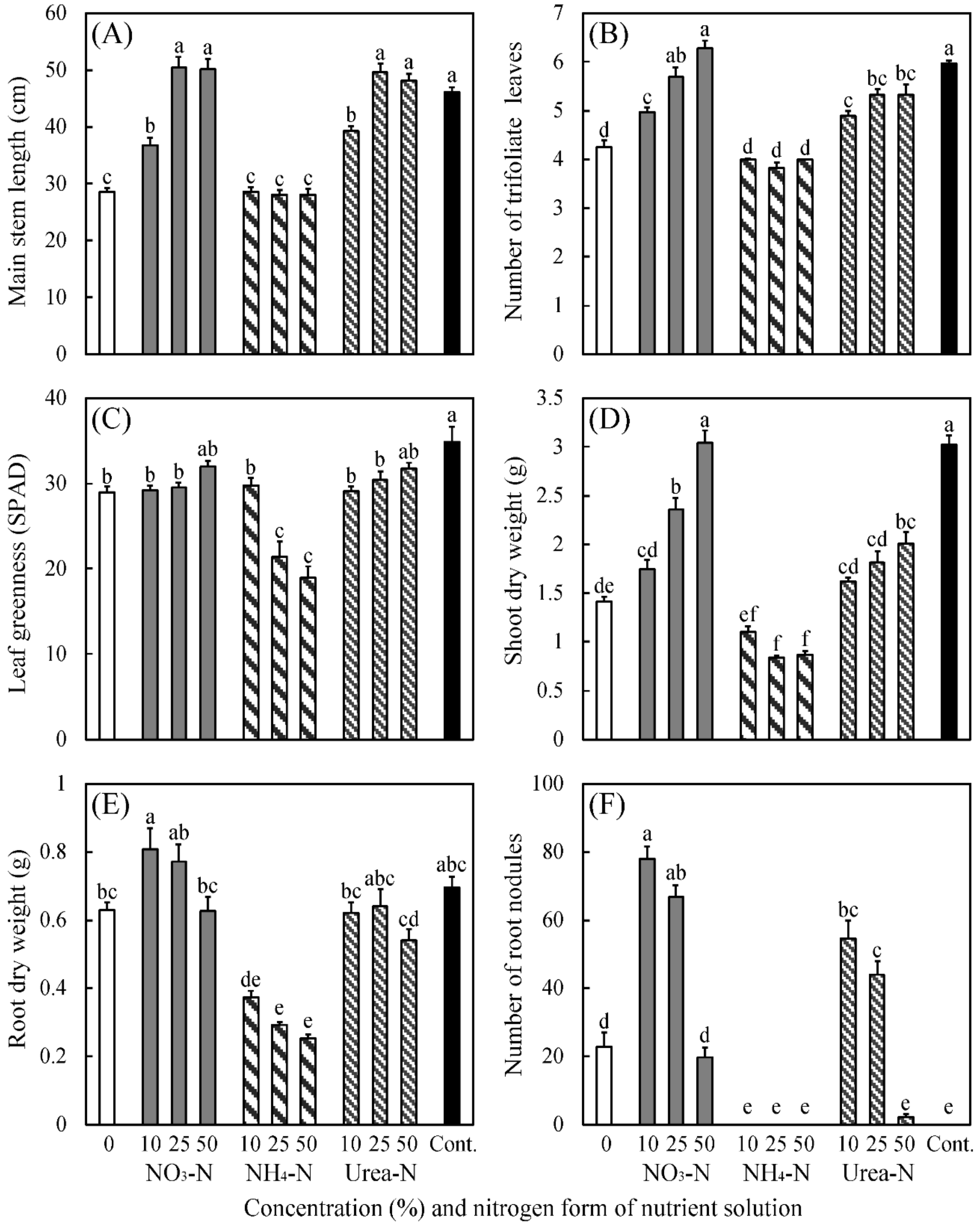

Fig. 6. Effects of the nitrogen form and nutrient solution concentration on the main stem length (A), number of trifoliate leaves (B), green leaf color intensity (C), shoot dry weight (D), root dry weight (E), and number of root nodules (F) of the inoculated soybean plants at 3 weeks after cutting. Values represent means ± S.E. (n = 10). Different letters above bars represent significant differences as determined with Tukey-Kramer's multiple range test ($p < 0.05$)

DISCUSSION

Subirrigation system for rooting and inoculation

The culture vessel used in this study, consisting of a PVC tube and a root-resistant and water-permeable sheet, has been used for growing tomato (Suzuki et al. 2011) and melon (Kawahara & Masuda 2012) seedlings in a subirrigation system. The results of the current study indicate that such a system can also be used to produce soybean transplants from seedling stem cuttings. Adventitious root formation was detected on all the cuttings within 7 days of cutting without any auxin treatment, suggesting that the adventitious rooting of soybean cuttings is promoted by large amounts of storage compounds in the cotyledons and not inhibited by the moisture status of silica sand under subirrigation system with a 1 cm depth.

The *Rhizobium* biofertilizer ‘Mamezo’ is usually applied as a pre-sowing seed dressing, but this study indicated that it can be incorporated in the rooting media under subirrigation as an inoculant. In a previous study on soybean plants under flooding conditions, the adventitious roots represented approximately 90% of the total root length, with root nodules on the adventitious roots, but not on the primary and lateral roots (Hattori et al. 2013). Accordingly, inoculating the adventitious roots of seedling stem cuttings may be better than inoculating seedling roots. Moreover, nodulated roots were not detected on uninoculated plants, implying that the silica sand used in this study, which was manufactured by crushing rocks, was nearly sterile, including rhizobial bacteria. Such conditions might provide *Rhizobium* species with an ecological niche suitable for root nodulation.

On the basis of our findings, we concluded that the subirrigation system may be used for the rooting and inoculation of soybean cuttings to produce transplants in a substrate-based hydroponic system.

Necessity of nitrogen applications

According to Saito et al. (2014), the application of combined nitrogen, especially nitrate, inhibits the root nodule formation, growth, and nitrogen fixation activity of soybean plants. Therefore, we assumed that a nitrogen-free nutrient solution can provide other essential minerals without inhibiting nodulation, which can help induce the growth of inoculated plants that obtain nitrogen generated by symbiotic nitrogen fixation. However, we unexpectedly observed that the green leaf color intensity and the number of nodules per root system decreased with increasing concentrations of the nitrogen-free nutrient solution, implying the nitrogen-free nutrient solution did not promote the growth of inoculated plants (Fig. 3 & 4).

Soybean seeds are large and the storage compounds in the cotyledons can provide the nutrients required for the initial root and shoot growth (Ohyama et al. 2017). The relatively large amount of the storage compounds can partly explain why inoculated plants subirrigated with tap water were able to grow without any symptoms associated with a deficiency in essential minerals, including nitrogen, for 3 weeks (Fig. 3). However, when treated with a nitrogen-free nutrient solution at a concentration of 25% or more, the yellowing of leaves increased from the base to the top of the plants, which exhibited symptoms typically associated with nitrogen deficiency. These results suggest that the internal ratio of nitrogen to other essential minerals in plants following a nitrogen-free nutrient solution treatment might be lower than that resulting from tap water treatment. This decreased ratio may trigger stress responses to nitrogen deficiency.

The decrease in the number of root nodules was correlated with the decrease in the green leaf color intensity (Fig. 4). The yellowing of leaves indicates a loss of chlorophylls, which leads to a decrease in photosynthetic ability. Because nodule formation and activity require a considerable abundance of photosynthetic products provided by the host plant (Finn & Brun 1982), the number of root nodules and growth did not increase for the inoculated plants treated with high nitrogen-free nutrient solution concentrations. To avoid growth inhibition due to leaf yellowing accompanied by decreased nodule formation and activity, we propose that *Rhizobium*-inoculated plants should be subirrigated with a nutrient solution containing sufficient nitrogen to maintain an appropriate internal nitrogen ratio and to promote early growth up to the level of uninoculated control plants.

Optimal form and concentration of nitrogen

As expected, the green leaf color intensity of inoculated plants did not decrease with increasing nutrient solution concentrations when nitrogen was supplied as nitrate (Fig. 5 & 6C). Furthermore, urea also enhanced the green leaf color intensity. In both nitrate- and urea-fed plants, the internal nitrogen status, reflected by leaf greenness, may be enough to maintain photosynthetic ability. However, the chlorotic and/or necrotic lesions of the upper leaves of ammonium-fed plants increased in severity with increasing nutrient solution concentrations, likely because of ammonium toxicity (Tadano & Tanaka 1976; Ikeda & Osawa 1979; Yoneyama et al. 1985) rather than nitrogen deficiency. Although Xia et al. (2017) reported that the growth of soybean seedlings in a sand substrate was promoted by irrigation with a nutrient solution containing up to 100 ppm NH_4-N (7.14 mM), our results suggest that silica sand is nearly sterile, with minimal nitrifying bacteria that convert ammonium to nitrate, and the excess of ammonium remaining in the rhizosphere prior to absorption might result in ammonium toxicity, even when plants are subirrigated with a 25% treatment solution (4.33 mM NH_4-N).

Because the number of root nodules following the tap water treatment may have been underestimated more in Experiment 2 than in Experiment 1, it is unclear whether 10–25% nitrate fertilization will lead to more root nodules than the tap water treatment. However, our data indicate that nutrient solution concentrations up to 25% (4.33 mM NO_3-N) do not adversely influence nodulation (Fig. 6F). Although nitrate has long been known to strongly inhibit nodulation and the N_2 fixation activity in legumes, low concentrations of nitrate (1–2 mM) actually promote nodulation by ensuring early, rapid growth of the plant and the development of a healthy root system able to nodulate profusely (Giller & Wilson 1991; Ohyama et al. 2011). Furthermore, Xia et al. (2017) reported that the nodulation and N_2 fixation activity of soybean plants initially increase and then decrease with increasing nitrate concentrations, with the activity peaking at 50 ppm nitrate (3.57 mM). These results imply that the nitrate concentration can be increased to approximately 4 mM to promote the early plant growth and nodulation of inoculated soybean plants under subirrigation conditions. A treatment with 5 mM nitrate reportedly inhibits the growth and nitrogen fixation activity of soybean nodules by decreasing the amount of photosynthetic products supplied to the nodules (Ohyama et al. 2011; Saito et al. 2014). Thus, it is reasonable that the number of root nodules decreased when plants were treated with the 50% dilution (8.65 mM NO_3-N). Accordingly, nitrate concentrations exceeding 5 mM are unsuitable for cultivating inoculated soybean plants, even if they promote shoot growth (Fig. 6A–D).

According to Yoneyama et al. (1985) and Ohyama et al. (2013), the inhibitory effect of nitrate is stronger than that of ammonium or urea for the nodulation and nitrogen fixation activity of soybean plants. However, to our surprise, ammonium-fed plants never formed root nodules irrespective of the ammonium concentration (Fig. 6F), although soybean seedlings grown in a sand substrate can form many nodules, even when irrigated with 100 ppm NH_4-N (7.14 mM) (Xia et al. 2017). The excess ammonium in the rhizosphere due to the limited nitrification in semi-aseptic silica sand might have contributed to the plant injury observed as decrease in plant biomass (Fig. 6A–D) in addition to leaf chlorosis and/or necrosis. This is supported by the previous reports describing the severe injuries to hydroponically grown soybean plants caused by ammonium toxicity, even at low concentrations (2–3 mM NH_4-N) (Tadano & Tanaka 1976; Ikeda & Osawa 1979). Therefore, the decreased growth due to ammonium toxicity likely severely disrupts nodulation, even if the ammonium weakly inhibits nodulation.

Unlike the effects of ammonium, the urea treatment promoted the shoot growth (Fig. 6A–D) and nodulation (Fig. 6F) of inoculated plants, but less effectively than nitrate. The growth retardation of urea-fed plants may be partially explained by a previous report indicating that reduction of plant growth at the beginning of the growing cycle is likely related to the low use efficiency of urea in soybean seedlings (Paradiso et al. 2014b). Additionally, the long-term supply of urea as the sole nitrogen source results in a lower vegetative biomass and seed yield of hydroponically grown soybean plants compared with the effects of nitrate (Paradiso et al. 2014b, 2015).

Regarding nodulation, several reports indicated that urea-fed plants formed more and larger root nodules than nitrate-fed plants (Yoneyama et al. 1985; Paradiso et al. 2014b, 2015), but these observations were following treatments with high nitrogen concentrations (7.5–20 mM), which are highly inhibitory to nodulation, especially when supplied as nitrate. Therefore, urea can be used as the nitrogen source for promoting the early growth of inoculated soybean plants, but may not be necessarily superior to nitrate for nodulation at relatively low nitrogen concentrations.

In this study, nitrate or urea fertilization at 25% dilution enhanced the growth and nodulation of inoculated plants, but the shoot growth at the transplanting stage was not comparable to the growth of uninoculated plants treated with the 50% nutrient solution (Fig. 6). The diversity in shoot growth may have been caused by differences in the supply of essential minerals other than nitrogen. Accordingly, the optimum ratio of the nitrogen content in 25% solution to the content of the other essential minerals should be determined. However, if the growth retardation is mainly due to a limited fixed nitrogen supply from the nodules during the early growth stage (Hamawaki & Kantartzi 2018; Cafaro La Menza et al. 2020), it may be restored in the later growth stages by increasing the amount of biologically-fixed nitrogen supplied to plants, and further improvements, except for enhanced nodulation, may not be required for the management of inoculated plants, at least during the early growth stage.

CONCLUSIONS

In this study, we proved that inoculated soybean transplants can be effectively produced in a hydroponic system by rooting seedling cuttings in a medium containing a *Rhizobium* inoculant. Additionally, the inoculated plants should be subirrigated with an approximately 25% Enshi nutrient solution supplemented with nitrate or urea to promote early growth and nodulation prior to the transplantation. However, further improvements to the ratio of nitrogen to the other essential minerals in the nutrient solution may be possible.

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